Supplemental Data

EXPERIMENTAL PROCEDURES

Pulse-chase analysis- Stable PCSK9-expressing HepG2 cells were plated on day 0 at 5.0 x 10^5 cells in 60-mm dishes and cultured in Medium B. On day 2, cells were refed with 3 mL medium E. On day 3, cells were preincubated for 60 min in L-methionine- and L-cysteine-free Medium E, prior to pulse-labeling for 20 min with 200 μCi/ml EasyTag Express [^{35}S] Protein Labeling Mix (Perkin Elmer) in the same medium. Cells were then washed twice with PBS and incubated for various times in 1.5 ml chase medium consisting of Medium E containing 10 mM and 3 mM amounts of unlabeled L-methionine and L-cysteine, respectively. Immunoprecipitation of cell and medium extracts was performed using anti-FLAG M2 mAb (Sigma) as described (1). For analysis of LDLR processing, HuH7 cells were seeded on day 0 at 2.0 x 10^5 cells in 60 mm dishes and cultured in Medium D. On day 2, cells were transfected with empty vector (pCMV5), LDLR-WT (pLDLR-17), or LDLR(H306Y) expression plasmids (1 μg) using Lipofectamine 2000 transfection reagent (Invitrogen) as per manufacturer's instructions. On day 3, cells were refed with sterol-supplemented Medium F. On day 4, pulse-chase analysis was performed as described above except Medium E was replaced with Medium F and cell extracts were immunoprecipitated using mouse anti-LDLR mAb (IgG-C7) (2).

^{125}I-Uptake- Purified PCSK9 and EGF-AB proteins were radioiodinated as previously described (3) with the exception that the labeling reaction was carried out using IODO-GEN pre-coated iodination tubes (Pierce). HepG2 cells were incubated for 18 h in Medium E with human lipoprotein-poor serum (HLPPS) used in place of NCLPDS. Cells were then pulse-labeled for 1 h in DMEM with 5% HLPPS and 300,000 cpm of radiolabeled protein (100-150 cpm/mg protein). ^{125}I-labeled protein incubation was performed in the absence or presence of a 50-fold excess of an unlabeled competitor protein to determine non-specific uptake, which was typically <30% of total counts. Cells were washed 2x with DMEM with 5% HLPPS and 0.5% BSA and then incubated for 2 h in DMEM with 5% HLPPS. Medium was harvested and the radioactivity of the trichloroacetic acid-soluble fraction measured following removal of free iodide as described (4). Cell monolayers were harvested in 0.5 N NaOH and radioactivity was measured directly.

REFERENCES

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2. Beisiegel, U., Kita, T., Anderson, R. G., Schneider, W. J., Brown, M. S., and Goldstein, J. L. (1981) *J. Biol. Chem.* **256**(8), 4071-4078
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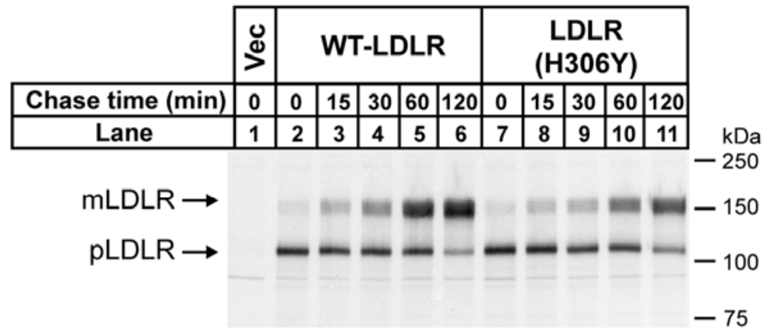
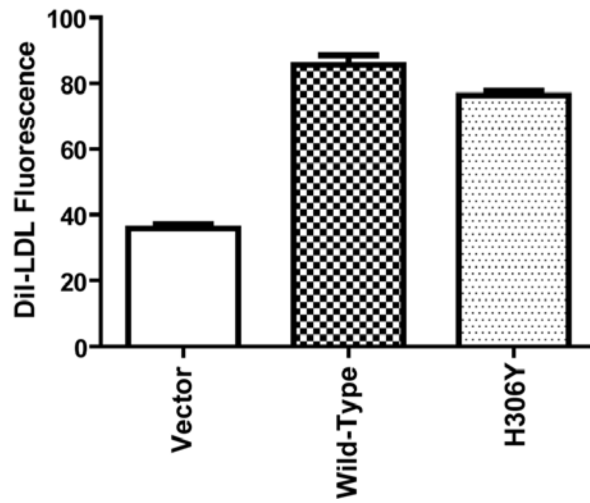
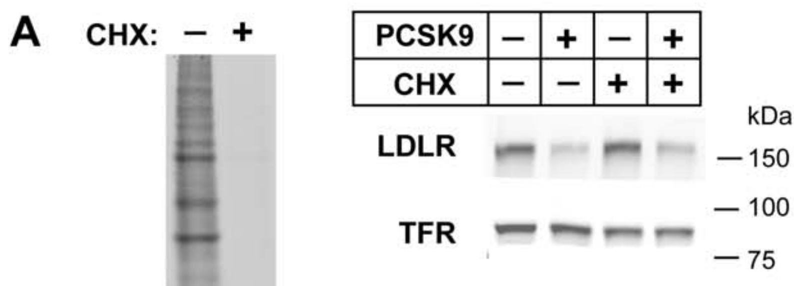
4. Goldstein, J. L., Basu, S. K., and Brown, M. S. (1983) *Meth. Enzymol.* **98**, 241-260

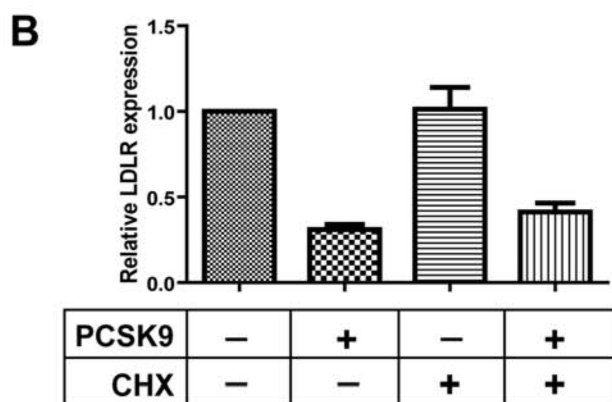
Supplemental Figure S1

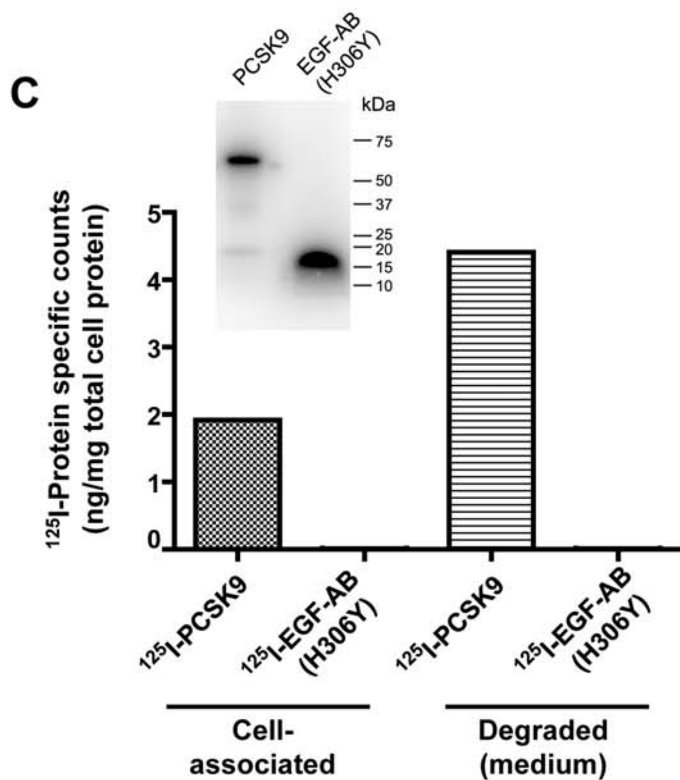




Supplemental Figure S2







Supplemental Figure S3

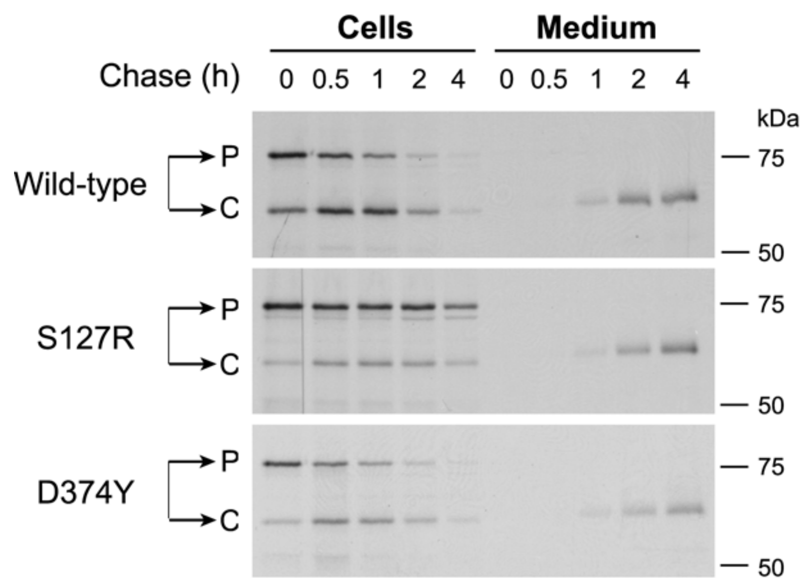


FIGURE LEGENDS

Supplemental Fig. S1. LDLR(H306Y) is not defective in processing or LDL uptake. HuH7 cells were transiently transfected with empty vector, LDLR-WT or LDLR(H306Y) expression constructs and cultured in sterol-supplemented medium (see *Experimental Procedures*) for 18 h prior to start of experiments. *A*. Cells were pulse-labeled for 20 minutes with [^{35}S]methionine/cysteine. After washing, cells were incubated in chase medium for the indicated times and LDLR was immunoprecipitated from cell lysates using an anti-LDLR mAb (IgG-C7). Samples were subjected to 8% SDS-PAGE and the gel was dried, exposed to a PhosphorImager plate and scanned to detect precursor (p) and mature (m) forms of LDLR. *B*. Cells were incubated 2 h with 100 µg/mL DiI-LDL. After incubation, cells were trypsinized and the mean fluorescence intensity of 10,000 cells for each sample was measured by flow cytometry as described in *Experimental Procedures.* Three replicate samples were used per condition.

Supplemental Fig. S2. HepG2 cells were cultured in sterol-depleting medium (see *Experimental Procedures*) for 18 h prior to start of experiments. *A & B*. Effect of exogenous PCSK9 on mature LDLR levels. Cells were preincubated for 2 h with 5 µg/ml cycloheximide (CHX) or vehicle control (DMSO; 0.05% v/v). To confirm CHX efficiency, a subset of dishes were labeled for 1 h with [^{35}S]methionine/cysteine. Cell lysates were subjected to 8% SDS-PAGE and the gel was dried and exposed to film. Other dishes were incubated for a further 4 h with 5 µg/ml purified PCSK9 in the presence (+) or absence (-) of CHX. Cells were lysed and subjected to SDS-PAGE and immunoblot analysis for LDLR and TFR, which were visualized and quantified using an IRDye800-labeled secondary antibody and the LI-COR Odyssey infrared imaging system. LDLR levels were normalized to TFR expression. Triplicate samples were used for each condition. Shown in *A* is a representative immunoblot. *C*. ^{125}I-labeled protein uptake/degradation in HepG2 cells. Cells were incubated for 1 h with equal counts of ^{125}I-labeled PCSK9 or EGF-AB(H306Y) proteins. Cells were washed and incubated for 2 h in fresh medium. Following extensive washing, cell-associated radioactivity was directly measured in cell extracts. The medium was collected and subjected to trichloroacetic acid precipitation. The acid-soluble medium fraction containing mono^{125}I-tyrosine was counted for radioactivity. *Inset* shows 4-15% SDS-PAGE of ^{125}I-labeled PCSK9 and EGF-AB(H306Y) proteins. The gel was dried, exposed to a PhosphorImager plate and scanned.

Supplemental Fig. S3. Pulse-chase analysis of PCSK9 overexpressing HepG2 cells. HepG2 cells stably transfected with FLAG-tagged versions of wild-type PCSK9, PCSK9(S127R), or PCSK9(D374Y) were labeled for 20 min with [^{35}S]methionine/cysteine. After washing, cells were incubated in chase medium for the indicated times. Cells were lysed and PCSK9 was immunoprecipitated from cell lysates and medium using anti-FLAG M2 mAb. Samples were subjected to 8% SDS-PAGE and the gel was dried, exposed to a PhosphorImager plate and scanned. P and C denote proprotein and cleaved forms of PCSK9, respectively.