The Transmembrane Domain Region of Nicastrin Mediates Direct Interactions with APH-1 and the γ-Secretase Complex*

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Nicastrin (NCT) is a type I integral membrane protein that is one of the four essential components of the γ-secretase complex, a protein assembly that catalyzes the intramembranous cleavage of the amyloid precursor protein and Notch. Other γ-secretase components include presenilin-1 (PS1), APH-1, and PEN-2, all of which span the membrane multiple times. The mechanism by which NCT associates with the γ-secretase complex and regulates its activity is unclear. To avoid the misfolding phenotype often associated with introducing deletions or mutations into heavily glycosylated and disulfide-bonded proteins such as NCT, we produced chimeras between human (hNCT) and Caenorhabditis elegans NCT (ceNCT). Although ceNCT did not associate with human γ-secretase components, all of the ceNCT/hNCT chimeras interacted with γ-secretase components from human, C. elegans, or both, indicating that they folded correctly. A region at the C-terminal end of hNCT, encompassing the last 50 residues of its ectodomain, the transmembrane domain, and the cytoplasmic domain was important for mediating interactions with human PS1, APH-1, and PEN-2. This finding is consistent with the fact that the bulk of the γ-secretase complex proteins resides within the membrane, with relatively small extramembranous domains. Finally, hNCT associated with hAPH-1 in the absence of PS, consistent with NCT and APH-1 forming a subcomplex prior to association with PS1 and PEN-2 and indicating that the interactions between NCT with PS1 may be indirect or stabilized by the presence of APH-1.

An important pathological feature of Alzheimer’s disease is the accumulation of senile plaques composed predominantly of amyloid-β (Aβ) peptide (reviewed in Refs. 1 and 2). The Aβ peptide is generated from proteolytic processing of the amyloid precursor protein (APP), a type I transmembrane glycoprotein (3). APP is cleaved by two distinct enzymatic activities, which result in the production of an array of Aβ species that differ only slightly in length but vary greatly in their abilities to aggregate (4). In the amyloidogenic pathway, APP is first cleaved by β-site APP cleaving enzyme, which cuts at a luminal site generating C-terminal APP fragments, and subsequently within its transmembrane domain by γ-secretase to generate Aβ peptides and an intracellular APP C-terminal domain (5, 6). In addition to APP, several other transmembrane proteins undergo regulated intramembranous cleavage by γ-secretase, including Notch, Nectin-1α, CD44, the tyrosine receptor ErbB4, and E-cadherin (7–12).

The γ-secretase is a complex of four noncovalently associated protein components that appear to be sufficient for enzymatic activity (13). The active site of the complex has been shown to reside in presenilin-1 (PS1) or its closely related isoform presenilin-2 (PS2), which are predicted to traverse the membrane eight times. However, for PS1 to attain enzymatic function, it must associate with three other integral membrane proteins, nicastrin (NCT) (14–17), APH-1 (18), and PEN-2 (18, 19), and it must undergo an activating cleavage event, resulting in C- and N-terminal PS fragments (20, 21). PEN-2 is a protein of 101-amino acid travel length that traverses the membrane twice, with both its C- and N-terminal domains facing the lumen of the endoplasmic reticulum (ER) (22). APH-1 is predicted to traverse the membrane seven times and regulates the levels of mature NCT and PS1 proteolysis (23, 24). NCT is a type I transmembrane protein containing multiple glycosylation sites, and is essential for APP processing (25) and Notch signaling (16, 26). Studies performed in Drosophila melanogaster have shown that NCT appears to stabilize PS1 and to be critical for trafficking of PS1 to the cell surface (14). Conversely, PS1 is required for the maturation and trafficking of NCT (27–29).

Relatively little is known about the role of NCT in the γ-secretase complex, except that it is a required component (30). Formally, it is not even known if NCT interacts directly with PS1, because communoprecipitation results could be due to indirect PS1-NCT interactions mediated by APH-1, PEN-2, or other molecules. Studies designed to investigate how NCT associates with γ-secretase have largely relied on the introduction of deletions within NCT. Deletion of a 29- or a 58-residue region in the NCT ectodomain that encompasses a conserved DYIGS sequence reduced Aβ secretion, whereas a double-point mutation within this sequence (D336A/Y337A) increased Aβ peptide

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§The abbreviations used are: Aβ, amyloid-β; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonate; APP, amyloid precursor protein; PS1, -2, presenilins 1 and 2; ER, endoplasmic reticulum; NCT, nicastrin; hNCT, human NCT; ceNCT, C. elegans NCT; TMD, transmembrane domain; aa, amino acid(s); FBS, fetal bovine serum; ES, embryonic stem; HA, hemagglutinin; PBS, phosphate-buffered saline.
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production (17). A recent study found that multiple deletions within the ectodomain of NCT, each encompassing at least 50 amino acids, prevented its association with PS1 (31). However, large deletions within the ectodomains of extensively disulfide-bonded proteins that bear multiple N-linked glycosylation sites frequently result in misfolding (43). Failure of a misfolded protein to associate with its normal assembly partners may not reflect the loss of a region or sequence that is in fact important for mediating relevant protein-protein interactions. In the absence of evidence that modified proteins fold normally, it is difficult to draw conclusions about domains that are important for function.

To investigate determinants in human NCT (hNCT) that mediate its interaction with PS1, APH-1, and PEN-2, we developed a strategy in which we produced chimeras by exchanging domains between hNCT and Caenorhabditis elegans NCT (ceNCT), and examined their ability to interact with human γ-secretase complex components as judged by communoprecipitation. Importantly, we found that each chimera folded correctly, because each colocalized in part with the Golgi marker GM130, and each associated with correctly, because each colocalized in part with the Golgi

Finally, studies performed in PS1/PS2-negative (PS

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cause the bulk of these three proteins resides within the mem-

brane domain (TMD), the TMD, and the C-terminal do-

sidered with 10% fetal bovine serum (FBS) and 1% penicillin/strepto-

mycin. Mouse embryonic stem (ES) cells, either wild-type (PS

−/−) or deficient for both PS1 and PS2 (PS

−/−), were described previously (21). The PS

−/− ES cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 15% FBS, nonessential amino acids, 2 mM l-gluta-

mine, 1 mM sodium pyruvate, 100 μM β-mercaptoethanol, 1% penicillin/ streptomycin, and 10 μg/ml of antileukemic factor (Chemicon, Temecula, CA). For transient expression, quadruple transfe-

sions with PS1-HA, PEN-2-HA, and hNCT or ceNCT or chimera were performed in 10-cm dishes of HEK293T cells or ES cells (1 × 10^6 cells), and 2-cm dishes of HeLa cells. Transfections were performed with Gene Porter I Lipid transfection reagent (Gene Therapy System, San Diego, CA) according to the supplier’s protocol.

Protein Analysis—For protein analysis, 24-h post-transfection cell extracts were prepared in 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, and protease inhibitor mixture (Roche Applied Science, Indianapolis, IN). Cell lysates were incubated at 4 °C for 30 min and centrifuged at 10,000 × g for 10 min. SDS-PAGE was carried out using 10% SDS-PAGE gels (Bio-Rad, Hercules, CA), followed by Western blot analysis using horse-radish peroxidase-conjugated secondary antibody followed by visualization with ECL-Plus Western blotting Detection Reagents (Amersham Biosciences, Piscataway, NJ).

Commmunoprecipitation—Communoprecipitation assays were performed as previously described (22). Briefly, HEK293T and ES PS

−/− cells (1 × 10^6 cell) overexpressing combinations of hPS1, hAPH-1-HA, hPEN-2-HA, and epitope-tagged hNCT or ceNCT chimeras were lysed with 0.5 ml of lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% CHAPSO, 5 mM EDTA, and protease inhibitor mixture. Pre-cleared lysates were incubated for 1 h at 4 °C with anti-PS1M antibody, anti-THA tag, or anti-V5 tag antibodies previously coupled to protein A/G beads. Immunoprecipitated proteins were eluted at 100 °C for 5 min with 20 μl of Sample Buffer (0.08 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.005% bromphenol blue). Samples were analyzed by Western blot as described above.

Immunofluorescence Microscopy—HeLa cells grown to 50% conflu-

ence on glass coverslips were transfected with pcDNA3.1/V5, pcDNA3.1/APH-1-HA, pcDNA3.1/APH-1-HA, and hNCT-V5- or V5-

tagged chimeras. 24 h post-transfection, cells were treated with 5 μg/ml cyclohexamide for 3 h. Cells were washed with PBS containing 0.5 mM MgCl_2 and were fixed for 10 min with 2% (w/v) formaldehyde in PBS. Cells were then incubated for 1 h at room temperature with the primary antibodies, anti-v5 tag (Sigma) and anti-GM130 (BD Transduction Laboratories) in PBS containing 4% FBS and 0.02% saponin. Cells were washed with PBS and incubated for 1 h at room temperature with the secondary antibodies Alexa Fluor 488 (goat anti-rabbit) and Alexa Fluor 594 (goat anti-mouse) (Molecular Probes, Eugene, OR) diluted 1:500 in 4% FBS in PBS with 0.02% saponin. 4′,6-Diamidino-2-phenylindole was added to the secondary antibody mixture at a dilution of 1:1000. Cells were washed in PBS, mounted in Fluoromount-G (Southern Biotechnol-
ectodomain Cys residues. In addition, hNCT can partially rescue the genetic phenotype caused by ablation of ceNCT in *C. elegans* cells, the reverse situation may not occur due to the failure of ceNCT to stably associate with hPS1.

To determine if ceNCT could associate with human γ-secretase complex components, we transiently expressed ceNCT or hNCT in HEK293T cells along with hPS1, hAPH-1, and hPEN-2. Transfection efficiencies were monitored by the use of a green fluorescent protein expression plasmid and subsequent experiments to ensure comparable transfection efficiencies. Cells were lysed in 1% CHAPSO, and immunoprecipitated with an antibody to the V5-epitope tag present at the C terminus of hNCT and ceNCT (top panel) to monitor expression of both constructs. Alternatively, aliquots of cell lysate were immunoprecipitated with an antibody to PS1 (PS1-M, middle panel). In both cases, the immunoprecipitates were analyzed by Western blot with anti-V5 tag antibody to detect hNCT or ceNCT. Detection of hPS1 was performed with cell lysates in each case (bottom panel) with an antibody to PS1.

**Expression of Chimeras between hNCT and ceNCT**—To identify regions in hNCT that mediate interactions with γ-secretase complex components, chimeras between hNCT and ceNCT were constructed in which regions of ceNCT were replaced with the corresponding hNCT domains (Fig. 2). Chimeras were designed so that cysteine residues were conserved and, to normalize detection, a V5 epitope tag was added at the C terminus of each construct. The chimeras were transiently expressed in HEK293T cells along with hPS1, hAPH-1, and hPEN-2. Western blot analysis and immunoprecipitation (Fig. 3) of cell lysates showed that the chimeras were well expressed and that their electrophoretic mobilities were consistent with their predicted molecular weights, although chimaera ceNCT (h35-709) sometimes exhibited a lower molecular weight fragment. Mass differences were largely due to different numbers of N-linked glycosylation sites in the various chimeras (Fig. 2).

**Association of Chimeras with Human γ-Secretase Complex Components**—To determine if the chimeras were able to interact with γ-secretase components, coimmunoprecipitation studies with HEK293T cells overexpressing hPS1, hAPH-1, and hPEN-2, and the indicated chimeras were performed (Fig. 4). The efficiency of the immunoprecipitation assay conditions used throughout this report were ~90% (data not shown). Only chimaera ceNCT (h628-721) was coimmunoprecipitated with an antibody to PS1 (Fig. 4A). This chimera could also be coimmunoprecipitated with antibodies to either PEN-2 or APH-1 (Fig. 4, B and C). Transient expression levels of hPS1, hPEN-2, hAPH-1, and corresponding NCT chimeras (Fig. 4, A–D; bottom portion of each panel) were monitored by Western blot. These results suggest that ceNCT is capable of establishing a stable interaction with human γ-secretase complex components when amino acids 628–721 were replaced with the corresponding hNCT (620–709) region. This region contains a 50-amino acid stem region adjacent to the TMD, the TMD itself, and the entire intracellular C-terminal domain.

**Chimeras That Fail to Associate with Human γ-Secretase Complex Components Bind to C. elegans γ-Secretase**—The failure of most chimeras to associate with hPS1, hAPH-1, and hPEN-2 could be due to the absence of important hNCT sequences in a largely ceNCT background. Alternatively, some chimeras may not fold correctly, making their failure to associate with γ-secretase components largely irrelevant. To inves-
tigate this possibility, we monitored the transport of each chimera by immunofluorescence microscopy and measured their ability to interact with cePS1, ceAPH-1, and cePEN-2. Immunofluorescence confocal microscopy studies of HeLa cells expressing each chimera showed that each partially colocalized with GM130, a cis-Golgi marker (Fig. 5). This result suggests that all of the chimeras folded sufficiently well so that they were able to pass the quality control mechanisms resident in the ER that prevent transport of misfolded proteins (43).

To more directly monitor protein folding, we determined if the chimeras interacted with C. elegans γ-secretase components cePS1, ceAPH-1, and cePEN-2. HEK293T cells expressing these components (Fig. 6A) and the indicated chimeras were lysed and either subjected to Western blot analysis against the V5 epitope tag present on the chimeras to monitor expression (Fig. 6B) or coimmunoprecipitation using the HA epitope tag present on the C. elegans γ-secretase components (Fig. 6C). All of the chimeras could be efficiently coimmunoprecipitated, indicating that each folded correctly and could stably interact with C. elegans γ-secretase complex components. Chimeric ceNCT-h(521–628) was coimmunoprecipitated inefficiently in the experiment shown, but was also expressed at relatively low levels. In other experiments, this chimera was coimmunoprecipitated as efficiently as the other chimeras. To confirm this specificity, we also performed coimmunoprecipitations with the anti-HA tag antibody using cell lysates overexpressing V5-tagged cePS1, ceAPH-1, and cePEN-2. None of the chimeras were detected under these conditions (data not shown). Taken together, our results show that all of the chimeras folded sufficiently well to allow exit from the ER and for the specific association with human γ-secretase, C. elegans γ-secretase, or both. Thus, we conclude that the failure of some chimeras to associate with hPS1 cannot be attributed to a misfolded phenotype but, rather, is due to the absence of residues that are important for mediating stable interactions with hPS1. It should be noted that all of the chimeras associated with C. elegans components, including chimera ceNCT-h(628–721), which also bound to hPS1. Thus, none of the modifications introduced by placing hNCT sequences in a ceNCT background were sufficient to prevent binding to C. elegans components.

Further Analysis of Chimera ceNCT-h(628–721).—We further investigated the role that the stem (first 50 residues of the NCT ectodomain adjacent to the TMD), TMD, and cytoplasmic domain regions play in mediating interactions with hPS1, hAPH-1, and hPEN-2 by constructing additional chimeras (Fig. 2). We individually introduced the stem region, the TMD, and the cytoplasmic domain of hNCT into a ceNCT background. We found that the chimera containing the TMD of hNCT (ceNCT-h(680–701)) could be efficiently coimmunoprecipitated with antibodies to hPS1 (Fig. 7A), hAPH-1 (Fig. 7C), and to a lesser extent hPEN-2 (Fig. 7B). Transient expression levels of hPS1, hPEN-2, and hAPH-1 were monitored by Western blot, as previously shown in Fig. 4 (data not shown). Chimera ceNCT-h(628–680), which contains the stem region of hNCT, was coimmunoprecipitated less efficiently, whereas the chimera containing only the cytoplasmic domain (ceNCT-h(701–721)) was the least efficiently coimmunoprecipitated (Fig. 7). Therefore, it appears that the 50-aminoc acid stem region adjacent to the TMD, the TMD itself, and to a lesser extent the C-terminal domain are capable of mediating stable interactions with hPS1, hAPH-1, and hPEN-2, in the context studied. Furthermore, each of these chimeras was transported to the Golgi, and each could associate efficiently with C. elegans γ-secretase complex components (data not shown), indicating that these chimeras also folded correctly.

Direct Interactions between NCT and APH-1.—The ability of NCT to be coimmunoprecipitated with antibodies to hPS1,
hPEN-2, or hAPH-1 does not mean that NCT directly interacts with each of these proteins. Under our detergent lysis conditions, the γ-secretase complex remains structurally and functionally intact (33). Thus, NCT could interact with PS1 indirectly via contacts with APH-1 and/or PEN-2. To begin to address the capacity of NCT to interact with specific γ-secretase components, we coexpressed hNCT with either hAPH-1 or hPEN-2 in a PS/− murine cell line. We found that under these conditions hNCT could be coimmunoprecipitated with an antibody to APH-1, although not as efficiently as when hPS1 was present (Fig. 8A). Transient expression levels of hNCT and ceNCT (Fig. 8A, bottom panel), as well as hPS1, hAPH-1, and hPEN-2 (data not shown) were monitored by Western blot. Although we cannot rule out the possibility that endogenous PEN-2 plays a role in this interaction, other studies have shown that PEN-2 expression is down-regulated in the absence of PS1 (19, 34). Thus, it is likely that endogenous PEN-2 levels in the PS/− cells are very low. This, and the efficiency of coimmunoprecipitation, leads us to conclude that NCT and APH-1 can interact with each other without PS as an intermediate.

When the NCT chimeras were expressed along with hAPH-1 in the PS/−, only chimera ceNCT(16628–721) was coimmunoprecipitated with an antibody to APH-1 (Fig. 8B), indicating that the transmembrane domain of NCT plays an important role in mediating direct interactions with the largely membrane-embedded APH-1 molecule. Chimeras containing only the stem region or cytoplasmic domain of hNCT either did not associate with hAPH-1 under these conditions or did so very weakly. This is in contrast to the ability of these chimeras to coimmunoprecipitate with hAPH-1 when hPS1 was present. We therefore conclude that, although NCT can interact directly with APH-1, this noncovalent association is rendered more stable by the presence of other γ-secretase components. Finally, we attempted to coexpress NCT and PEN-2 in the PS/− cells but were unable to obtain detectable levels of PEN-2. Thus, we could not determine if NCT can interact with PEN-2 in the absence of PS1.

**Discussion**

Nicastrin (NCT) is one of four known components of the γ-secretase complex (35, 36). Expression of hNCT, hPS1, hAPH-1, and hPEN-2 in yeast imparts γ-secretase activity, strongly suggesting that these four proteins are both necessary and sufficient for this enzymatic activity (13). Although PS1 contains the catalytic residues responsible for proteolytic activity, the roles of the other three components in the structure and activity of this tightly regulated enzyme complex are uncertain. Studies have shown that PEN-2 and APH-1 may be involved in coordinately regulating proteolytic processing of PS1 (34, 37). Nicastrin has been shown to form a complex with PS1 (38), and its presence is required both for the cleavage of PS1 into its active C- and N-terminal fragments and for its transport beyond the ER (39, 40). Likewise, in the absence of PS1, NCT is not transported suggesting that interactions between these proteins are needed for them to either fold correctly, making it possible to exit the ER, or perhaps to mask an ER retention signal (27–29).

Deletions within the ectodomain of NCT have been shown to block interactions with PS1 as judged by coimmunoprecipitation and to reduce or ablate γ-secretase activity (17, 31, 41, 42). It is not possible to conclude if these results are due to the genetic deletion of residues or regions in NCT that are needed for specific interactions with one or more γ-secretase components, or if they are due to gross misfolding of the modified NCT constructs. Deletions within the ectodomains of large, heavily glycosylated proteins that contain multiple disulfide bonds often result in protein misfolding, particularly if Cys residues and N-linked glycosylation sites are eliminated (43). Information regarding assembly of the γ-secretase complex can only be gained if an NCT molecule folds properly. Because the only known function of NCTs seems to be to associate and
regulate the γ-secretase complex and promote its maturation, structure-function studies are made more difficult. If the only assay that can be used to probe NCT structure is its assembly with PS1, APH-1, and/or PEN-2, then it cannot be readily determined if a mutation that blocks assembly does so specifically by modifying residues important in NCT interactions with γ-secretase components or nonspecifically by inducing misfolding of the molecule with concomitant loss of structures needed to mediate protein-protein interactions. The formation of disulfide-linked aggregates or prolonged association with molecular chaperones such as BiP/GRP78 and calnexin can be taken as presumptive evidence of misfolding (43), although this has not been examined for the NCT deletion mutants published to date. One way to avoid the misfolding phenotype so often associated with deletion mutagenesis of proteins such as NCT is to construct chimeras between the protein of interest and a related molecule that shares conserved features, yet differs sufficiently in primary amino acid sequence so as to allow identification of residues important for a given function. Thus, we sought a homolog of hNCT that would fulfill this requirement and found one in the form of ceNCT. Genetic evidence indicates that ceNCT has functions that are broadly similar to those of its human counterpart, particularly with regards to γ-secretase-mediated cleavage of Notch (11, 17, 26, 42). In addition, while sharing only 21% amino acid identity, the large majority of Cys residues are conserved between the two molecules, suggesting a conserved overall structure. We found that ceNCT did not stably interact with human γ-secretase components when expressed in HEK293T cells. However, it should be stressed that our conclusions apply only to interactions that are stable in the presence of CHAPSO, it is possible that ceNCT does interact with human γ-secretase components, albeit with less stability than hNCT.

We found that all hNCT/ceNCT chimeras retained the ability to interact with γ-secretase components derived from one and sometimes both species, providing strong evidence that all chimeras attained native or near-native conformations. Thus, the failure of many chimeras to associate with hPS1, hAPH-1, or hPEN-2 cannot be attributed to a failure to fold, a conclusion that usually cannot be made with deletion mutants that fail to assemble properly. Our overall conclusion is that the NCT TMD, as well as regions immediately adjacent to it, plays a particularly important role in mediating γ-secretase complex interactions. Thus, the TMD of hNCT, when introduced into ceNCT, resulted in stable interactions with hPS1, hAPH-1, and hPEN-2. If the cytoplasmic domain and the last 50 residues of the hNCT ectodomain (here referred to as the stem region)
were included, interactions appeared to be more efficient. Consistent with this, both the stem region and the cytoplasmic domain alone were able to confer to ceNCT the ability to interact with human γ-secretase proteins when all four components were expressed, at least to some degree. However, our results should not be taken to mean that more proximal N-terminal regions of NCT play no role in γ-secretase interactions. The hNCT and ceNCT ectodomains do share some conserved features, such as the DYIGS and Y(D/Q)M(E/K)GFKP sequences, that would obviously not be altered through the construction of chimeras between the two molecules; therefore, their roles in complex assembly cannot be fully assessed using our experimental approach. Additional mutagenesis studies, employing both chimeras and site-directed mutagenesis, will be needed to find more finely map domains that mediate interactions between NCT and other molecules.

Our finding that the TMD of NCT, as well as regions that immediately adjoin it on either side of the membrane, play important roles in interactions with multiple γ-secretase components is perhaps not surprising given that PS1, APH-1, and PEN-2 reside largely within the membrane. Using currently existing models of membrane topology, ~33% of PS1, 40% of PEN-2, and 61% of APH-1 reside within the membrane, with a total of 17 distinct TMDs. That adjoining TMDs can interact with each other is well documented and can involve interactions between TMDs within a single protein or between different molecules (44). The transmembrane domains of hNCT and ceNCT share surprisingly little amino acid identity, with only 1 of 20 residues from Leu-721 to Tyr-739 being identical. When the TMD of ceNCT was replaced with that of hNCT, association with hPS1, hAPH-1, and hPEN-2 was readily detected. Thus, in this context, replacing 19 hydrophobic residues in the ceNCT TMD with the hydrophobic residues present in the hNCT TMD were sufficient to mediate stable interactions with the human γ-secretase complex.

Although NCT has been shown to be coimmunoprecipitated with antibodies to PEN-2, APH-1, and PS1, it is not clear if it directly interacts with one or more than one of these proteins, because lysis with CHAPSO enables the entire complex (33). Low levels of endogenous proteins, even in the absence of PS1 (19, 34), so we would anticipate that endogenous PEN-2 were also coexpressed, at least to some degree. However, our results cannot rule out the possibility that NCT-APH-1 association is not just how NCT associates with APH-1 and other γ-secretase components but the role NCT may play in modulating enzymatic activity.

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