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PCSK9 IS REQUIRED FOR THE DISPOSAL OF NON-ACETYLATED INTERMEDIATES OF THE NASCENT MEMBRANE PROTEIN BACE1

SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Catalog numbers of the antibodies employed in this study

Anti-acetylated lysine (monoclonal; Abcam ab409); anti-BACE1 (polyclonal; Abcam ab2077); anti-calreticulin (polyclonal; Abcam ab12227); anti-ERGIC 53 (polyclonal; Sigma E1031); anti-syntaxin (monoclonal; Abcam ab12370); anti-EEA1 (monoclonal; BD Transduction Laboratories 610456); anti-c-myc (polyclonal; Sigma C3956); anti-actin (polyclonal; Cell Signaling 4967); anti-PDI (Protein Disulfide Isomerase-ER marker; monoclonal; Abcam 2792); anti-FLAG (monoclonal; Sigma F1802); anti-PCSK9 (polyclonal; Cayman 10008811 and 10007185); anti-LDLr (generous gift from Dr. Alan Attie; University of Wisconsin-Madison); anti-NGF (polyclonal; Chemicon AB5743); anti-flotillin 2 (polyclonal; Cell Signaling 3244); anti-C99 (monoclonal; MBL M066-3); anti-APP C-terminal (polyclonal; Chemicon 5352); anti-BSA (monoclonal; Abcam ab3781).

Cell cultures and Animals

CHO (Chinese Hamster Ovary) and H4 (human neuroglioma) cells were grown in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (Mediatech, Inc.) as described before (Costantini *et al*, 2007; Ko and Puglielli, 2007; Puglielli *et al*, 2003; Puglielli *et al*, 2001). Cells stably transfected with PCSK9, PCSK9-H226A, or LDLr were grown in media supplemented with G418 sulfate (Mediatech). BACE1 expressing cells have been described before (Costantini *et al*, 2007). Cells were maintained in a humidified atmosphere with 5% CO_2. All transfections were performed with Lipofectamine2000^{TM} (Invitrogen) as suggested by the manufacturer.

Non-transgenic and homozygous *Pcsk9*$^{-/-}$ mice (B6;129S6-*Pcsk9*^{tm1Jdh}/J; stock n. 005993) were obtained from The Jackson Laboratory. Animals (males) were 2.5-month-old when sacrificed. The brains were immediately removed for the isolation of neocortex and hippocampus. Tissue was immediately processed for protein extraction in GTIP buffer (100 mM Tris pH 7.6, 20 mM EDTA, 1.5 M NaCl) supplemented with 1% Triton X-100 (Roche), 0.25% NP40 (Roche), complete protease

inhibitors cocktail (Roche), as described before (Costantini *et al*, 2007; Costantini *et al*, 2006; Costantini *et al*, 2005). Animal experiments were carried out in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

Subcellular fractionation

Subcellular fractionation of cultured cells was performed using a protocol that has been previously described (Costantini *et al*, 2007; Costantini *et al*, 2006; Ko and Puglielli, 2007; Puglielli *et al*, 2001). Briefly, confluent cells were washed twice each with ice-cold phosphate-buffered saline and homogenization buffer (10 mM triethanolamine, 10 mM acetic acid, 250 mM sucrose, 1 mM EDTA, and 1 mM dithiothreitol; pH 7.4) plus a protease inhibitor cocktail (Roche) and homogenized using a 25-gauge needle and a tight-pestle Dounce homogenizer. A post-nuclear supernatant resulting from low speed centrifugation (1000 g for 15 min at 4^{o}C) was separated by differential centrifugation at 14000 × *g*, and 100000 × *g* to yield membrane fractions P2 and P3, respectively. Pooled membrane fractions were layered on top of a step gradient consisting of 24, 19.33, 14.66, and 10% isotonic Nycodenz solutions (made in 0.75% NaCl, 10 mM Tris-pH 7.4, 3 mM KCl, and 1 mM EDTA) and centrifuged at 100000 × *g* in a SW41 rotor for ~18 h at 4^{o}C. Fractions were collected from the top to the bottom of the gradient and concentrated by centrifugation at 45,000 for 30 min. or at 25 p.s.i. (100000 x *g*) for 15 min in an air-driven ultracentrifuge (Airfuge; Beckman). An average of 50 confluent 150 mm Petri dishes was processed for each gradient.

Anti-myc and anti-FLAG affinity purification, *in vitro* interaction, and classical immunoprecipitation

BACE1-myc and LDLr-myc were purified from total cell extracts of stably-transfected cells by using anti-myc antibodies covalently attached to aldehyde-activated agarose beads (ProFound c-Myc-Tag IP/Co-IP Kit; Pierce), as suggested by the manufacturer and already described in our previous work (Costantini *et al*, 2007). Secreted (C-form) PCSK9-FLAG was purified from the conditioned media whereas full-length PCSK9-H226A-FLAG was purified from total cell extracts of stably-transfected cells. In both cases we used anti-FLAG antibodies covalently attached to immobilized agarose beads (EZview^{TM} red anti-FLAG$^{®}$ M2 affinity beads; Sigma). For the *in vitro* interaction of immobilized BACE1 and PCSK9, 10 mM $CaCl_2$ was added to the input material before loading on to the column.

For classical immunoprecipitation of LDLr, cell extracts were pre-cleared with BioMag Protein A (Polysciences); beads were used at a concentration of 15 µl of the slurry per sample and separated with a magnetic holder. LDLr was then immunoprecipitated with anti-LDLr antibodies and recovered with BioMag Protein A, as described (Costantini *et al*, 2007). The immunoprecipitate-bead complexes were washed three times and boiled in sample buffer. Beads were then separated and the samples were analyzed by reducing SDS/PAGE (on a 4–12% Bis/Tris NuPAGE system) and Western blotting.

Aβ determination

Aβ in brain homogenates was determined by standard sandwich ELISA as described before (Costantini *et al*, 2006; Costantini *et al*, 2005; Puglielli *et al*, 2003). We used antibodies 9131 (for Aβ 1-40) and 9134 (for Aβ 1-42) as capture antibodies, and 9154 and 4G8 as biotinylated reporter antibodies. The above antibodies were all from Signet Laboratories/Covance. For each sample, the levels of $A\beta_{40}$, $A\beta_{42}$, and $A\beta_{total}$ were quantified in triplicate based upon standard curves run (on every ELISA plate) and then expressed as pmol Aβ/mg of protein. The values reported here are in the range of previously found values for endogenous murine Aβ using a similar approach (Bowen *et al*, 2004; Costantini *et al*, 2006; Costantini *et al*, 2005). $A\beta_{42}$ was constantly found to be ~20 to 30% of total Aβ values.

References to Supplementary Methods

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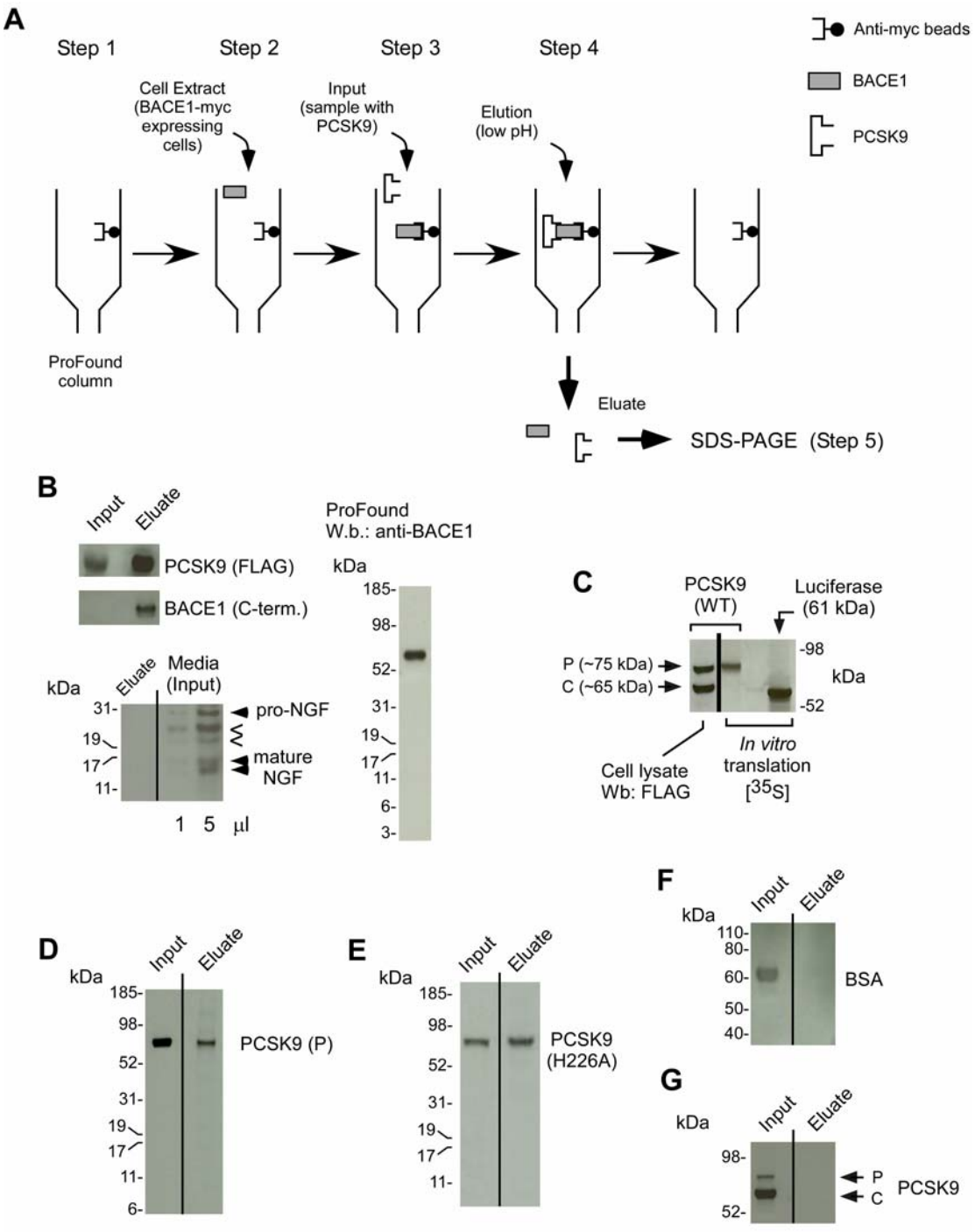
SUPPLEMENTARY FIGURES



Supplementary Fig S1. PCSK9 can interact with BACE1 *in vitro*. (**A**) Schematic description of the experimental approach used. We first assembled an affinity column with anti-myc antibodies covalently attached to aldehyde-activated agarose beads (ProFound column; Step 1). The column was then used to immobilize BACE1-myc (Costantini *et al*, 2007) from cell extracts of stably-expressing cells (Step 2).

After extensive washing to remove unbound material, the column received different samples (see below) as source of the different forms of PCSK9 (Step 3) and underwent several more rounds of washing prior to elution (Step 4). Finally, the eluate was analyzed by SDS-PAGE and immunoblotting (Step 5).

(**B**) Western blot analysis of the eluate resolved after the experiment described in (A). Top panel. Input: conditioned media from PCSK9-expressing cells showing the presence of PCSK9 (the C-form) in the material loaded onto the column. Eluate: eluate generated after Step 4 showing the presence of both BACE1 and PCSK9. The migration of BACE1 from a typical eluate of the ProFound column in the absence of PCSK9 is shown for comparison in the right panel. The migration of secreted PCSK9 in the conditioned media of stably-expressing cells is shown in Fig 3A (*left panel*) of the main text. Bottom panel. Western blot showing that NGF and pro-NGF, which are also present in the conditioned media loaded onto the BACE1-immobilized column, could not be detected in the eluate. Both mature NGF and pro-NGF are indicated (arrow-heads). Symbols (<) indicate commonly observed NGF high-molecular species (Mouri *et al*, 2007).

(**C**) SDS-PAGE and autoradiography showing the migration of PCSK9 after *in vitro* translation. *In vitro* translated PCSK9 migrates as the full-length, uncleaved form of PCSK9. The left lane shows a Western blot of total cell lysates from PCSK9-expressing cells. The migration of *in vitro* translated luciferase (~61-kDa), which migrates similarly to the C-form of PCSK9 (~65-kDa), is shown for comparison.

(**D**) The experiment described in (**A**) was repeated by loading the *in vitro* translated product shown in (**C**) onto the BACE1 immobilized column. Input: autoradiography showing the presence of *in vitro* translated PCSK9 in the material loaded onto the column. Eluate: autoradiography showing the presence of *in vitro* translated PCSK9 (P-form) in the eluate.

(**E**) The experiment shown in (**D**) was repeated with the H226A mutant form of PCSK9.

(**F**) The experiment shown in (**D** and **E**) was repeated with pure bovine serum albumin (BSA; negative control) followed by Western blotting. For BSA Western blot, membrane was blocked with SeaBlock (Invitrogen) and detected with anti-BSA mouse antibody (Abcam).

(**G**) The experiment shown in (**D** and **E**) was repeated in the absence of BACE1 (negative control). In this case, a cell extract from PCSK9 stably-transfected cells were loaded onto a ProFound column that did not contain BACE1. Input: Western blot showing the presence of both forms of PCSK9 in the cell extract that was loaded onto the column. Eluate: Western blot showing that neither the P-form nor the C-form of PCSK9 could be found in the eluate when BACE1 was omitted as "bait" (please, see (**A**)).

Comment to Fig S1.

The experiments described in Fig S1 were performed to assess whether the different forms of PCSK9 employed in this study (P-form, C-form, and H226A mutant) were able to bind to BACE1 *in vitro*. We decided to employ an *in vitro* system because the interaction of a protease with its substrate is difficult to detect *in vivo* under endogenous conditions. Therefore, we generated an affinity column that employed immobilized BACE1 as "bait" for the pull-down of PCSK9 (schematically described in Fig S1A).

Fig S1B shows that PCSK9 (C-form) was successfully purified from the conditioned media indicating functional interaction with immobilized BACE1. Most importantly, this interaction was highly specific because unrelated proteins that were abundantly present in the conditioned media did not bind to the column (Fig S1B; *lower panel*). Next, we decided to assess whether the full-length and uncleaved P-form of PCSK9 could also bind to BACE1. We first generated an *in vitro* translated form of PCSK9, which migrated as the full-length form of the protease (Fig S1C). Interestingly, we never observed the cleaved form of PCSK9 *in vitro* (data not shown), even after long incubation at 30^{o}C or after translation in the presence of isolated microsomes (Fig S2), suggesting that the autocatalytic processing of PCSK9 *in vivo* has additional requirements. However, when *in vitro* translated full-length PCSK9 (P-form) was loaded onto the BACE1 immobilized column, we successfully purified PCSK9, indicating functional interaction with BACE1 (Fig S1D). Similar results were obtained with the H226A mutant (Fig S1E). More importantly, purified bovine serum albumin (BSA) did not bind to immobilized BACE1 (Fig S1F) indicating that the PCSK9-BACE1 *in vitro* interaction is specific. Finally, PCSK9 could not bind to the anti-myc column in the absence of BACE1 (Fig S1G). When taken together, the above results indicate that PCSK9 can interact with BACE1 *in vitro*. This is consistent with previous work showing that pro-PCSK9 can interact with the immature form of LDLr in the ER (Nassoury *et al*, 2007).

References to Fig S1

Costantini C, Ko MH, Jonas MC, Puglielli L (2007) A reversible form of lysine acetylation in the ER and Golgi lumen controls the molecular stabilization of BACE1. *Biochem J* **407**: 383-395.

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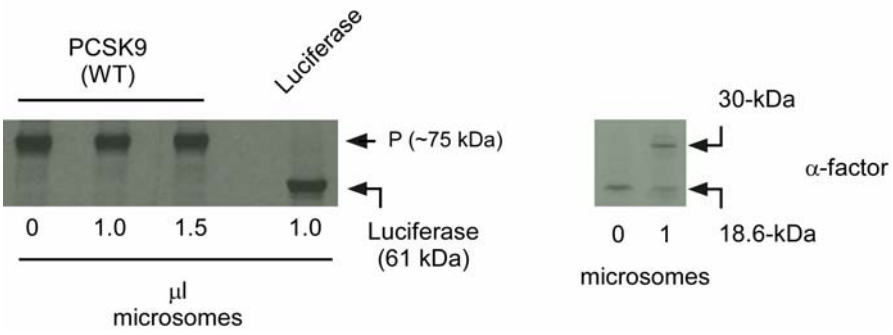

Supplementary Fig S2. *In vitro* translated PCSK9 is not processed to the cleaved form. The *in vitro* translation of PCSK9 was performed as described in the Supplementary Methods section and shown in Fig S1C with the only difference that canine pancreatic microsomes (Promega; cat. #Y4041) were added to the reaction mixture to ensure proper folding *in vitro*. *In vitro* translated PCSK9 was then analyzed by SDS-PAGE and autoradiography as in Fig S1C. *In vitro* translated PCSK9 migrates as the full-length, uncleaved form of PCSK9. The migration of *in vitro* translated luciferase, which migrates similarly to the C-form of PCSK9, is shown for comparison. The right panel shows successful glycosylation of *in vitro* translated *S. cerevisiae* α-mating factor in the presence of microsomes (internal positive control provided by the manufacturer). The 18.6-kDa band corresponds to the immature (non-glycosylated) form whereas the 30-kDa band corresponds to the glycosylated form of the protein. The immature (non-glycosylated) form is visible both in the absence and presence of microsomes whereas the glycosylated form is only visible in the presence of microsomes.

The inability of *in vitro* translated PCSK9 to undergo processing to the C-form -even when microsomes were added to the reaction mixture- supports the conclusion that the autocatalytic processing of PCSK9 *in vivo* has additional requirements. This conclusion is consistent with recent work performed with the Familial Hypercholesterolemia-associated S127R mutant form of PCSK9, which suggests that the interaction of PCSK9 (P-form) with accessory proteins is required for processing to the C-form (Pandit *et al*, 2008)

References to Fig S2

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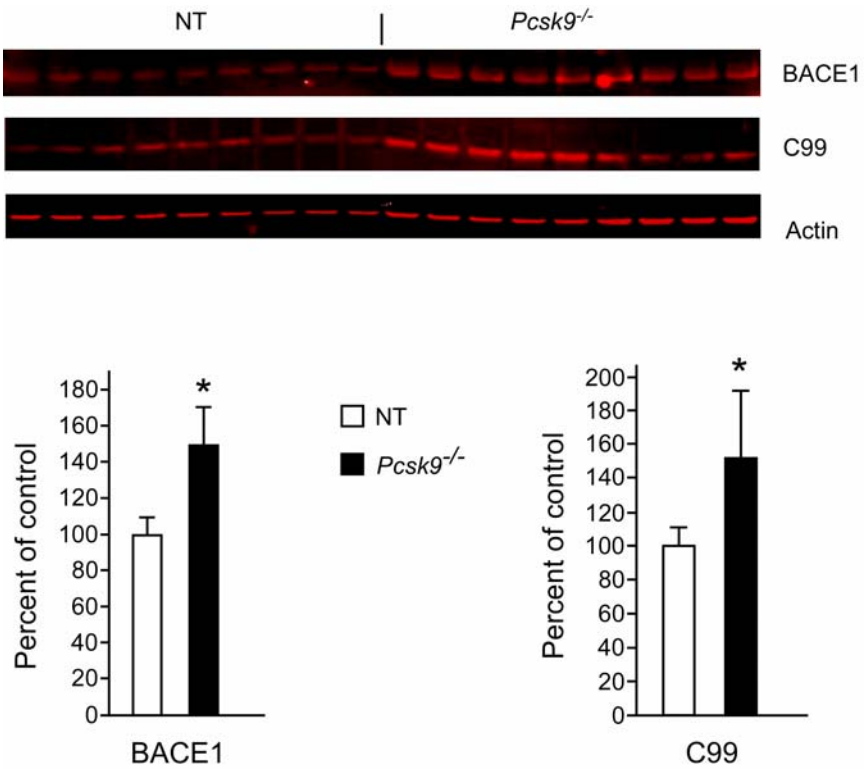

Supplementary Fig S3. *Pcsk9*$^{-/-}$ mice have increased levels of BACE1 and C99 in the neocortex. Western blot images (*upper panel*) were generated with the LiCor Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE). Images were obtained by near-infrared fluorescence detection of IRDye-labeled (Alexa-680) antibodies. A representative Western blot generated by classical chemiluminescent detection is shown in Fig 2E and 2F. Appropriate quantification of images is shown in the *lower panel*. Results are expressed as percent of non-transgenic (NT) mice and represent the average $\pm$ S.D. C99 represents the immediate product of BACE1-mediated cleavage of APP. *, $P<0.05$