

Rer1p competes with APH-1 for binding to nicastrin and regulates γ -secretase complex assembly in the early secretory pathway

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The γ -secretase complex, consisting of presenilin, nicastrin, presenilin enhancer-2 (PEN-2), and anterior pharynx defective-1 (APH-1) cleaves type I integral membrane proteins like amyloid precursor protein and Notch in a process of regulated intramembrane proteolysis. The regulatory mechanisms governing the multi-step assembly of this “proteasome of the membrane” are unknown. We characterize a new interaction partner of nicastrin, the retrieval receptor Rer1p. Rer1p binds preferentially immature nicastrin via polar residues within its transmembrane domain that are also critical for interaction with APH-1. Absence of APH-1 substantially increased

binding of nicastrin to Rer1p, demonstrating the competitive nature of these interactions. Moreover, Rer1p expression levels control the formation of γ -secretase subcomplexes and, concomitantly, total cellular γ -secretase activity. We identify Rer1p as a novel limiting factor that negatively regulates γ -secretase complex assembly by competing with APH-1 during active recycling between the endoplasmic reticulum (ER) and Golgi. We conclude that total cellular γ -secretase activity is restrained by a secondary ER control system that provides a potential therapeutic value.

Introduction

γ -Secretase catalyzes the final cleavage of the amyloid precursor protein (APP), liberating the amyloid β (A β) peptide (Annaert and De Strooper, 2002) probably causing Alzheimer's disease (Hardy and Selkoe, 2002; Walsh and Selkoe, 2004). Besides APP, γ -secretase cleaves other biologically important substrates like Notch and many more type I membrane proteins and is involved in that way in development, neurogenesis, and cancer (Brown et al., 2000; Selkoe and Kopan, 2003; Kopan and Ilagan, 2004). γ -Secretase is a multisubunit complex consisting of presenilin (PS), nicastrin (NCT), PS enhancer-2 (PEN-2), and anterior pharynx defective-1 (APH-1; De Strooper, 2003).

These proteins are minimally required to generate a functional active complex. It is far from fully understood where in the cell the complex is assembled and activated and what the sequence of events are leading to full assembly. Nevertheless, given its complexity and indispensable role in intramembrane proteolysis, mechanisms must be present to tightly control and regulate this assembly. The prevailing hypothesis suggests that assembly is initiated by the NCT-APH-1 subcomplex and is followed by the sequential incorporation of PS and PEN-2 or, alternatively, the PS-PEN-2 subcomplex (Hu and Fortini, 2003; LaVoie et al., 2003; Fraering et al., 2004; Capell et al., 2005). Because all four hydrophobic components are synthesized and translocated in the ER, this is the earliest membrane compartment where assembly can theoretically be initiated, but whether the complex is already activated in this organelle remains a question. This has been formulated originally as the spatial paradox (Annaert and De Strooper, 1999): although most of the endogenous PS1 resides in the ER and in the intermediate compartment (IC; Annaert et al., 1999) in coat protein complex I (COPI)-coated areas, (Rechards et al., 2003),

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Abbreviations used in this paper: A β , amyloid β ; AICD, APP intracellular domain; APH, anterior pharynx defective; APP, amyloid precursor protein; BN-PAGE, blue native PAGE; COP, coat protein complex; CTF, C-terminal fragment; DTBP, dimethyl 3,3'-dithiobispropionimidate; endoH, endoglycosidase H; FL, full-length; IC, intermediate compartment; KO, knockout; MEF, mouse embryonic fibroblast; NCT, nicastrin; NTF, N-terminal fragment; PEN, PS enhancer; PS, presenilin; Rer1p, retrieval to ER 1 protein; TLN, telencephalin; TMD, transmembrane domain; WT, wild-type.

activity seems mainly to occur at the cell surface or close to the cell surface in endosomal compartments (Kaether et al., 2006; Rajendran et al., 2006). The slow maturation of NCT in the Golgi (Yang et al., 2002; Herreman et al., 2003) and the relative long turnover of this and other γ -secretase complex partners once they are incorporated in the complex indicate that these components are actively retrieved from Golgi compartments or retained in the ER. In support of this, Kaether et al. (2004) suggested that a hydrophobic stretch in the C terminus of PS1 constitutes a retention signal for unassembled PS1 in the ER, the basis of which remains to be investigated. Moreover, absence of one component results in destabilization or reduced expression of other components and impaired maturation (NCT) or PS endoproteolysis (Li et al., 2003; Nyabi et al., 2003; Zhang et al., 2005), predicting a substantial role of early biosynthetic compartments in complex assembly.

The intriguing question, therefore, is what the molecular mechanism is that governs active recycling of γ -secretase components. This knowledge is of utmost importance, as it may converge with the regulation of the stepwise assembly of the complex in early compartments (Capell et al., 2005). Some proteinous components have been identified recently that modulate γ -secretase activities, including CD147 (Zhou et al., 2005), TMP21 (Chen et al., 2006), and phospholipase D1 (Cai et al., 2006). However, they emerged as binding partners of mature complexes, acting likely in later trans-Golgi network and endosomal compartments. On the contrary, not a single binding

partner or factor has been identified that mediates through its interaction early steps of complex assembly.

In this paper, we show the interaction of NCT with Rer1p (retrieval to ER 1 protein), a membrane receptor operating in Golgi retrieval. Binding to Rer1p requires critical residues in the transmembrane domain (TMD) of NCT also used by APH-1, thereby lifting a first important corner of the veil in the molecular regulation of complex assembly in early compartments. This interaction highlights the requirement of secondary ER quality control in complex assembly.

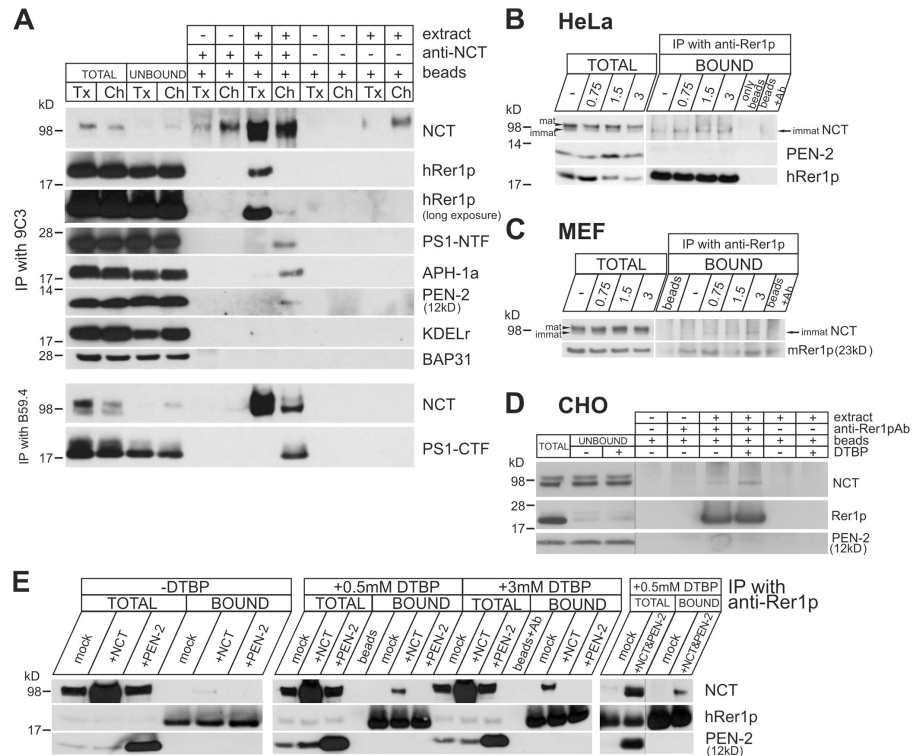
Results

Rer1p preferentially interacts with uncomplexed immature NCT

Several signal motifs in membrane proteins mediate retrieval from IC or Golgi compartments, including dilysine and -arginine motifs in intracellular or the KDEL motif in luminal domains (Lee et al., 2004; Michelsen et al., 2005). However, none of these "classical" motifs are identified in individual γ -secretase components. We therefore hypothesized that individual components are retrieved through interaction with membrane proteins bearing such motifs. Candidate retrieval proteins were tested for their interaction with NCT in HeLa cells by coimmunoprecipitation using anti-NCT mAb 9C3 or affinity-purified pAb B59.4. Because CHAPS extraction preserves the integrity of the complex, mAb 9C3 pulled down NCT with all γ -secretase complex

Figure 1. Endogenous and overexpressed NCT and hRer1p coimmunoprecipitate.

(A) mAb 9C3 and affinity-purified pAb B59.4 against NCT coimmunoprecipitate preferentially γ -secretase complex components (PS1-NTF, PEN-2, APH-1 α , and PS1-CTF) from CHAPS-extracted HeLa cells (right lane; bound fraction), as this detergent preserves the integrity of the complex. Although hRer1p was also recovered in very small amounts, levels of coimmunoprecipitating hRer1p increased strongly when Triton X-100 was used for cell extraction (left lane; bound fraction). Other membrane proteins involved in ER-Golgi trafficking, like KDELr and BAP31, were not pulled down by NCT antibodies. Immunoprecipitations in the absence of extract, primary antibodies, or both were used as additional controls. (B–D) pAb against Rer1p coimmunoprecipitates small amounts of immature NCT from HeLa (B) and MEF (C) Triton X-100 extracts. DTBP cross-linking (0.75, 1.5, and 3 mM) before extraction slightly increased the coimmunoprecipitated immature NCT. In CHO that bears higher levels of immature NCT, the selective interaction with immature NCT was even more obvious (D). No PEN-2 (or other components; not depicted) coimmunoprecipitated with Rer1p. (E) Endogenous hRer1p interacts with overexpressed NCT but not PEN-2. Immunoprecipitation was performed with anti-hRer1p antibody upon transient overexpression of mouse NCT or PEN-2 in HeLa cells. When DTBP cross-linker was applied at two different concentrations (0.5 and 3 mM) before coimmunoprecipitation (right), the amount of NCT bound to hRer1p was increasing in a dose-dependent manner. Transiently overexpressed PEN-2 protein was used as a control and showed no interaction with hRer1p. When co-overexpressed, only NCT, not PEN-2, coimmunoprecipitated with anti-Rer1p (rightmost panel), again underscoring the specificity of the interaction. Black lines (D and E) indicate that total/unbound and bound lanes are anti-Rer1p from the same experiment/blot but different exposure times.



members (Fig. 1 A). Although only little hRer1p coimmunoprecipitated from CHAPS extracts, these levels increased dramatically in Triton X-100 extracts, i.e., under conditions that disrupt the interaction between γ -secretase components. Other retrieval proteins like BAP31 (Annaert et al., 1997) and the KDEL receptor did not interact with NCT, underscoring the specificity of the pull down. In a reciprocal experiment, anti-hRer1p pAb pulled down small amounts of endogenous immature NCT from Triton X-100 HeLa and mouse embryonic fibroblast (MEF) extracts (Fig. 1, B and C). Adding the chemical cross-linker dimethyl 3,3'-dithiobispropionimidate (DTBP) before solubilization and coimmunoprecipitation even slightly increased NCT levels in the precipitate. The specific interaction with immature NCT is even more apparent from CHO extracts that express higher levels of endogenous immature NCT (Fig. 1 D). Other γ -secretase complex components like PS1 C-terminal fragment (CTF; unpublished data) and PEN-2 did not bind, underscoring the selectivity of the interaction. Similar coimmunoprecipitations were performed upon overexpression of either NCT or PEN-2 in HeLa cells (Fig. 1 E). Although overexpression levels were similar for both proteins, anti-hRer1p pAb pulled down NCT but not PEN-2. The amount of immature NCT bound to hRer1p increased dose dependently with increasing concentrations of DTBP (0.5–3 mM; Fig. 1 E). Collectively, we can conclude that hRer1p specifically interacts with immature NCT, most likely in its uncomplexed status.

Rer1p is an integral membrane protein of \sim 23 kD, including four TMDs with both termini facing the cytosol. The *Rer1* gene emerged originally from a yeast screen for mutants defective in retention of Sec12, a protein involved in COPII-coated vesicle formation (Boehm et al., 1994; Sato et al., 1995). Since then, several yeast proteins have been identified next to Sec12p (Sato et al., 1996) that use the Rer1p-dependent retrieval system, including subunits of the translocation machinery, like Sec63p and Sec71p (Sato et al., 2003), and the iron transporter subunit Fet3p (Sato et al., 2004). The latter is of particular interest for the current study, as yRer1p only binds Fet3p in its unassembled state.

Like in yeast, epitope-tagged hRer1p localized to the Golgi in HeLa cells (Fullekrug et al., 1997). However, at the endogenous level, hRer1p only partially colocalized with the cis-Golgi marker GM130, as demonstrated by high-resolution confocal microscopy (Fig. 2). Instead, a far better colocalization was observed with ERGIC-53, indicating that, at steady state, hRer1p essentially localizes in the vesicular tubular elements of the IC. This was confirmed by subcellular fractionation (unpublished data) and by incubating cells at 15°C, a condition that blocks transport from the IC. Under this condition, hRer1p remained entirely colocalized with ERGIC-53-positive structures (Fig. 2). Interestingly, highly overexpressed hRer1p accumulates in the ER (Fullekrug et al., 1997; unpublished data), indicating that hRer1p requires other (limiting) sorting determinants enabling it to cycle rapidly between ER and Golgi. In conclusion, the localization of hRer1p in the IC predicts a functional role for the hRer1p–NCT interaction in the transport of NCT between early biosynthetic compartments. To better understand the physiological relevance of this interaction, we first decided to analyze in detail the binding characteristics.

hRer1p interacts with NCT via its TMD

As the Rer1p interactions in yeast are mediated through the TMD of the reported binding proteins, we generated a series of NCT deletion constructs to delineate possible interaction sites, focusing on the TMD of NCT (Fig. 3 A). We transiently coexpressed these constructs with hRer1p in HeLa cells followed by coimmunoprecipitation using anti-hRer1p pAb (Fig. 3 B). Deleting the intracellular domain (NCT Δ IC) considerably reduced binding compared with full-length (FL) NCT. Removing the TMD in addition (NCT Δ [TMD + IC]) further interfered with

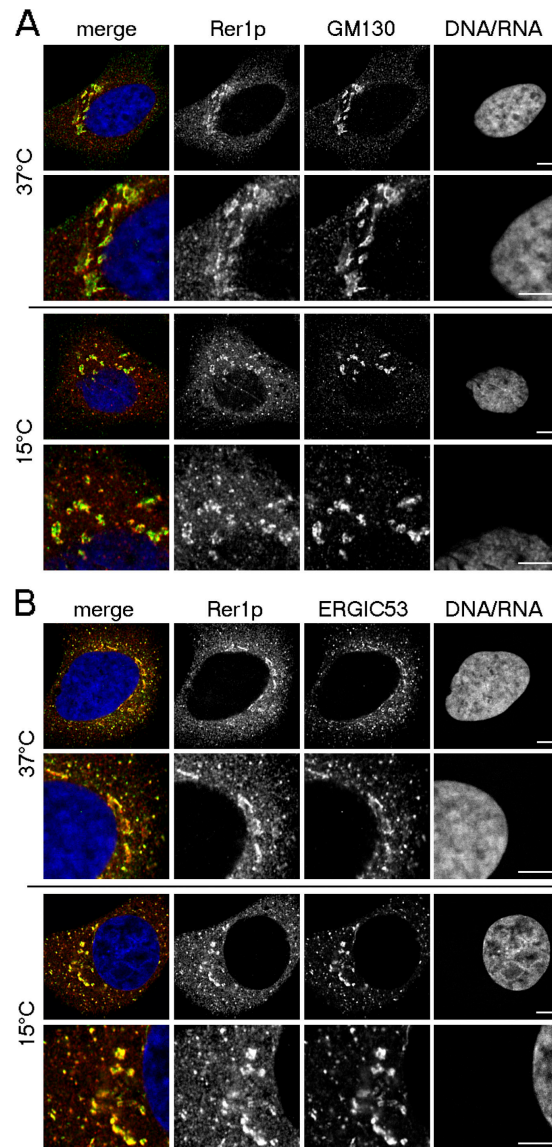
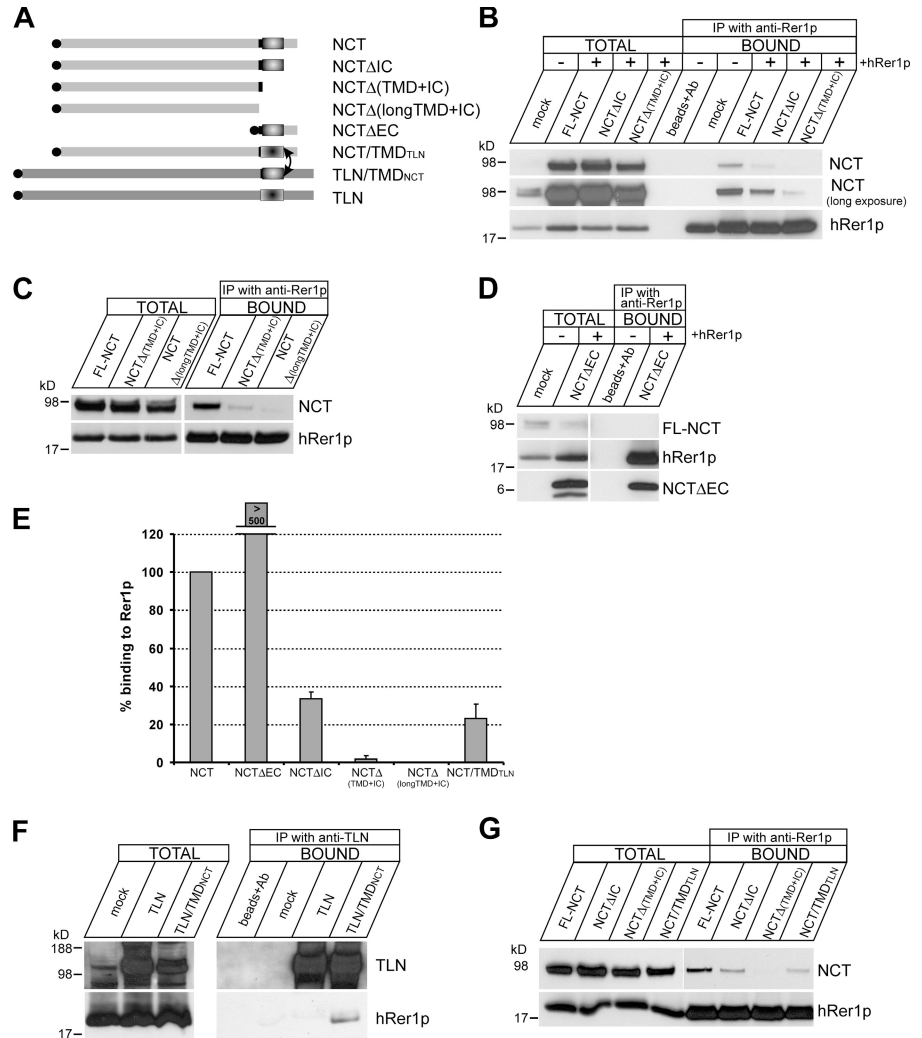


Figure 2. **Endogenous hRer1p resides mainly in the IC.** Fixed HeLa cells were processed for indirect double immunofluorescence labeling using antibodies against hRer1p and GM130 (A) or ERGIC-53 (B). Detection was done with Alexa 488 and 568 conjugated secondary antibodies followed by confocal laser-scanning microscopy. Immunostaining for GM130, a cis-Golgi matrix protein, and hRer1p were intimately but not identically distributed. Almost a complete overlap was found with anti-ERGIC-53, which marks the vesiculotubular elements of the IC. Colocalization was even more obvious when cells were preincubated at 15°C for 3 h (a condition that blocks transport from the IC; Saraste and Svensson, 1991) before fixation (A and B, bottom). Bars, 5 μ m.

Figure 3. hRer1p interacts with NCT through its TMD. (A) Schematic overview of the different NCT deletion constructs as well as chimeric TLN and NCT with swapped TMD. (B–D) Western blot analysis of coimmunoprecipitations with anti-hRer1p pAb from Triton X-100 extracts of HeLa cells transiently co-overexpressing hRer1p and different NCT deletion constructs. As a control, incubations without extract or extracts from mock-transfected cells were used. Total inputs are compared with bound fractions. Deletion of the IC slightly decreases binding, whereas ablation of IC and TMD almost completely abrogated interaction (B). Additional deletion of a short hydrophobic stretch upstream of the TMD abolished interaction completely (C). The interaction of hRer1p with a construct lacking the coding region for the entire ectodomain (NCT Δ EC) demonstrates that the ectodomain is dispensable for the interaction with hRer1p. (E) Densitometric scanning and semiquantification of the hRer1p binding efficiencies toward the different NCT constructs, normalized to the binding of FL-NCT. Notice that NCT Δ EC displays increased binding compared with intact NCT (mean \pm SEM of three to four independent experiments). (F) The NCT TMD is sufficient for binding to hRer1p. Co-overexpression of the chimeric TLN construct, bearing only the TMD of NCT, with hRer1p followed by coimmunoprecipitation. TLN/TMD_{NCT} but not FL-TLN coimmunoprecipitates with hRer1p using anti-hRer1p pAb. (G) Reciprocal experiment using chimeric NCT/TMD_{TLN} (NCT bearing the TMD of TLN) co-overexpressed with hRer1p. Coimmunoprecipitation using anti-hRer1p pAb shows a greatly diminished interaction between NCT/TMD_{TLN} and hRer1p compared with FL-NCT (semiquantified in E).



binding (Fig. 3, B–E), whereas interaction with hRer1p was completely abolished when an additional short hydrophobic stretch before the putative TMD region was deleted (NCT Δ [long TMD + IC]; Fig. 3, C–E). On the other hand, NCT Δ EC, a construct lacking the entire ectodomain, displayed a relatively increased interaction compared with wild-type (WT) NCT, indicating that the ectodomain is dispensable for Rer1p interaction (Fig. 3, D and E). We next swapped the TMD of NCT and from another unrelated type I transmembrane protein, namely, telencephalin (TLN; Annaert et al., 2001), which does not bind hRer1p (Fig. 3 F) and coexpressed the two chimeric proteins each with hRer1p. hRer1p interacted with TLN harboring the TMD of NCT (TLN/TMD_{NCT}) but not with NCT containing the TMD of TLN (Fig. 3, F and G). However, in the latter, some binding was still observed, further confirming the contribution of regions adjacent to the NCT TMD to hRer1p interaction. In conclusion, this experiment demonstrates that the NCT TMD is required and sufficient for efficient binding to hRer1p.

hRer1p binding is mediated through polar residues in the NCT TMD

In yeast it has been shown that interaction with yRer1p (e.g., Sec71p) critically depends on precisely spaced polar residues

within the TMD. We identified in the NCT TMD several polar residues that generate a charged patch on one side in an α -helical wheel model (Fig. 4 A, left, orange residues). When aligned, it becomes clear that the spacing of polar residues in the NCT TMD is similar to that seen in other Rer1p binding partners, including Sec12p and Sec71p (Fig. 4 A, right, red boxed residues). Polar residues can also be found in the TMD of the other γ -secretase components, e.g., in the second TMD of PEN-2; however, these lack the critical spacing. This striking structural resemblance of the NCT TMD with the TMD of other Rer1p binding partners urged us to investigate the polar residues of NCT in more detail. Therefore, polar residues were mutated individually or together to leucine residues using site-directed mutagenesis (Fig. 4 B). All mutants were coexpressed with hRer1p in HeLa cells and tested for their ability to coimmunoprecipitate with anti-Rer1p pAb. To determine the extent to which each mutation affects binding to Rer1p, FL-NCT and NCT/TMD_{TLN} were used as positive and negative controls for binding, respectively (Fig. 4 C).

Single (S₆₈₁/L) or double mutations (T₆₈₅Y₆₈₆/LL and S₆₈₁T₆₈₅/LL) already substantially interfered with hRer1p binding, indicating that hRer1p may use mechanisms similar to yeast Rer1p in the recognition of binding partners. Excitingly,

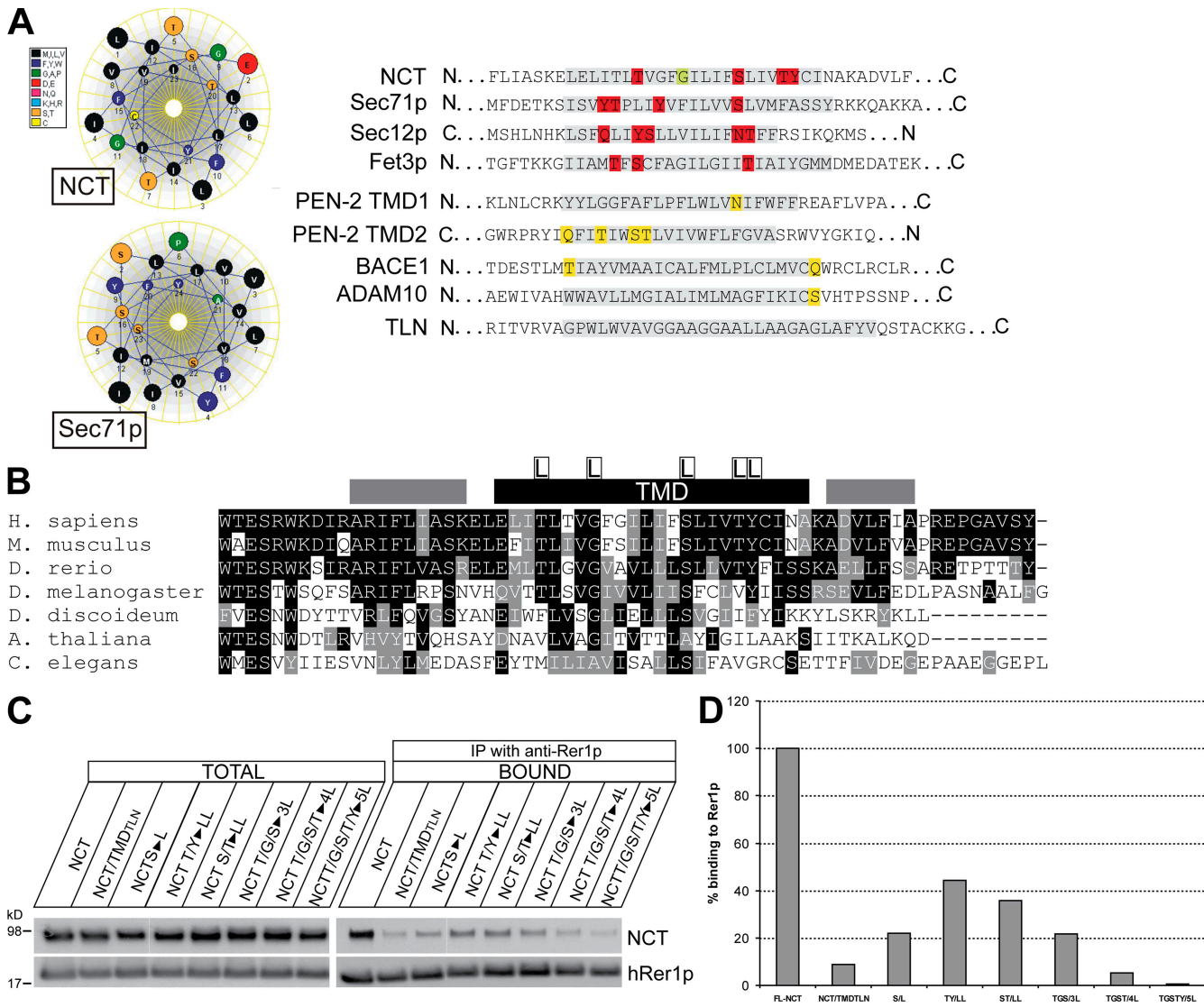


Figure 4. Polar residues in the NCT TMD are critical for the interaction with Rer1p. (A, left) α -Helical wheel projection (using DNASTAR) of the TMD of NCT and Sec71p reveals a similar distribution of polar amino acid residues on one side of the helix (orange residues). These residues in Sec71p (but also Sec12p) have been shown to be critical for Rer1p binding. (right) Amino acid sequence of the TMDs of Rer1p interacting proteins as well as those of PEN-2, secretases, and TLN. The critical spacing of polar residues is only found in Rer1p interacting proteins (red boxed residues) and is not present in other TMDs (yellow boxed residues). The single Gly (green box) in NCT has been related to the binding of Aph-1 (Capell et al., 2003). (B) Sequence alignment of the extended TMD of NCT from different species (black/gray indicates identical/conserved residues). The indicated conserved polar amino acid residues, including Gly and Tyr within the NCT TMD (T₆₇₀, G₆₇₄, S₆₈₁, T₆₈₅, and Y₆₈₆), are mutated to leucine. (C) Co-overexpression of FL-NCT and NCT bearing one or more point mutations within the TMD with hRer1p in HeLa cells, followed by coimmunoprecipitation using anti-hRer1p pAb. FL-NCT and NCT/TMD_{1LN} are used as positive and negative control, respectively. Mutating individual polar residues extensively interferes with binding to hRer1p. Changing four or all indicated residues brings binding levels down to those of the chimeric NCT/TLN_{TMD}. (D) Densitometric scanning and semiquantification of the Western blots from C. hRer1p binding for the different mutants is normalized to FL-NCT.

the same polar residues, S₆₈₁, T₆₈₅, and Y₆₈₆, but also T₆₇₀ and G₆₇₄, have been shown to be critical for the interaction of NCT with APH-1 (Capell et al., 2003). We reasoned that a competition at the level of this binding motif between APH-1 and Rer1p could provide a molecular mechanism that couples ER-Golgi trafficking of NCT (through its interaction with Rer1p) to sub-complex assembly with its cognate APH-1 partner. As such, this would constitute a secondary ER quality control for the multi-subunit assembly of the γ -secretase complex. To test the requirement of these residues, we generated NCT TMD mutants with three to all five residues mutated to leucine (T₆₇₀G₆₇₄S₆₈₁/3L,

T₆₇₀G₆₇₄S₆₈₁T₆₈₅/4L, and T₆₇₀G₆₇₄S₆₈₁T₆₈₅Y₆₈₆/5L). In our co-immunoprecipitation assay, these mutants displayed a gradual decrease of interaction with hRer1p (Fig. 4 C), indicating that these five amino acids are required for hRer1p binding.

Rer1p binding to NCT is increased in APH knockout (KO) MEFs

Our data demonstrate that Rer1p and APH-1 require the same structural features (or the same residues) in the TMD that mediate binding to NCT and that both proteins would be in competition for binding to this motif. If this holds true, binding of Rer1p

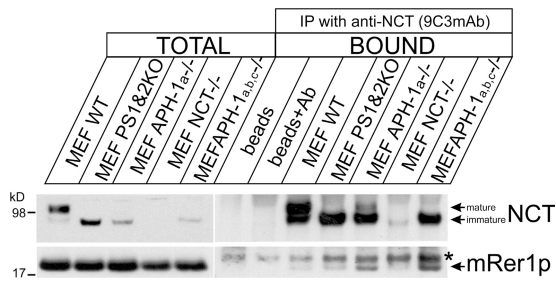


Figure 5. Rer1p competes with APH-1 for binding to NCT. Triton X-100 extracts of WT MEFs and MEFs deficient in PS1 and -2, APH-1a, APH-1a,b,c, and NCT were used for coimmunoprecipitation experiments with mAb 9C3 against NCT to test whether Rer1p interacts with NCT in the absence of other γ -secretase complex components. In WT and PS1 and -2 KO MEFs, equal levels of endogenous Rer1p coimmunoprecipitated, and these levels clearly increased in APH-1a single and even more in APH-1a,b,c KO MEFs (arrow). As expected, no binding is observed in NCT^{-/-} MEFs. This suggests that APH-1 isoforms compete with Rer1p for binding to NCT. The asterisk indicates a nonspecific band.

to NCT should increase in the absence of APH-1. We therefore used MEFs deficient for PS, NCT, APH-1a, or APH-1a,b,c (Fig. 5) to check this intriguing possibility. As shown in Fig. 5, a substantial increase of mRer1p coimmunoprecipitating with NCT is observed in single APH-1a and triple APH-1 KO MEFs, respectively, strongly arguing in favor of our interpretation that APH-1 and Rer1p compete for binding to the same site in NCT. The absence of any effect in PS1 and -2 KO MEFs moreover underscores the specific interaction of Rer1p with NCT and not with any other γ -secretase component. These findings extend our previous conclusion that Rer1p not only mediates the retrieval of NCT from Golgi compartments but also may couple this retrieval of NCT with the assembly of the γ -secretase complex in early biosynthetic compartments. To test this, we investigated the effect of changing Rer1p expression levels on the trafficking of NCT and γ -secretase complex formation.

hRer1p down-regulation affects the glycosylation and localization of NCT

The functional relevance of the interaction between NCT and Rer1p becomes apparent after knock down of hRer1p in HeLa cells. Specific RNAi duplexes efficiently suppressed hRer1p levels up to 80–90% at 48 h after transfection (Fig. 6 A). Interestingly, we noticed a slight shift in the mobility of mature glycosylated (endoglycosidase H [endoH] resistant) NCT. This hypoglycosylation suggests that Rer1p knockdown affects the residence time of NCT in early compartments. To test this, we performed metabolic pulse-chase experiments that confirmed the higher mobility of newly synthesized mature NCT after Rer1p down-regulation (Fig. 6 B). Moreover, quantification of the ratio of mature over total NCT at different time points revealed that Rer1p knockdown considerably delayed mature glycosylation. This is opposite from what is expected if Rer1p mediates retrieval of NCT from cis-Golgi or IC. After all, ablation of such a retrieval mechanism would result in higher kinetics of NCT passaging to post-Golgi compartments. Our results, therefore, suggest that NCT is controlled by additional sorting mechanisms that become apparent after scaling down

the Rer1p-dependent retrieval. This is confirmed in NCT^{-/-} MEFs stably expressing NCT/TMD_{TLN}, i.e., an NCT variant bearing the TMD of TLN (see also Fig. 3). In contrast to NCT, NCT/TMD_{TLN} failed to become mature glycosylated, indicating that it is fully retained in the ER. Moreover, NCT lacking its TMD fails to restore any aspect of γ -secretase, including PS1 endoproteolysis, APH-1 and PEN-2 stabilization, complex formation, and activity (Fig. 6 C). On the other hand, introducing the NCT TMD into TLN (TLN/TMD_{NCT}) sharply decreased mature glycosylation, suggesting that Golgi passage of TLN/TMD_{NCT} was substantially delayed as compared with TLN (Fig. 6 D). Excitingly, Rer1p knockdown (72 h) partially restored mature glycosylation, demonstrating that the decrease in TLN/TMD_{NCT} glycosylation is directly mediated through interaction of Rer1p with the NCT TMD. Hence, although the NCT TMD mediates an Rer1p-dependent retrieval of NCT in early compartments, other yet-to-be-determined domains in NCT confer more ER retention independently of Rer1p.

Finally, we explored the effect of Rer1p knockdown on the post-Golgi localization of NCT applying cell surface biotinylation. Surprisingly, Rer1p down-regulation resulted in substantially more mature (though hypoglycosylated) NCT, but not the transferrin receptor, at the cell surface (Fig. 6 E). This raises the question of how a longer residence time and slower kinetics in pre-Golgi compartments still results in increased surface expression. Interestingly, the co-increase of other γ -secretase components, like PS1 N-terminal fragment (NTF) and APH-1a, fosters the idea that Rer1p-dependent and -independent trafficking of NCT in early compartments may be directly linked to the degree of γ -secretase complex assembly.

Modulating Rer1p expression levels affects γ -secretase complex assembly

To explore this, we applied blue native PAGE (BN-PAGE) to study the effect of Rer1p expression on complex formation. Rer1p was down-regulated using RNAi (48 h) in HeLa, WT, and PS1 and -2 KO MEFs or, alternatively, up-regulated by transient overexpression (36 h) in HeLa cells (Fig. 7, A and B). In mock-transfected cells, NCT immunostaining typically reveals four bands on BN-PAGE. The ~140-kD band is stained with only anti-NCT antibody, whereas the other two bands between 150 and 440 are subcomplexes of NCT–APH-1 and NCT–APH-1–PS1-CTF, respectively, and the ~440-kD band is the mature complex. When Rer1p is down-regulated, the relative amount of mature γ -secretase complex is clearly increased both in HeLa cells and in WT MEFs, in line with the cell surface biotinylation data discussed in the previous paragraph. In addition, we observe a decrease of the NCT–APH-1 subcomplex and an increase of the NCT–APH-1–PS1-CTF subcomplex. This suggests that in the absence of Rer1p, the NCT–APH-1 subcomplex is more rapidly converted to more complete complexes. This was confirmed in an overexpression experiment showing that high expression of Rer1p in HeLa cells resulted in relatively lower levels of mature complexes and an increase of subcomplexes (Fig. 7 B). Because altering the levels of Rer1p affects the NCT–APH-1 subcomplexes, we hypothesized that Rer1p interacts with NCT at a very early stage in γ -secretase

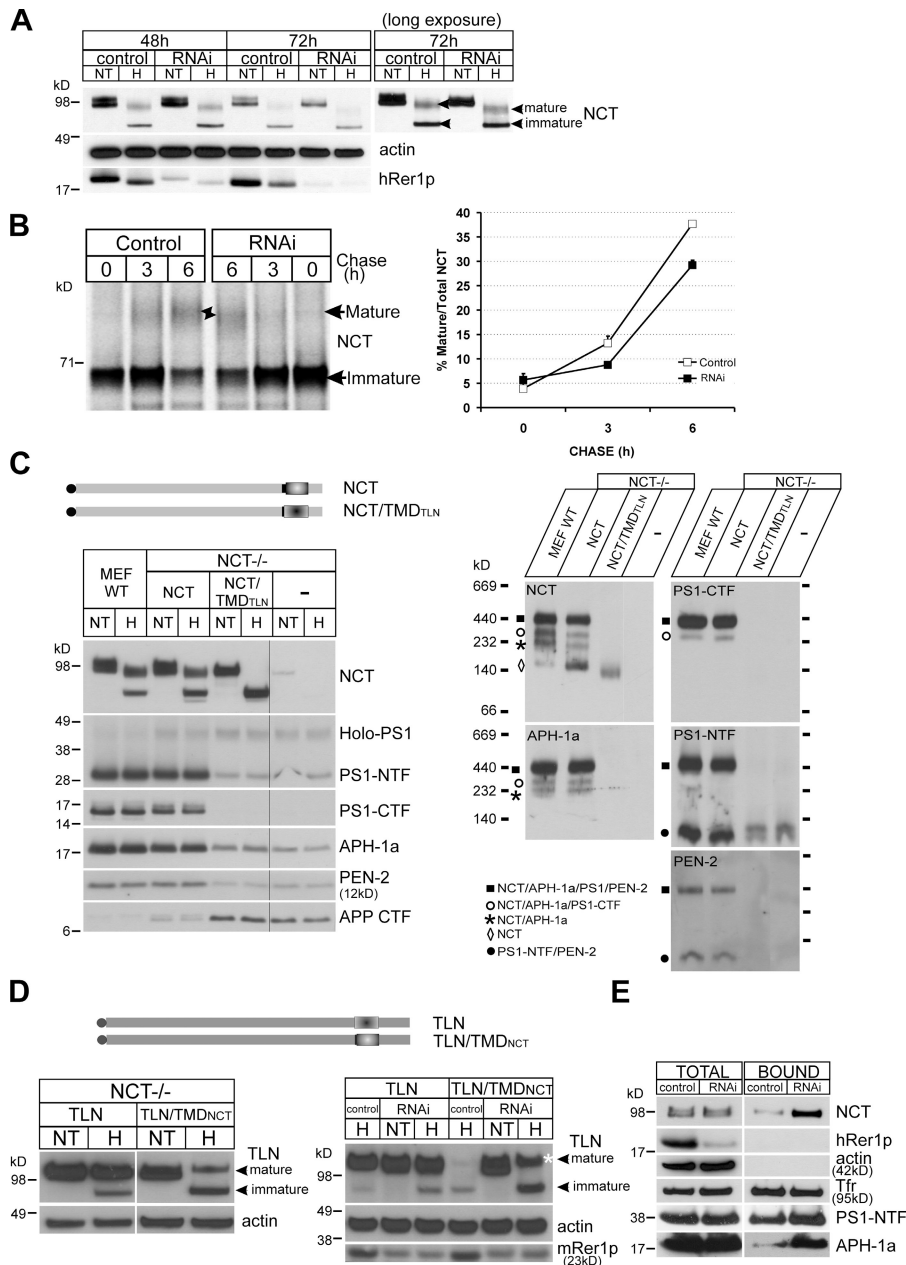


Figure 6. Rer1p functions in NCT recycling between ER and Golgi. (A) hRer1p down-regulation affects NCT glycosylation. Western blot analysis of extracts from control and RNAi-treated HeLa cells pretreated without (NT) or with endoH (H) before SDS-PAGE. Knock down of hRer1p by >80% revealed an increased mobility of endoH-resistant (mature glycosylated) NCT (48 and 72 h, RNAi, H lane). (B) Representative metabolic pulse-chase experiment showing that Rer1p knockdown results in a higher mobility of the mature endoH-resistant NCT (arrowhead). Quantification of the endoH-resistant/total NCT over time (mean \pm SEM) shows that the maturation of newly synthesized NCT is considerably delayed upon Rer1p knockdown, suggesting a longer residence time in early biosynthetic compartments. (C) NCT/TMD_{TLN} is retained in the ER. Western blot of MEF WT, NCT^{-/-} stably transduced with NCT, or NCT/TMD_{TLN} (Fig. 3 A). In contrast to NCT, NCT/TMD_{TLN} remains fully immature and fails to rescue PS1 endoproteolysis, PEN-2 and APH-1 stability, and APP-CTF processing. BN-PAGE clearly demonstrates that the TMD of NCT is required for interaction and γ -secretase complex formation. The black line indicates that the NCT^{-/-} lanes were run on remote slots of the same gel. (D) The NCT TMD mediates an Rer1p-dependent ER-Golgi retrieval when introduced in a TLN reporter. TLN/TMD_{NCT}, when stably transduced in NCT^{-/-} MEF, presents with a sharp decrease in the levels of mature glycosylated chimeric TLN as compared with WT TLN. Subsequent down-regulation of endogenous Rer1p rescues mature glycosylation of TLN/TMD_{NCT} (indicated by asterisk), demonstrating that Rer1p mediates its recycling/retrieval in early compartments through TMD interactions. (E) Western blot analysis of cell surface biotinylated proteins after 48 h of hRer1p knockdown. Down-regulation of hRer1p substantially increased the cell surface levels of mature NCT, PS1-NTF, and APH-1 in comparison to control treated cells, whereas transferrin receptor (Tfr) levels were unaffected. Equal inputs were used for all conditions, as shown by the total extracts.

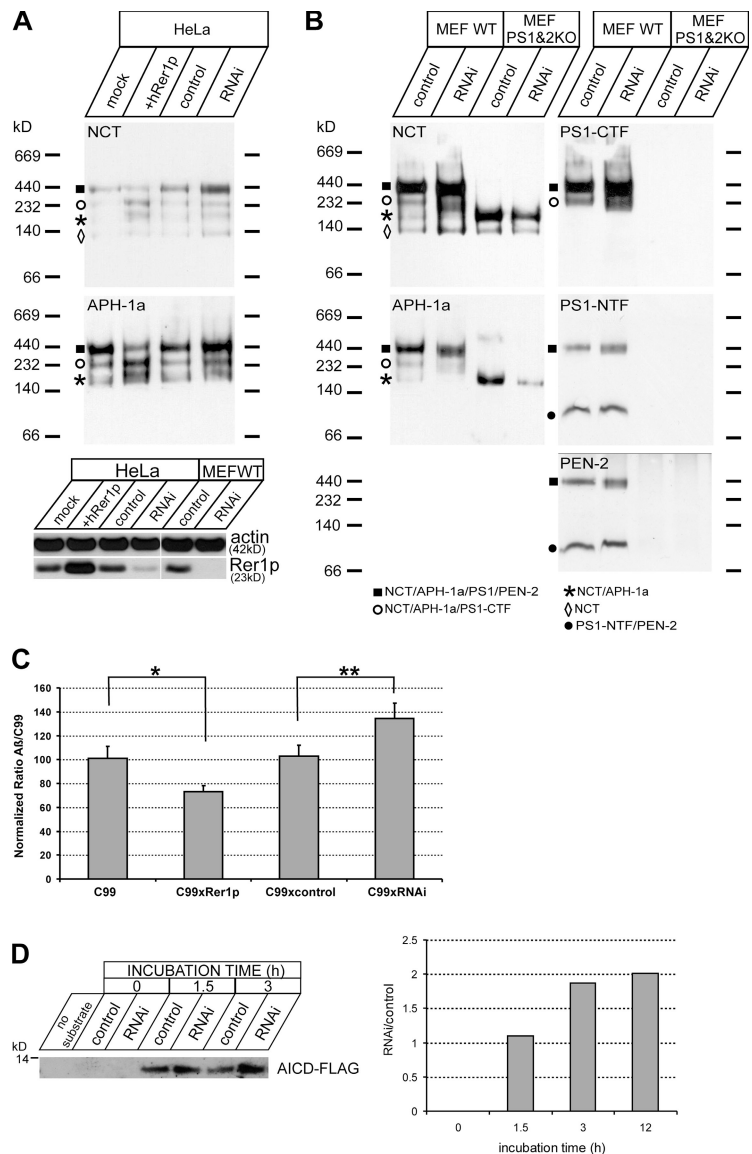
complex assembly. The simplest interpretation of our data is that the Rer1p–NCT interaction titrates the amount of NCT available for binding to APH-1. Further support for this idea comes from an experiment in PS1 and -2 KO MEFs, in which no mature complex is generated. Because Rer1p down-regulation results in a sharp decrease of remaining NCT–APH-1 subcomplexes, and no PS and almost no PEN-2 is present in these cells, Rer1p must interfere at the level of the NCT–APH-1 subcomplex. In conclusion, the longer residence time in early compartments as a result of Rer1p knockdown (Fig. 6 B) appears to also promote complex assembly (Fig. 7). Hence, we provide strong evidence that the regulation of full complex assembly is tightly linked with ER-Golgi recycling. Consequently, complex assembly may ultimately mask Rer1p-dependent and -independent retrieval/retention motifs, enabling complexed NCT to escape

quality control in early compartments. Hence, increased complex formation due to Rer1p knockdown may therefore also explain the increased surface expression of γ -secretase components, as these are stable and long-lived components (Fig. 6 E).

hRer1p expression levels also modulate γ -secretase activity

If hRer1p is rate limiting for the assembly of the complex, it becomes important to know whether it is also rate limiting for γ -secretase activity. To test this, APP-C99, a direct γ -secretase substrate, was overexpressed in HeLa cells together with hRer1p or in conjunction with down-regulation of hRer1p, followed by metabolic labeling and immunoprecipitation of newly produced A β (Fig. 7 C). Significantly more A β was secreted from hRer1p down-regulated cells, whereas the reverse effect could be observed

Figure 7. Changing the expression levels of Rer1p affects γ -secretase complex assembly and activity. (A and B) MEF WT, PS1 and -2 KO, and HeLa cells with normal and down-regulated Rer1p (48 h) and HeLa cells with transiently overexpressed hRer1p (36 h) were extracted in 0.5% dodecylmaltoside and processed for BN-PAGE. Control cells were transfected with empty vector (mock) or nonspecific duplex oligonucleotides (control). Western blot analysis for the different γ -secretase components indicates that down-regulation results in relatively increased levels of mature complexes (indicated by black box) in both HeLa (A; RNAi vs. control lane) and MEF WT cells (B). In contrast, overexpression of Rer1p in HeLa cells resulted in relatively less of the mature complex and more of the intermediate complexes. Suppression of hRer1p in MEF PS1 and -2 KO cells affects the stability of the NCT-APH-1a subcomplex (A). Identification of different dissociated complexes was done according to Fraering et al. (2004). Routinely, extract samples were tested on SDS-PAGE followed by Western blotting to verify the efficiency of Rer1p overexpression or down-regulation in HeLa and MEFs (bottom left). (C and D) hRer1p levels inversely correlate with cellular γ -secretase activity. (C) After 24 h of overexpression or 48 h of down-regulation (RNAi, specific duplex; NS, nonspecific control) of hRer1p in combination with overexpression of APP-C99, HeLa cells were metabolically labeled for 4 h as described previously (Annaert et al., 1999). Total secreted A β and APP-C99 were, respectively, immunoprecipitated from media and extracts and quantified by phosphorimaging. The ratio of A β to APP-C99 is significantly decreased or increased when hRer1p levels are up- or down-regulated (mean \pm SEM; $n = 5$; t test *, $P < 0.03$; **, $P < 0.05$). (D) Cell-free γ -secretase assay. Extracts from control and hRer1p knockdown HeLa cells were mixed with affinity-purified recombinant APP-C99-FLAG (from transfected γ -secretase-deficient MEFs) and incubated at 37°C. Newly produced AICD-FLAG is clearly increased after hRer1p knockdown, indicating enhanced levels of γ -secretase activity.



when hRer1p was overexpressed. However, the possibility that these changes in A β production are indirectly caused by an altered trafficking of APP-C99 due to a change in hRer1p levels cannot be excluded. Therefore, direct γ -secretase activity was tested in a cell-free assay (Fig. 7 D). Here, cell extracts were mixed with recombinant flag-tagged APP-C99 (Kakuda et al., 2006). More APP intracellular domain (AICD; Fig. 7 D) was produced from membranes generated from cells that were down-regulated for hRer1p, arguing for a higher total γ -secretase activity in these cells. In conclusion, the change in mature γ -secretase complex levels caused by altering the expression of hRer1p as observed by BN-PAGE correlated well with the changes in γ -secretase activity, indicating that hRer1p regulates γ -secretase activity.

Discussion

In this study, we identify Rer1p as a novel limiting factor in the stepwise assembly of the γ -secretase complex. Rer1p binds NCT in a region that also involves the binding with other

γ -secretase components, notably, PS1 and APH-1. We also show that Rer1p is a resident protein of the IC/cis-Golgi, indicating that Rer1p acts as a retrieval receptor for ER-Golgi recycling of NCT. We propose that it negatively regulates the availability of NCT for assembly in the NCT-APH-1 subcomplex. By interfering with the initial steps of complex assembly, Rer1p expression controls total levels of γ -secretase complexes and, hence, activity in the cell.

The evidence that Rer1p plays a key role in complex assembly is, in the first place, based on the identification of important binding requirements in the TMD of NCT. Although short regions adjacent to the TMD contribute to binding, especially the polar residues within the NCT TMD are crucial, and mutating one or more of them affects binding to Rer1p to a large extent. These residues are also important for the interaction of NCT with APH-1 (Capell et al., 2003) and indicate that both proteins compete for binding to NCT. This is strongly supported by our findings that Rer1p binding to NCT is substantially increased in APH-1 KO MEFs.

The presence of polar residues alone, however, is not sufficient for interaction, as the second TMD of PEN-2 bears three polar residues but fails to interact with Rer1p (Fig. 1). The additional structural feature becomes apparent when we project the TMD of NCT in a helical wheel. Now the critical residues form a hydrophilic patch on one side of the α -helix, implying that their spacing in the TMD is critical for binding. When aligned, the polar residues within the TMD of NCT flank a highly hydrophobic core domain, and this spaced distribution is also encountered in the TMD of other Rer1p-binding proteins, like Sec12p, Sec71p, and Fet3p. Moreover, in the case of Sec71p-Rer1p, the length of this hydrophobic core is indeed essential for binding (Sato et al., 2003). Collectively, it can be easily envisaged, also in our study, that mutating any of the flanking polar residues expands the length of the hydrophobic core as well as decreases the size of the hydrophilic patch, both accounting for a decreased interaction with Rer1p (Fig. 4). Our study not only extends the findings in yeast but also proves that the mechanism Rer1p uses to target TMDs is conserved from yeast to mammals.

Mechanistically, the binding of Rer1p to NCT may decrease its availability to bind APH-1 and form the initial NCT-APH-1 subcomplex. Conversely, the binding of APH-1 can sterically mask Rer1p interaction, allowing the NCT-APH-1 subcomplex to escape the Rer1p-dependent retrieval mechanism. However, to get a full complex assembled, mechanisms must exist that secure the NCT-APH-1 binding, preventing it from shifting back to Rer1p interaction. Because the NCT-APH-1 subcomplex likely constitutes the earliest step in assembly, it may act as a scaffold for the sequential association of PS1 and PEN-2 (Capell et al., 2005; Niimura et al., 2005). PEN-2, eventually in coordination with APH-1 (Luo et al., 2003), acts to stabilize and promote PS1 endoproteolysis. Here, the binding of the C terminus of PS1 to the TMD of NCT (Kaether et al., 2004) could provide a molecular mechanism to “lock” the NCT-APH-1 interaction into a maturing complex. The incorporation of PS1 could definitively prevent Rer1p from binding to NCT and reversing the interaction with APH-1. This idea is supported by the effects of modulating Rer1p expression on complex formation. Overexpression of Rer1p predicts a more active deprivation of NCT for APH-1 binding and a slowing down of complex formation, as indicated by a decrease in mature complexes. On the other hand, Rer1p knockdown may facilitate the binding of APH-1 to NCT, thereby shifting the equilibrium and promoting a transition to more complex formation (Fig. 7). The fact that we do not see the NCT-APH-1 subcomplex accumulating in these cells is in agreement with the very rapid association of PS1 (and PEN-2) with NCT-APH-1 and fast transition to mature complexes.

Furthermore, we demonstrate that Rer1p knockdown in PS1 and -2 KO MEFs results in substantially lower levels of the NCT-APH-1 subcomplexes. Apparently, the combined absence of active recycling by hRer1p and the stabilizing PS1-PEN-2 interactions cause rapid degradation of both NCT and APH-1 under those conditions. Although we have no proof, we find it probable that NCT and APH-1 in these cells are degraded, not unlike what is observed in yeast deficient for Rer1p (Δ *rer1*) and Ftr1p (Δ *ftr1*; Sato et al., 2004). Here, unassembled Fet3p,

is more rapidly transported to the vacuole for degradation.

We show that endogenous Rer1p resides at steady state in the IC/early cis-Golgi, indicating that Rer1p contributes in complex assembly during ER-Golgi recycling. This localization is, however, dynamic, as Rer1p readily exits the ER in COPII vesicles in a cell-free ER-budding assay (unpublished data). This is in agreement with its rapid recycling and function as a retrieval receptor in yeast and mammals (Boehm et al., 1994; Sato et al., 2001). Interestingly, in γ -secretase-deficient MEFs (PS1 and -2 or APH-1 KO), NCT still exits the ER (unpublished data), and the fact that it remains fully immature can only be explained by an active retrieval mechanism preventing it from passing through the Golgi. That Rer1p is involved herein is indicated by its effects on complex formation outlined in the Results section. Moreover, the observed slow maturation of NCT (Herreman et al., 2003) indicates that NCT retrieval is a repetitive process, increasing or even titrating its chance to bind the cognate APH-1. Also, PS1 has a long half-life and is concentrated over COPI-coated areas of the IC, indicating that it is actively retrieved (Reichards et al., 2003). Except for an ER-retention sequence in the C terminus (Kaether et al., 2004), no retrieval motifs are recognized in PS1, suggesting that PS1, like NCT, also depends on retrieval receptors. If so, Rer1p is most likely not involved here because we did not coimmunoprecipitate PS1 (and also PEN-2) with Rer1p antibodies. A potential candidate is TMP21, a member of the p24 family of retrieval receptors that was found to interact with PS1 (Chen et al., 2006). Surprisingly, the functional relevance of this interaction was apparently not related to the established role of TMP21 in protein transport in the early secretory pathway.

Nevertheless, at this stage, our study unequivocally demonstrates that γ -secretase complex assembly extends from ER to IC and cis-Golgi compartments and is at least under the active control of a Rer1p-dependent recycling mechanism. Our findings may, therefore, explain the existing controversy on the subcellular location of γ -secretase assembly ranging from the ER (Capell et al., 2005) to Golgi/trans-Golgi network compartments (Baulac et al., 2003).

Although the yeast homologue Rer1p has been known for quite some time (Nishikawa and Nakano, 1993; Boehm et al., 1994; Sato et al., 1996, 1997, 2003), NCT is the first mammalian membrane protein identified that utilizes this Rer1p-dependent recycling. Irrespective of the topology, Rer1p binds to similar structural motifs within the TMD of identified targets (Sato et al., 1996, 2003, 2004), except for Mns1p (Massaad and Herscovics, 2001). A surprising observation, however, is that depending on the ligand the Rer1p retrieval mechanism serves different purposes. Rer1p functions in the retrieval of escaped ER-resident proteins, such as Sec12p, Sec71p, and Sec63p or can recycle components of the vesicle fusion machinery, like Sed4p. Strikingly, Rer1p also interacts with proteins that are not yet assembled in their corresponding multisubunit complexes, for example, Fet3p (Sato et al., 2004) and NCT (this study).

Fet3p, a subunit of the yeast iron transporter, is only retrieved by Rer1p as long as it is not assembled with its cognate subunit Ftr1p (Sato et al., 2004). These authors proposed that

interaction with Ftr1p conceals Rer1p from binding to Fet3p, thereby triggering escape from the retrieval mechanism. This mechanism constitutes a secondary ER quality-control system (Ellgaard and Helenius, 2003) that couples the assembly stage of multimeric protein complexes to forward transport. Control at the stage of complex assembly has also been demonstrated for other multisubunit complexes, including major histocompatibility complex class II, K_{ATP} channels, and cystic fibrosis transmembrane conductance regulator (Heusser and Schwappach, 2005). In addition to detecting the TMDs of unassembled subunits, a common alternative mechanism is based on masking of Arg-based ER-sorting motifs. However, these motifs are localized in cytosolically exposed regions of individual subunits (Michelsen et al., 2005). In any case, the quality-control systems ensure that only correctly assembled complexes could leave their place of synthesis and reach the compartments where their action is required. Although the Rer1p-dependent retrieval of NCT is most reminiscent of that of unassembled Fet3p, it appears to be more complicated. Indeed, and aside from the Rer1p-dependent retrieval, we show that NCT trafficking is counterbalanced by an Rer1p-independent ER retention-based mechanism mediated through its ecto- or intracellular domain (Fig. 6). Therefore, NCT recycling in early compartments is subject to at least a dual ER quality-control system that serves to control the residence time for NCT in early compartments. This time window defines the chance to interact with properly folded APH-1 and, thus, the amount of initial NCT-APH-1 subcomplex formation. Excitingly, our findings clearly demonstrate that these secondary ER quality controls provide the cell with a mechanism to tightly control the quantitative levels of the γ -secretase complex and, hence, activity in distal compartments, such as the cell surface and endosomes (Kaether et al., 2006; Rajendran et al., 2006).

In conclusion, our data establish Rer1p as a limiting factor and transport regulator in initial complex assembly through binding to NCT. Moreover, we demonstrate the feasibility to interfere with complex assembly and activity by altering Rer1p expression levels. Controlling $A\beta$ production via modulation of γ -secretase is an important therapeutic strategy in Alzheimer's disease. Herein, PS1 has caught most of the focus partly because it harbors the catalytic site of the complex. Additionally, the fact that proteolysis and binding/docking occurs in spatially distinct domains has increased at least the chance to develop specific inhibitors (Annaert et al., 2001). Our findings, together with the role of NCT as a substrate receptor (Shah et al., 2005), definitely augment the critical role of NCT in the complex. The identification of the binding motif for Rer1p in NCT and the competition with APH-1 may open opportunities for drug development yet to be explored.

Materials and methods

Antibodies and cell lines

Rabbit pAb against human PS1-NTF (B14.5), mouse PS1-NTF and -CTF (B19.2 and B32.1, respectively; Annaert et al., 1999), NCT (pAb B59.4; Herremans et al., 2003), APH-1a₁ (B80.2; Nyabi et al., 2003), PEN-2 (B95.1), APP (B63.3; Esselens et al., 2004), $A\beta$ (B104.1; Spasic et al., 2006), TLN (B36.1 and biotinylated B36.1; Annaert et al., 2001), and

mAb 9C3 against NCT (Esselens et al., 2004) have been characterized before. pAbs SB129 (anti-PS1), anti-KDELr (erd2), and -BAP31 were provided by C. Van Broeckhoven (University of Antwerp, Antwerp, Belgium), H.-D. Schmitt (Max Planck Institute for Biophysical Chemistry, Göttingen, Germany), and M. Reith (Max Planck Institute for Immunology, Freiburg, Germany). mAbs 5.2 against PS1 and Ergic-53 were obtained from B. Cordell (Scios, Fremont, California) and H.-P. Hauri (University of Basel, Basel, Switzerland), respectively. Polyclonal anti-murine Rer1p was generated in New Zealand white rabbits using the C-terminal sequence CKRRYK-GKEDVGKTFAS coupled to KLH (Pierce Chemical Co.) as the antigen (PickCell Laboratories). Additional antibodies used follow: mAb against NCT ectodomain (amino acids 168–289; BD Biosciences), the transferrin receptor (Tfr; cl H68.4; Zymed Laboratories), GM130 (BD Biosciences), and actin (cl AC-15; Sigma-Aldrich). Studies were performed in HeLa cells, WT MEFs, and MEFs deficient for PS (PS1 and -2 KO; Nyabi et al., 2003), APH-1a, APH-1a,b,c (Serneels et al., 2005), or NCT (a gift from P. Wong, Johns Hopkins University, Baltimore, MD; Li et al., 2003). All cell lines were routinely grown and maintained in DME/F12 supplemented with 10% FCS.

Confocal laser-scanning microscopy and immunofluorescence

After HeLa cells were incubated at 37 or 15°C for 3 h, they were fixed in 4% paraformaldehyde and processed for double immunofluorescent labeling as described before using Alexa 488 and 568 conjugated secondary antibodies (Invitrogen) and TOPRO-3 to label nuclei (Esselens et al., 2004). Images were captured on a confocal microscope (Radiance 2100; Carl Zeiss MicroImaging, Inc.) connected to an upright microscope (Eclipse E800; Nikon) and using an oil-immersion plan Apo 60 \times A/1.40 NA objective lens. Final processing was done using Lasersharp 2000 (Carl Zeiss MicroImaging, Inc.) and Photoshop (Adobe) and restricted to limited linear color balance adjustments to interpret merged pictures.

Cloning

To obtain NCT without intracellular domain (NCT Δ IC) and NCT without both intracellular and transmembrane domains (NCT Δ [TMD + IC]), FL mouse NCT, subcloned in pGEM-T (Promega), was used as a template in PCR reactions with the same sense primer (including a Sall restriction site) and two different antisense primers, including a stop codon, a flag-tag sequence, and NcoI restriction site. In NCT Δ IC, 16 C-terminal amino acids are missing, and in NCT Δ (TMD + IC), an additional 24 amino acids are omitted. PCR products were cloned first in pGEM-T to generate Sall ends and then further into the XhoI restriction site of pSG5** (pSG5 with extended multiple cloning site). The NCT construct lacking the ectodomain, except the signal peptide (NCT Δ EC), was generated using gene splicing by overlap extension reaction PCR (SOE-PCR; Horton et al., 1993). Restriction enzymes Sall and NcoI were used for subcloning the final PCR product from SOE reaction into pMSCV (CLONTECH Laboratories, Inc.); using BglII and BamHI restriction enzymes, the NCT Δ EC construct was further subcloned from pMSCV into the BamHI restriction site of pSG5**. NCT Δ EC contains the last 43 amino acids of NCT. The NCT Δ (long TMD + IC) construct was made by introducing a stop codon at position 656 of mouse NCT (subcloned in pSG5**) using site-directed mutagenesis (Stratagene).

cDNAs encoding mouse NCT and TLN, subcloned in pSG5**, were used for introducing restriction sites MluI and AgeI by site-directed mutagenesis at codon positions 660 and 690 of NCT and at codon positions 828 and 858 of TLN, respectively. These codon positions correspond to the flanking residues of the NCT and TLN TMDs. The introduced restriction sites were used to swap the coding sequence of the TMDs of the two proteins (NCT/TMD_{TLN} and TLN/TMD_{NCT}).

Amino acids T670, G674, S681, T685, and Y686 were mutated to L by site-directed mutagenesis using NCT subcloned in pSG5** as a template. Human *Rer1* is subcloned in pCB6 using HindIII and XbaI. All constructs were transiently overexpressed in HeLa cells for interaction studies. The cDNAs encoding TLN, NCT, NCT/TMD_{TLN}, and TLN/TMD_{NCT} were cloned in the retroviral vector pMSCV and packaged in retroviruses, which were used to transduce MEF NCT^{-/-} cells, all according to established procedures of virus production and puromycin selection (Spasic et al., 2006).

Coimmunoprecipitation

For coimmunoprecipitation experiments, cells were harvested in PBS, centrifuged (800 g for 10 min), and lysed in 125 mM NaCl, 50 mM Hepes, pH 7.4 (supplemented with 1% Triton X-100 or CHAPS and Complete protease inhibitors [Roche]) for 30 min at 4°C. After centrifugation (16,000 g for 15 min), cleared cell extracts were incubated overnight at 4°C with protein A beads and specific antibodies (anti-NCT mAb 9C3 or affinity-purified

pAb B59.4) or with anti-rabbit IgG beads (Rabbit IgG TrueBlot set) and anti-hRer1 pAb. In the case of biotinylated B36.1 pAb, streptavidin-Sepharose beads (GE Healthcare) were used (Esselens et al., 2004). Immunoprecipitates were solubilized in NuPAGE sample buffer (Invitrogen) under reducing (for anti-hRer1 and biotinylated B36.1) or nonreducing conditions (for anti-NCT), electrophoresed on 4–12% NuPAGE Bis-Tris gels in MES running buffer (Invitrogen) and processed for Western blotting and immunodetection using ECL (PerkinElmer). When mature glycosylation was studied, fractions were treated with endoH (10 mU for 18 h at 37°C) as described previously (Spasic et al., 2006) before SDS-PAGE.

Protein cross-linking

Cells were harvested in PBS, centrifuged (800 g for 10 min), and homogenized in 250 mM sucrose, 10 mM Hepes, and 1 mM EDTA, pH 7.4, supplemented with protease inhibitors using a ball-bearing cell cracker (10 passages; clearance 10 μ m; Isototec). After low-speed centrifugation (400 g for 10 min), the postnuclear supernatant was ultracentrifuged (100,000 g for 1 h). Microsomal membranes resuspended in 125 mM NaCl and 50 mM Hepes, pH 7.4, were used for reaction with DTBP (Pierce Chemical Co.) at room temperature for 30 min. DTBP is thiol-cleavable, water-soluble cross-linker. The reaction was quenched with 50 mM Tris, pH 7.5, at room temperature for 15 min. Proteins were extracted by adding Triton X-100 to a final concentration of 1%, followed by a 30-min incubation at 4°C. After centrifugation (16,000 g for 15 min), cleared cell extracts were used for coimmunoprecipitation as indicated in the previous paragraph. To cleave DTBP, immunoprecipitates were solubilized in NuPAGE sample buffer with 4% β -mercaptoethanol and incubated at 100°C for 10 min before electrophoresis.

RNAi

For RNAi, the target sequence 5'-AATATCAGTCCTGGCTAGACA-3' was used in human *Rer1* cDNA and 5'-CCTGGTGATGACTTCATCATGCTT-3' in mouse *Rer1* cDNA. The GL2 luciferase RNAi duplex was used as a non-specific control. Oligonucleotides (human *Rer1* and Stealth [Dharmacon Research, Inc.] and mouse *Rer1* [Invitrogen]) were annealed and transfected using Lipofectamine (MEF) or Oligofectamine (Invitrogen; HeLa) as described previously. The cells were analyzed 48 or 72 h after transfection or further processed for metabolic labeling. In this case, control and RNAi down-regulated HeLa cells were pulse labeled with [³⁵S]methionine/cystein (Translabel; MP Biomedicals) for 30 min, washed, and chased in DME supplemented with 1% FCS for 0, 3, and 6 h. Cleared 1% Triton X-100 extracts were subjected to immunoprecipitation with 9C3 mAb (anti-NCT). Bound fractions were denatured (10 min at 70°C) and incubated with endoH (1 U/50 μ l for 18 h at 37°C). Labeled proteins were separated by SDS-PAGE on 7% Tris-Acetate gels (Invitrogen) and quantified using phosphorimaging (Typhoon; Molecular Dynamics) and ImageQuant software.

BN-PAGE

Microsomal membranes were resuspended in buffer containing 0.5% dodecylmaltoide, 20% glycerol, 25 mM Bis-Tris/HCl, pH 7.0, and protease inhibitors and solubilized for 1 h on ice. After ultracentrifugation (100,000 g at 4°C for 30 and 15 min), the cleared supernatant was collected. For each sample, the same amount was mixed with 5 \times BN sample buffer (2.5% Coomassie brilliant blue G-250, 50 mM BisTris-HCl, 250 mM δ -amino-caproic acid, pH 7.0, and 15% sucrose) and stored overnight at 4°C. BN-PAGE was performed as described previously (Herreman et al., 2003) with some modifications. Samples were loaded on a 5–16% polyacrylamide gel and electrophoresed (100 V for 30 min followed by 1 h at 200 V). For the final run (1 h 50 min), the Coomassie brilliant blue G-250 was omitted from the cathode buffer.

Cell surface biotinylation

For cell surface biotinylation, 0.5 mg/ml NHS-SS-biotin (Pierce Chemical Co.) was used as described previously (Spasic et al., 2006), and biotinylated proteins were isolated overnight (4°C) using streptavidin-Sepharose beads (GE Healthcare). Bound material was processed for Western blotting as described.

γ -Secretase activity assays

Subconfluent (70–80%) HeLa cells were cotransfected with pcDNA3.1-APP-C99 and either a control empty pSG5 vector, pCB6-hRer1 using GeneJuce (VWR), or RNAi duplex oligonucleotides for hRer1p (see RNAi section) using Lipofectamine 2000 according to the manufacturer's instructions. After 24 h of hRer1p overexpression and 48 h of hRer1p down-regulation, cells

were metabolically labeled (100 μ Ci 35S-Translabel/dish in Met/Cys-free DME) for 3–4 h in a CO₂ incubator. Next, conditioned media were collected while cell layers were scraped and extracted in TBS containing 1% Triton X-100. Both cleared media and cell extracts were subjected to immunoprecipitation using 20 μ l protein G-Sepharose beads, including anti-APP pAb B104.1 (Spasic et al., 2006) or anti-APP B63.3 (1:200), respectively, and bound material was separated on 10% NuPAGE gels in MES buffer (Invitrogen), dried, and analyzed by phosphorimaging (Typhoon) and ImageQuant software (Molecular Dynamics).

For the cell-free γ -secretase assay, CHAPS-extracted microsomal fractions derived from HeLa cells either overexpressing hRer1p or with down-regulated hRer1 levels (see RNAi section) were mixed with recombinant APP-C99-FLAG affinity purified from transiently transfected APH-1 α , β , γ MEFs exactly as described previously (Kakuda et al., 2006). After incubation, newly produced AICD was separated on 10% NuPAGE gels in MES buffer and processed for Western blotting and ECL.

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