THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 284, NO. 42, pp. 28995–29004, October 16, 2009

The Sterol-sensing Endoplasmic Reticulum (ER) Membrane Protein TRC8 Hampers ER to Golgi Transport of Sterol Regulatory Element-binding Protein-2 (SREBP-2)/SREBP Cleavage-activated Protein and Reduces SREBP-2 Cleavage*[S]

Received for publication, July 5, 2009, and in revised form, August 11, 2009 Published, JBC Papers in Press, August 25, 2009, DOI 10.1074/jbc.M109.041376

Masato Irisawa[‡], Jun Inoue[‡], Nozomi Ozawa[‡], Kazutoshi Mori[§], and Ryuichiro Sato[‡1]

From the [‡]Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo 113-8657 and the [§]Department of Biophysics, Graduate School of Science, Kyoto University, Kyoto 606-8502, Japan

TRC8 (translocation in renal cancer from chromosome 8) is an intrinsic protein of the endoplasmic reticulum that contains a sterol-sensing domain and a RING finger motif encoding an E3 ubiquitin ligase. Here we show that TRC8 overexpression hinders sterol regulatory element-binding protein-2 (SREBP-2) processing, thereby reducing SREBP-2 target gene expression, TRC8 depletion has the opposite effect. Mutation analyses of TRC8 reveal that the ubiquitin ligase activity is dispensable for these effects. Activating transcription factor 6 (ATF6) is also processed in the Golgi by the same two proteases as those for SREBP, but ATF6 processing is not affected by TRC8. TRC8 is capable of binding both SREBP-2 and SREBP cleavage-activated protein (SCAP), thereby forming a TRC8·SREBP-2·SCAP complex. This complex formation hampers the interaction between SCAP and Sec24, one of the COPII proteins that are involved in SREBP-2 transport to the Golgi, thereby reducing SREBP-2 cleavage. TRC8 conjugated by ubiquitin is unstable, whereas the mutant TRC8, lacking the E3 ubiquitin ligase activity and only slightly modified by ubiquitin, is quite stable. TRC8 becomes stable when cells are cultured with a proteasome inhibitor or under a lipoprotein-depleted condition. Lipoprotein depletion impairs ubiquitination of TRC8. Taken together, TRC8 is a novel sterol-sensing endoplasmic reticulum membrane protein that hinders SREBP-2 processing through interaction with SREBP-2 and SCAP, regulating its own turnover rate by means of its E3 ubiquitin ligase activity.

The sterol regulatory element-binding protein (SREBP)[2] family members SREBP-1 and SREBP-2 are localized on the ER as membrane proteins after being synthesized, and thereafter are processed to liberate the N-terminal halves that function as transcription factors in the nucleus. The proteolytic processing of SREBPs is highly controlled by the interaction between two ER membrane proteins, SCAP and the insulin-inducing gene (INSIG). Once the ER membrane cholesterol content increases, SCAP binds cholesterol, thereby leading to a conformational change in the membrane domain, the so-called sterol-sensing domain (SSD). This domain resembles sequences in certain other proteins that are postulated to interact with sterols: 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the Niemann-Pick C1 protein, and Patched (1). Subsequently, the conformational change promotes the binding of SCAP to the resident ER protein INSIG. In contrast, when the ER membrane cholesterol content decreases, the SREBP·SACP complex binds to COPII proteins, which cluster the complex into transport vesicles that move to the Golgi where SREBPs are processed sequentially by two proteases, designated site-1 protease (S1P) and site-2 protease (S2P). These cleavage steps release the mature forms of SREBPs that enter the nucleus and activate genes related to cholesterol and fatty acid metabolism (2).

Because SREBP-1 primarily regulates the transcription of genes related to fatty acid metabolism and SREBP-2 preferentially controls cholesterol metabolism, under certain circumstances these two proteins are distinctively activated through proteolytic cleavage in the ER and Golgi. The difference between SREBP-1 and SREBP-2 processing is observable in the liver of rats or mice refed after fasting. The amount of the nuclear active form of SREBP-1, predominantly SREBP-1c in the liver, increases enormously with the consumption of a high carbohydrate/low fat diet as compared with nonfasted levels, whereas the nuclear SREBP-2 protein levels remain unaltered (3, 4). Increased SREBP-1c processing is thought to be in part due to insulin effects after feeding. Insulin greatly suppresses the genetic expression of *INSIG-2a*, a predominant isoform of INSIG-2 active in the liver, but reciprocally augments *INSIG-1* gene expression, thereby stimulating the ER to Golgi transport of the SREBP-1c·SCAP complex (5). Although the INSIG-2a to INSIG-1 switch is thought to play a critical role in insulin-trig-

* This work was supported by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S7.

[1] To whom correspondence should be addressed. E-mail: aroysato@mail.ecc.u-tokyo.ac.jp.

[2] The abbreviations used are: SREBP, sterol regulatory element-binding protein; ALLN, *N*-acetyl-Leu-Leu-norleucinal; ATF6, activating transcription factor 6; ER, endoplasmic reticulum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, hemagglutinin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; INSIG, insulin-inducing gene; LPDS, lipoprotein-deficient serum; PLAP, placenta alkaline phosphatase; S1P, site-1 protease; S2P, site-2 protease; SCAP, SREBP-cleavage-activated protein; SQS, squalene synthase; SSD, sterol-sensing domain; TRC8, translocation in renal cancer from chromosome 8; siRNA, small interfering RNA; GST, glutathione *S*-transferase; HEK, human embryonic kidney; FBS, fetal bovine serum.

gered SREBP-1c processing, the precise mechanism for the division of roles between the two INSIG family members remains unclear. A recent paper demonstrated that insulin-mediated phosphorylation of SREBP-1c enhances the association between the SREBP-1c·SCAP complex and COPII proteins, and subsequently, ER to Golgi transport and proteolytic cleavage (6). At the same time, changes in the levels of both SCAP and INSIG also play a crucial role in controlling SREBP processing. SCAP deficiency in the mouse liver results in severe reductions in SREBP processing and lipid synthesis (7, 8). Overexpression of one of the INSIG family members, INSIG-1, in the liver also brings about parallel declines in SREBP processing and lipid synthesis (9). On the other hand, *INSIG-1*/*-2* double deficiency provokes a marked increase in SREBP-1 processing, but surprisingly not SREBP-2, or fatty acid and triglyceride synthesis (10). This finding prompts us to speculate on the existence of an as yet unidentified regulatory factor other than INSIGs that determines the fate of the SREBP·SCAP complex by distinguishing between SREBP-1c and SREBP-2.

TRC8 encoding an E3-ubiquitin ligase disrupted in a family with hereditary renal cell carcinoma is a putative tumor suppressor (11, 12). TRC8 is thought to localize to the ER as a membrane-associated protein with multiple membrane spanning domains containing a putative SSD, and is ubiquitously expressed in all human tissues (11). A previous paper (13) demonstrated that overexpression of TRC8 suppressed genes involved in cholesterol and fatty acid biosynthesis, consistent with the notion that growth suppression induced by TRC8 is linked to reduced cholesterol and fatty acid synthesis required for cell growth. However, little is known concerning the molecular mechanism of the inhibitory effect of TRC8 on lipid synthesis or the importance of its SSD. In the current investigation we confirmed that TRC8 suppresses SREBP processing, especially SREBP-2, and reduces the expression of SREBP target genes. Conversely, RNA interference-mediated depletion of endogenous TRC8 enhanced SREBP-2 processing and transcription of its target genes. Interaction between TRC8 and the SREBP-2·SCAP complex hindered the ER to Golgi transport of the complex through an inhibition of the binding of SCAP to a component of the COPII protein, Sec24. The TRC8 protein was rapidly degraded, whereas mutant TRC8 lacking its E3 ubiquitin ligase activity was quite stable. TRC8 protein levels gradually increased after cells were cultured with a medium containing lipoprotein-deficient serum (LPDS) instead of normal serum, suggesting that the SSD that senses a decline in lipoprotein supply determined its own turnover rate. Taken together, TRC8 is a novel sterol-sensing ER membrane protein in addition to SCAP and INSIGs, which are involved in SREBP processing.

EXPERIMENTAL PROCEDURES

Materials—Cholesterol, 25-hydroxycholesterol, LPDS, and cycloheximide were purchased from Sigma. *N*-Acetyl-Leu-Leu-norleucinal (ALLN) and tunicamycin were from Nacalai Tesque.

Cultured Cells—HepG2 and HEK293 cells were maintained in medium A (Dulbecco's modified Eagle's medium containing 100 units/ml penicillin and 100 μg/ml streptomycin) supplemented with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 atmosphere.

Antibodies—Anti-FLAG (M2), anti-GST, and anti-β-actin antibodies were purchased from Sigma. Anti-SREBP-2 (1C6) antibody (anti-SBP-2(C)) was from Santa Cruz. Anti-hemagglutinin (HA) antibody was from Covance. Anti-TRC8 antibody was from Abnova. Anti-SREBP-2 polyclonal antibody (anti-SBP-2(N)) has been described previously (14). Monoclonal antibody against SREBP-2 (1D2) was from MBL.

Plasmids—Expression plasmids for FLAG-TRC8, FLAG-Sec24c, and FLAG-SREBP-2-(521–1141) were constructed by inserting PCR fragments encoding human TRC8, Sec24c, and SREBP-2-(521–1141), respectively, into pCMV-3×FLAG (Sigma). An expression plasmid for FLAG-TRC8ΔRING (amino acids 1–546) was constructed by inserting a PCR fragment encoding human TRC8 lacking the C terminus containing the RING finger domain. Several types of reporter plasmids and an expression plasmid for SREBP-2-(1–481) were described previously (15, 16). A mutant expression plasmid for FLAG-TRC8 RING mut (C547S and C550S) was generated using a site-directed mutagenesis kit (Stratagene). An expression plasmid for enhanced green fluorescent protein-ATF6 was described previously (17). An expression plasmid for GST-SCAP-Loop6 was constructed by inserting a PCR fragment encoding human SCAP-Loop6 (amino acids 441–518) into the pME GST vector (18). An expression plasmid for human SCAP-HA was constructed by inserting DNA oligonucleotides encoding an HA sequence into pCMV-SCAP. An expression plasmid for human HA-SREBP-2-(1–1141) was constructed by inserting DNA oligonucleotides encoding six repeats of HA sequence into pME-SREBP2 (15). An expression plasmid for HA-ubiquitin was described previously (19). The expression plasmid for human TRC8 without any tags was generated using an expression vector, pME-18S (15). An expression plasmid for the placenta alkaline phosphatase (PLAP)-SREBP-2-(513–1141) fusion protein was generated as previously reported (20).

Stable Cells—HEK293 cells were transfected with one of the expression plasmids for human FLAG-TRC8 and FLAG-TRC8 RING mut, or pCMV-3×FLAG, together with an expression plasmid for blasticidin deaminase. Surviving cells were obtained in a culture medium containing blasticidin (8 μg/ml) for 2 weeks. Stable clones expressing TRC8 (WT4 for FLAG-TRC8 and MUT24 for Flag-TRC8 RING mut) were selected after determining the protein expression by immunoblot analysis. Mock cells resistant to blasticidin were also obtained.

In Vivo GST Pull-down and Immunoprecipitation Experiments—HEK293 cells were transfected with the indicated plasmids by a calcium phosphate method (21). Forty-eight h later, the cells were harvested and lysed with RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, and 0.25% sodium deoxycholate) supplemented with 1 mM phenylmethylsulfonyl fluoride, 10 mM *N*-ethylmaleimide, 50 μM ALLN, and 0.1% protease inhibitor mixture on ice for 30 min. The lysates were centrifuged at 15,000 × *g* for 10 min at 4 °C, and the supernatant was incubated with 50 μl of 50% slurry of glutathione-Sepharose 4B (GE Healthcare), or immunoprecipitated with the indicated antibody with 50 μl of a 50% slurry of protein G-Sepharose CL-4B (GE Healthcare). The

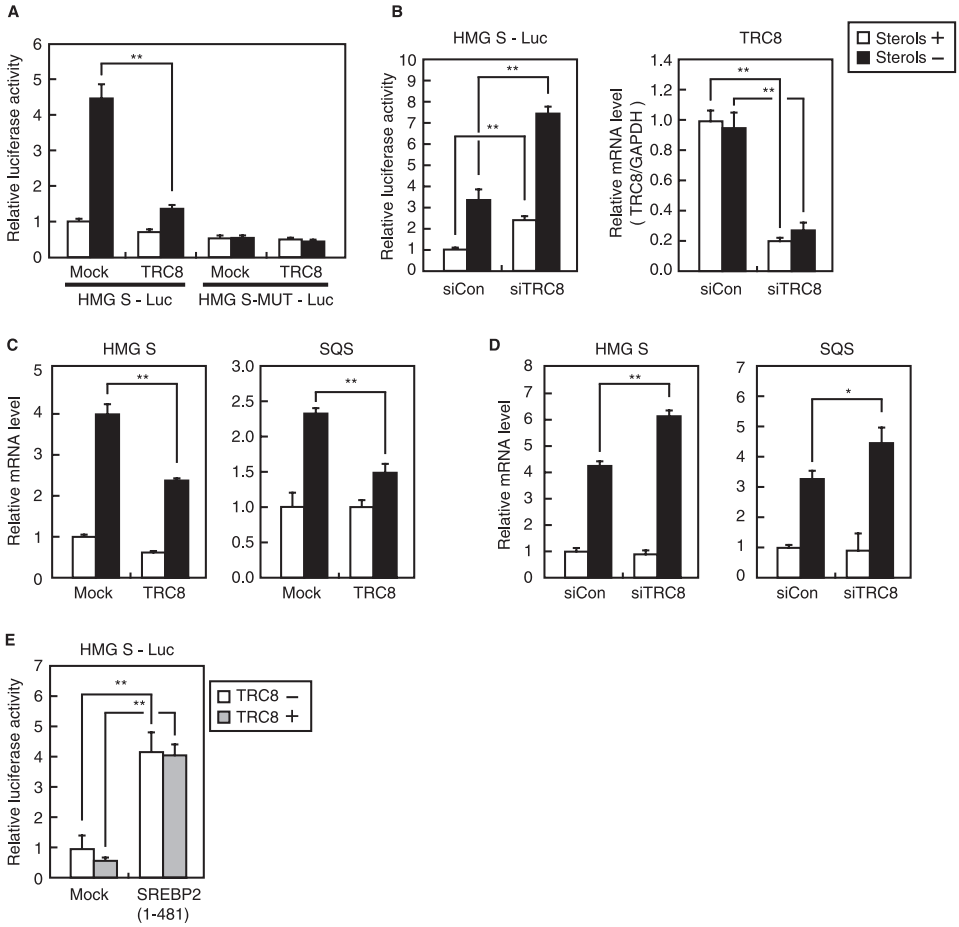

FIGURE 1. **TRC8 affects the expression of SREBP target genes.** *A*, HEK293 cells were transfected with expression plasmids for FLAG-TRC8 and β-galactosidase, and a reporter gene containing either the human *HMG-CoA synthase* promoter (*HMG S-Luc*) or its mutant promoter (*HMG S-MUT-Luc*) lacking one of the functional SRE sequences. After transfection, the cells were cultured under either sterol-loaded (*Sterols* +) or sterol-depleted (*Sterols* −) conditions for 48 h. Luciferase activities were normalized to β-galactosidase activities and considered as 1.0 in the absence of TRC8 under the sterol-loaded condition. *B*, HEK293 cells were transfected with either control (*siCon*) or TRC8 siRNA oligonucleotides (*siTRC8*). After 24 h, the cells were transfected with HMG S-Luc and then cultured as described in *A*. Relative mRNA levels (*TRC8*/*GAPDH*) were determined by real time PCR after being normalized to *GAPDH* mRNA. *C*, HEK293 cells were transfected with an expression plasmid for FLAG-TRC8 and then cultured under either a sterol-loaded or -depleted condition for 24 h. Relative mRNA levels were determined by real time PCR after being normalized to GAPDH mRNA. *D*, HEK293 cells were transfected with either control (*siCon*) or *TRC8* siRNA oligonucleotides (*siTRC8*) and then cultured under either a sterol-loaded or -depleted condition for 48 h. Relative mRNA levels were determined by real time PCR after normalization to *GAPDH* mRNA. *E*, HEK293 cells were transfected with expression plasmids for FLAG-TRC8, a nuclear form of SREBP-2 (SREBP2-(1–481)), and β-galactosidase together with HMG S-Luc. Luciferase activities were normalized to β-galactosidase activities and considered as 1.0 in the absence of TRC8 and SREBP2-(1–481). All data (*A–E*) are presented as mean ± S.D. and represent at least three independent experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$.

beads were washed three times with 500 μl of RIPA buffer, and the specifically bound proteins were pelleted, resuspended with sample buffer, and subjected to immunoblot analysis. In the case of immunoprecipitation using anti-FLAG antibody, the specifically bound proteins were extracted from the beads with a 3×FLAG peptide (Sigma) solution.

Luciferase Assays—Reporter assays were performed as described previously (22). Cells were cultured with a medium containing 5% LPDS plus either 10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol (the sterol-loaded condition) or 50 μM pravastatin and mevalonate (the sterol-depleted condition) for the last 36 h (23).

Alkaline Phosphatase Assays—Aliquots of culture medium were incubated at 65 °C for 30 min to inactivate non-alkaline phosphatase, after which an assay for placental alkaline phosphatase activity was performed using the Phospha-Light™ System (Applied Biosystems) according to the manufacturer's instructions. To account for differences in transfection efficiency, the amount of alkaline phosphatase activity in medium was corrected for the amount of β-galactosidase in cells (20).

Small Interfering RNA Experiments—The siRNA (120 pmol/6-well plate) for human *TRC8* (nucleotides 1085–1105 (GCUCAGGCUACAG UGUUAAUG) in NM_007218) and control (GCGCGCUUUGUAGGAUUCG, the sequence of Scramble II Duplex by Darmacon) were transfected using Lipofectamine RNAiMAX (Invitrogen) into HEK293 cells according to the manufacturer's instructions (23).

Real Time PCR—Total cellular RNA was extracted and reverse transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems). Fluorescence real time PCR was performed using TaqMan Gene Expression Assays (Applied Biosystems) on an ABI PRISM 7000 system. The GAPDH (glyceraldehyde 3-phosphate dehydrogenase) transcript was used as an internal control to normalize the variations of RNA amounts.

RESULTS

TRC8 Affects SREBP Target Gene Expression—HEK293 cells were transfected with a reporter plasmid including the human *HMG-CoA synthase* gene promoter (HMG S-Luc) to evaluate the transcriptional activity of endogenous SREBPs. Under a sterol-depleted condition, the *HMG-CoA synthase* promoter activity was elevated almost 4-fold as compared with a sterol-loaded condition (Fig. 1*A*). With an increase in exogenous TRC8 expression, the activities, particularly when enhanced in response to sterol depletion, were dramatically reduced. Once a functional SRE in the *HMG-CoA synthase* promoter was mutated (HMG S-MUT-Luc), the promoter activity remained at a low level and no longer responded to TRC8. These results suggest that TRC8 suppresses the promoter activities driven by SREBPs. When endogenous TRC8 expression was reduced to ~20% of normal with gene-specific siRNA, the luciferase activities were significantly augmented under the sterol-depleted or -loaded conditions (Fig. 1*B*). The reporter

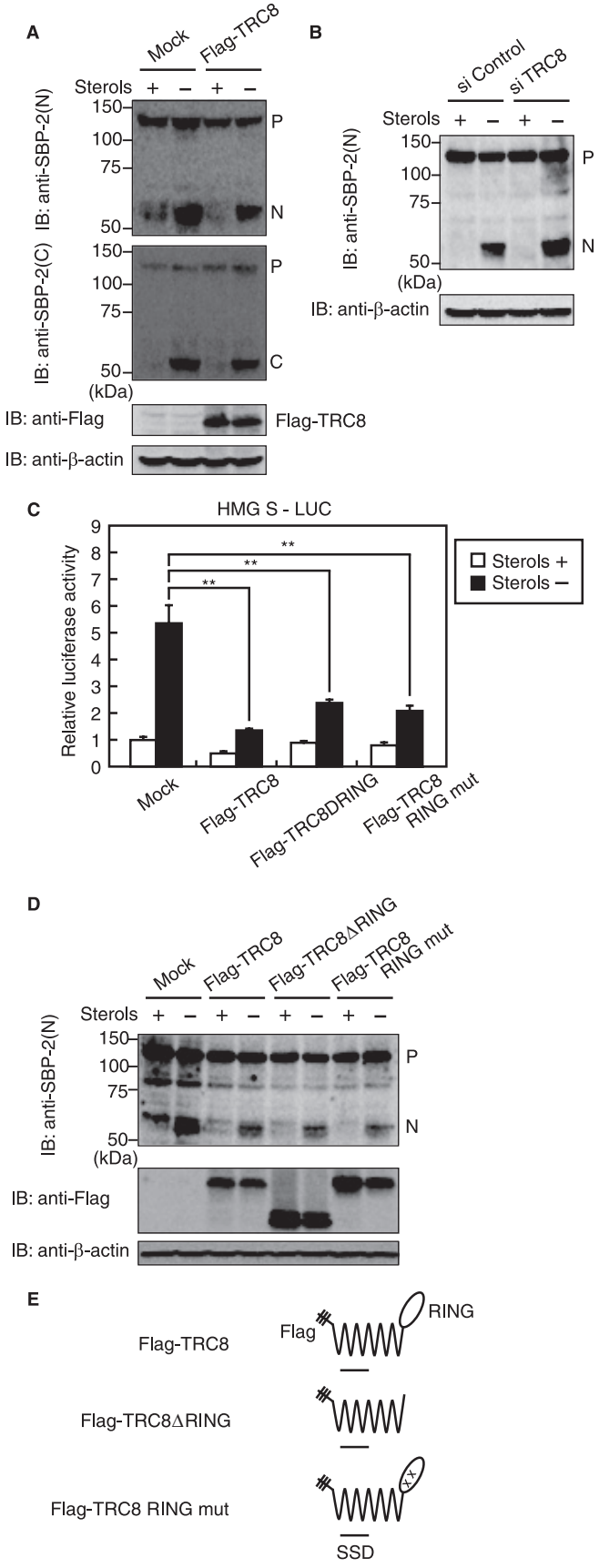

FIGURE 2. **TRC8 hinders SREBP-2 processing.** *A*, HepG2 cells were transfected with an expression plasmid for FLAG-TRC8 and then cultured for 48 h. The cells were cultured under either a sterol-loaded (*Sterols* +) or sterol-depleted (*Sterols* −) condition for the last 12 h, and harvested. Whole cell lysates were subjected to SDS-PAGE and immunoblot analysis using anti-SREBP-2 antibodies recognizing its N-terminal (*anti-SBP-2*(*N*)) and C-terminal portions (*anti-SBP-2*(*C*)), along with anti-FLAG or anti-β-actin antibody. *P*, *N*, and *C* denote the precursor form and the N-terminal and C-terminal fragments of SREBP-2, respectively. *B*, HepG2 cells were transfected with either control or TRC8 siRNA oligonucleotides and then cultured for 48 h. The cells were cultured under either a sterol-loaded (*Sterols* +) or sterol-depleted (*Sterols* −) condition for the last 12 h, and harvested. Immunoblot analysis was performed using anti-SBP-2(N) and anti-β-actin antibodies. *C*, HEK293 cells were transfected with one of the expression plasmids for FLAG-TRC8 and two mutant forms of TRC8, and β-galactosidase together with HMG S-Luc. The cells were cultured and luciferase assays were performed as described in the legend to Fig. 1*A*. *D*, three FLAG-TRC8 proteins expressed in these experiments are illustrated. All data are presented as mean ± S.D. and represent at least three independent experiments performed in triplicate. **, $p < 0.01$. *D*, HepG2 cells were transfected with one of the expression plasmids for FLAG-TRC8. Immunoblot analysis was performed as described in *A*. The same results (*A*–*C*) were obtained in three separate experiments. *IB*, immunoblot.

assays using the promoter of the human squalene synthase (*SQS*) gene, another SREBP target gene, also revealed that TRC8 suppresses the SREBP-mediated stimulation of the promoter activity (supplemental Fig. S1).

Next we examined the effect of TRC8 on endogenous SREBP target gene expression in HEK293 cells. Overexpression of TRC8 significantly suppressed the elevated mRNA levels of *HMG-CoA synthase* and *SQS* in response to sterol depletion (Fig. 1*C*). In contrast, the mRNA levels of acetyl-CoA carboxylase and stearoyl-CoA desaturase, the transcription of which is predominantly regulated by SREBP-1, were not altered (supplemental Fig. S2*A*). Based on these findings, we focused on the inhibitory effect of TRC8 on SREBP-2 functions in the following experiments. When endogenous TRC8 expression was reduced with siRNA, elevation in the mRNA levels of *HMG-CoA synthase* and *SQS* in response to sterol depletion was further increased (Fig. 1*D*). Taken together, we speculate that TRC8 deteriorates SREBP function, mainly SREBP-2, by either hindering SREBP processing or directly inhibiting the transcriptional activity of SREBP-2. It is, however, unlikely that the latter is the case because the transcription activity of the nuclear mature form of SREBP-2 (amino acids 1–481), when transiently expressed in HEK293 cells, was not affected by overexpression of TRC8 in the reporter assay (Fig. 1*E*). These results indicate that TRC8 affects SREBP processing that occurs in the ER and Golgi.

TRC8 Interferes with SREBP Processing—The above results prompted us to examine whether TRC8 influences proteolytic activation of SREBPs on the ER and Golgi membranes. To monitor SREBP processing, two types of antibodies against the N-terminal or C-terminal domains of human SREBP-2 were used in the following immunoblot analyses. The mature form of SREBP-2, which is recognized by a polyclonal antibody against the N-terminal domain of SREBP-2 (anti-SBP-2(N)), was increased in response to sterol depletion in mock transfected HepG2 cells (Fig. 2*A*, *upper panel*, *first* and *second lanes*), whereas in the presence of TRC8 the proteolytic activation of SREBP-2 was largely hindered (*second* and *fourth lanes*). When the C-terminal half of SREBP-2, which remained on the Golgi membrane after cleavage of the N-terminal half, was detected with a monoclonal antibody (anti-SBP-2(C)), it was found that overexpression of TRC8 reduced the level of the C-terminal

half of SREBP-2 (Fig. 2*A*, *lower panel*, *second* and *fourth lanes*). Furthermore, when endogenous TRC8 expression was knocked down by siRNA, more of the N-terminal, mature form of SREBP-2 was observed in response to sterol depletion (Fig. 2*B*, *second* and *fourth lanes*). These results clearly show that TRC8 has an inhibitory effect on proteolytic cleavage of SREBP-2. As long as the cleavage of SREBP-1 was analyzed using a monoclonal antibody against the N-terminal half of the protein, no reduction in the level of the mature form of SREBP-1, unlike SREBP-2, was found in the presence of exogenous TRC8 (supplemental Fig. S2*B*, *lanes 2* and *4*).

In an attempt to determine which domain in the TRC8 molecule was responsible for the inhibitory effect on SREBP-dependent gene expression, two types of mutant TRC8 expression plasmids, TRC8ΔRING, lacking the C-terminal RING-finger domain, and TRC8 RING mut, with substitution of serines for a pair of cysteines (Cys^{547} and Cys^{550}) in the RING domain, were constructed. The mutation of this pair of cysteines was shown to abolish the E3-ligase activity in a previous paper (24). In the luciferase assays using the *HMG-CoA synthase* promoter reporter gene, two mutant versions of TRC8 suppressed the reporter activities to the same degree as the wild type TRC8 (Fig. 2*C*). Consistent with these findings, all forms of TRC8 suppressed SREBP-2 processing in the presence or absence of sterols (Fig. 2*D*). All TRC8 proteins expressed in these experiments are illustrated in Fig. 2*E*. These results indicate that the membrane-spanning region is critical for the inhibitory effect of TRC8 on SREBP processing and the genetic expression of SREBP targets.

TRC8 Does Not Affect ATF6 Processing—The transcription factor ATF6 is also proteolytically activated in response to ER stress by two proteases, S1P and S2P, which are involved in SREBP processing (25). We next examined whether TRC8 interfered with ATF6 processing as well. When the green fluorescent protein-ATF6 fusion protein was expressed in HEK293 cells, a slight basal cleavage was observed in the absence of any stimuli (Fig. 3*A*, *lane 1, N*; nuclear forms). In the presence of the ER stress inducer tunicamycin, aberrant precursors with a lower molecular weight resulting from an inhibition of *N*-linked glycosylation (*P** in Fig. 3*A*) and an increase in the cleaved nuclear forms of green fluorescent protein-ATF6 (*N* in Fig. 3, *second* and *fourth lanes*) were observed. Overexpression of TRC8 did not affect the processing pattern at all (Fig. 3, *third* and *fourth lanes*). Expression of the CHOP protein, induced by tunicamycin-mediated ER stress, was not suppressed in the presence of TRC8 (*second* and *fourth lanes*). Moreover, ATF6 was not co-immunoprecipitated with TRC8, even when the two proteins were overexpressed in HEK293 cells (Fig. 3*B*, *third lane*). These results clearly show that there was no interplay between ATF6 and TRC8, and that SREBP-2 processing inhibition by TRC8 was not caused by the inactivation of S1P or S2P.

TRC8 Influences Secretion of PLAP-SREBP-2—In the SREBP processing pathway, translocation of the SREBP·SCAP complex from the ER to the Golgi is critical for subsequent proteolytic cleavage of SREBP in the pre-Golgi or Golgi compartments. To analyze the SREBP/SCAP translocation to the Golgi, proteolytic processing and secretion of the PLAP-SREBP-2 fusion protein were monitored in HEK293 cells transfected

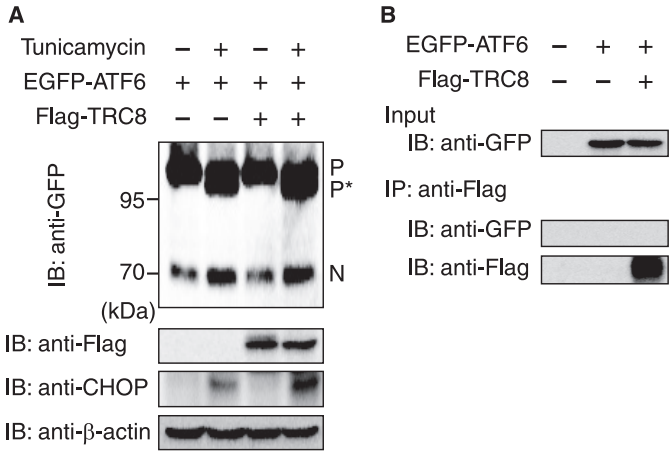

FIGURE 3. **TRC8 does not affect ATF6 processing.** *A*, HEK293 cells were transfected with expression plasmids for enhanced green fluorescent protein (*EGFP*)-ATF6 and FLAG-TRC8. After 48 h, the cells were incubated with 4 μM tunicamycin for 4 h and then harvested. Immunoblot analysis was performed using anti-green fluorescent protein, anti-FLAG, anti-CHOP, and anti-β-actin antibodies. *P*, *P**, and *N* denote the precursor, precursor without glycosylation, and nuclear form of enhanced green fluorescent protein-ATF6, respectively. *B*, HEK293 cells were transfected with expression plasmids for enhanced green fluorescent protein-ATF6 and FLAG-TRC8. After 48 h, the cells were harvested and whole cell lysates were subjected to immunoprecipitation using anti-FLAG antibody. Whole cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot (*IB*) analysis using anti-green fluorescent protein or anti-FLAG antibody. The same results (*A* and *B*) were obtained in three separate experiments.

with expression plasmids for the fusion protein and SCAP (20). PLAP was not processed in the absence of exogenous SCAP, because endogenous SCAP is insufficient to form a PLAP-SREBP-2·SCAP complex, but was secreted in the media when SCAP expression increased (Fig. 4*A*). The secretion was significantly inhibited in the presence of exogenous TRC8. It was also confirmed by immunoblot analysis that TRC8 expression did not affect the expression of SCAP, which was required for the ER to Golgi translocation of the fusion protein (Fig. 4*A*). Next we examined whether depletion of endogenous TRC8 by siRNA induces PLAP secretion in the medium. Greater secretion was observed after endogenous TRC8 expression had been depleted by specific siRNA oligonucleotides (Fig. 4*B*). Once higher levels of SCAP were expressed, depletion of endogenous TRC8 no longer affected the secretion of PLAP, simply because exogenous SCAP levels were extremely high compared with endogenous TRC8 levels (data not shown). These results indicate that TRC8 hinders the SREBP·SCAP translocation from the ER to the Golgi, leading to a reduced secretion of PLAP.

TRC8 Interacts with SREBP-2 and SCAP—It has been reported that the SREBP·SCAP complex interacts with another ER membrane protein, INSIG-1/-2, in response to excess intracellular cholesterol. To address whether TRC8 in place of INSIGs maintains the SREBP·SCAP complex on the ER membrane, we examined the protein-protein interaction between TRC8 and SREBP-2/SCAP. When TRC8 and full-length SREBP-2 were expressed in HEK293 cells, SREBP-2 was co-immunoprecipitated with TRC8 (Fig. 5*A*, *third lane*). Even when TRC8ΔRING was expressed, SREBP-2 interacted with this truncated form as well, which is consistent with the finding that TRC8ΔRING interfered with SREBP-2 processing (see Fig. 2). In these experiments as long as SREBP-2 and TRC8s were

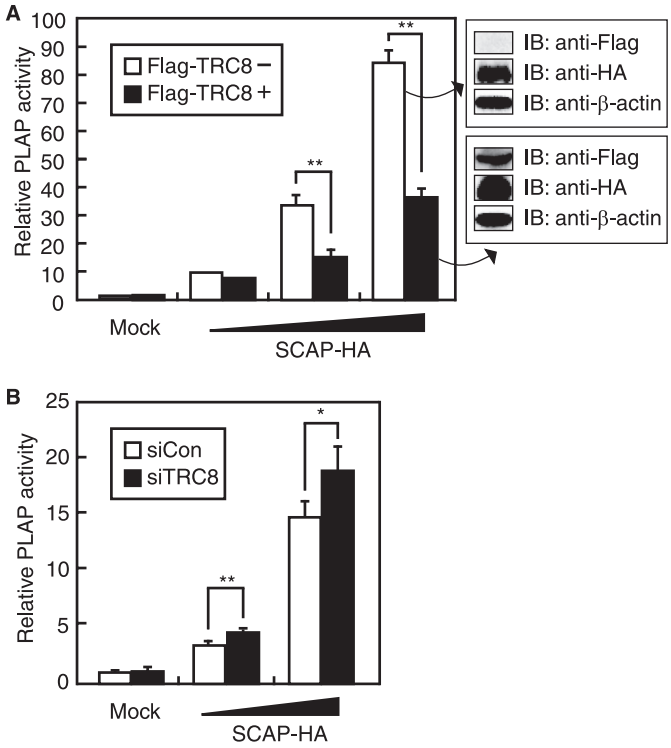

FIGURE 4. **TRC8 suppresses proteolytic processing and secretion of the PLAP-SREBP-2 fusion protein.** *A*, HEK293 cells were transfected with expression plasmids for PLAP-SREBP-2, SCAP-HA (15, 50, or 150 ng/12-well plate), FLAG-TRC8, and β-galactosidase. The cells were cultured under a sterol-depleted condition for 48 h. PLAP activities were normalized to β-galactosidase activities and considered as 1 in the absence of SCAP and TRC8. The cells transfected with the highest amount of SCAP-HA expression plasmid (150 ng) were harvested and immunoblot (*IB*) analyses was performed to confirm no interference of SCAP expression by TRC8. *B*, HEK293 cells were transfected with either control or *TRC8* siRNA oligonucleotides. The next day the cells were further transfected with the expression plasmids for PLAP-SREBP-2, SCAP-HA (10 or 30 ng/12-well plate), FLAG-TRC8, and β-galactosidase. The cells were cultured under a sterol-depleted condition for 48 h. PLAP activities were normalized to β-galactosidase activities and considered as 1 when the cells were transfected with control RNA oligonucleotides in the absence of SCAP-HA. All data (*A* and *B*) are presented as mean ± S.D. and represent at least three independent experiments performed in triplicate. *, $p < 0.05$; **, $p < 0.01$.

co-expressed, the SREBP-2-TRC8 interaction was not affected by the presence of excess sterols (Fig. 5*A*, *third* to *sixth lanes*). To determine which domain in SREBP-2 was involved in the interaction with TRC8, the N-terminal or C-terminal halves of SREBP-2 were expressed with TRC8 and immunoprecipitation assays were performed. SREBP-2-(1–481) lacking two transmembrane domains was not co-immunoprecipitated with TRC8 (Fig. 5*B*), whereas SREBP-2-(521–1141) including the second transmembrane domain interacted with TRC8 (Fig. 5*C*). These results show that at least the C-terminal half of SREBP-2 is required for the interaction with TRC8.

The C-terminal half of SREBP-2 interacts with the C-terminal domain of SCAP to form a complex. One can therefore speculate that TRC8 competes with SCAP for interaction with SREBP-2, or that TRC8 recruits SREBP-2 via endogenous SCAP by forming a SREBP-2·SCAP·TRC8 complex. Next we examined whether TRC8 directly interacts with SCAP. When both TRC8 and SCAP were expressed in HEK293 cells, SCAP was co-immunoprecipitated with TRC8 in a sterol-independent manner (Fig. 5*D*). The deletion of the RING domain (TRC8ΔRING) did not affect the interplay at all (Fig. 5*D*, *fifth* and *sixth lanes*). Moreover, when SCAP, SREBP-2, and TRC8 were all expressed in cells, both SCAP and SREBP-2 were co-immunoprecipitated with TRC8, suggesting that these three proteins form a complex (Fig. 5*E*). In HEK293 stable cells expressing FLAG-TRC8 (WT4 is described later), a complex including FLAG-TRC8 and a precursor form of endogenous SREBP-2 was co-immunoprecipitated with an anti-FLAG antibody (Fig. 5*F*). In terms of the complex formation of endogenous proteins, when SREBP-2 in HepG2 cells was immunoprecipitated, TRC8 was co-immunoprecipitated (Fig. 5*G*). Taken together, these results demonstrate the physical formation of the complex consisting of SREBP-2, SCAP, and TRC8.

TRC8 Hinders the Interaction between the SREBP·SCAP Complex and the COPII Protein Sec24—The SREBP·SCAP complex exits the ER in COPII protein-coated vesicles that bud from ER membranes (26). A component of COPII protein, Sec24, interacts with a specific sequence, MELADL, located in the cytoplasm loop between the 6th and 7th transmembrane domains of SCAP (27). To assess whether TRC8 interferes with the interaction between SCAP and Sec24, HEK293 cells were transfected with expression plasmids for GST protein fused with the cytoplasmic loop of SCAP (GST-SCAP-Loop6) and FLAG-Sec24, together with increasing amounts of a plasmid encoding TRC8. GST pull-down experiments revealed that Sec24 interacted with the SCAP loop and that the interaction was dose-dependently inhibited by an increase in TRC8 expression (Fig. 6*A*, *third panel*). The finding that TRC8 did not associate with the SCAP loop (supplementary Fig. S3) indicates that TRC8 interferes with the interaction between SCAP and Sec24.

We next examined whether TRC8 can associate with Sec24. When Sec24 and TRC8 were expressed in HEK293 cells, TRC8 was co-immunoprecipitated with Sec24 (Fig. 6*B*), suggesting that it seems likely that TRC8 is translocated between the ER and the Golgi via COPII protein-coated vesicles. To examine whether SREBP-2, which does not bind Sec24, interferes with association between Sec24 and TRC8, HA-SREBP-2, FLAG-Sec24, and TRC8 were expressed, and immunoprecipitation assays using anti-FLAG antibodies were carried out. TRC8 was co-immunoprecipitated with Sec24 (Fig. 6*C*, *second lane*) and this association was hindered by an increase in SREBP-2 expression (*third* to *fifth lanes*). This complex did not contain HA-SREBP-2 (Fig. 6*C*, *fourth panel*), suggesting that TRC8 associated with SREBP-2 no longer interacts with Sec24. Taken together, although TRC8 itself binds Sec24, it is possible that the TRC8·SREBP-2·SCAP complex is not associated with the COPII proteins.

TRC8 Was Rapidly Degraded—To analyze functions of the SSD and RING finger domain of TRC8, we established stable HEK293 cells expressing either FLAG-TRC8 (WT4) or FLAG-TRC8 RING mut (MUT24). In these cells the mRNA levels of *HMG-CoA synthase* and *SQS* declined, consistent with findings that transient expression of TRC8 or TRC8ΔRING suppressed transcription of these genes, as shown in Fig. 1 (see also supplementary Fig. S4). In addition, SREBP-2 processing was also reduced in these cells as compared with mock cells (see supplementary Fig. S5). Fig. 5*F* shows that endogenous SREBP-2 in WT4 cells was indeed co-immunoprecipitated with FLAG-

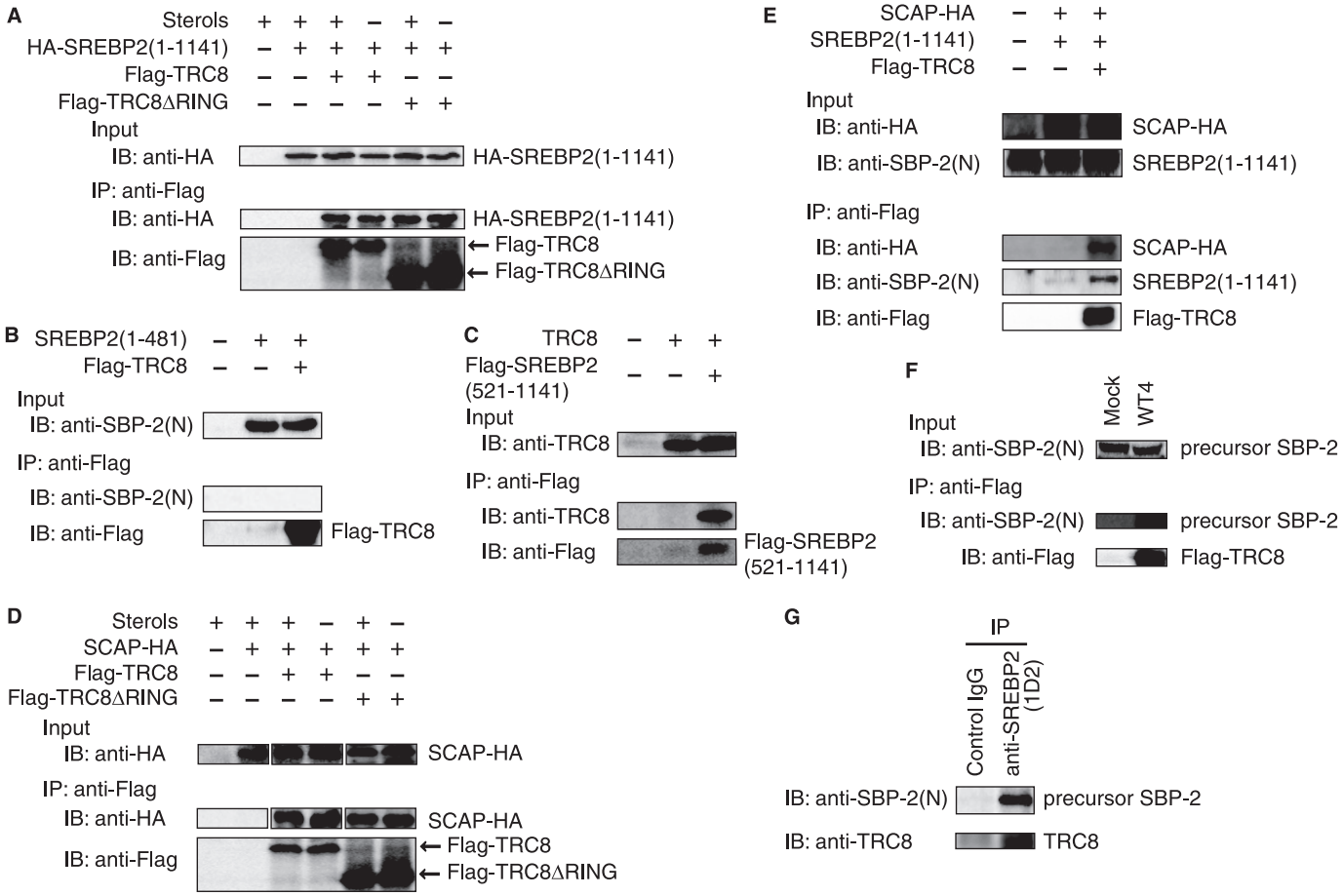

FIGURE 5. **TRC8 interacts with SREBP-2 and SCAP.** *A*, HEK293 cells were transfected with expression plasmids for HA-SREBP2-(1–1141) and either FLAG-TRC8 or FLAG-TRC8ΔRING. The cells were cultured under either a sterol-loaded or -depleted condition for 48 h. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot (*IB*) analysis with anti-HA and anti-FLAG antibodies. *B*, HEK293 cells transfected with expression plasmids for SREBP-2-(1–481) and FLAG-TRC8 were cultured with medium A containing FBS for 48 h. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot analysis with anti-SBP-2(N) and anti-FLAG antibodies. *C*, HEK293 cells transfected with expression plasmids for FLAG-SREBP2-(521–1141) and TRC8 were cultured with medium A containing FBS for 48 h. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot analysis with anti-TRC8 and anti-FLAG antibodies. *D*, HEK293 cells transfected with expression plasmids for SCAP-HA and either FLAG-TRC8 or FLAG-TRC8ΔRING were cultured under either a sterol-loaded or -depleted condition for 48 h. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot analysis with anti-HA and anti-FLAG antibodies. *E*, HEK293 cells transfected with expression plasmids for SREBP-2-(1–1141), SCAP-HA, and FLAG-TRC8 were cultured with medium A containing FBS for 48 h. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot analysis with anti-SBP-2(N), anti-HA, and anti-FLAG antibodies. *F*, whole cell lysates from stable mock HEK293 cells and WT4 cells expressing FLAG-TRC8 were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot analysis with anti-SBP-2(N) and anti-TRC8 antibodies. *G*, whole cell lysates from HepG2 cells were subjected to immunoprecipitation with either control IgG or anti-SREBP-2 monoclonal antibody (*1D2*). Immunoprecipitates were subjected to immunoblot analysis with anti-SBP-2(N) and anti-FLAG antibodies. The same results (*A–G*) were obtained in three separate experiments.

TRC8. These results indicate that all the observations in response to transient expression of TRC8 or TRC8 RING mut in Figs. 1 and 2 were reproducible in these stable cells. We first found that FLAG-TRC8 in WT4 cells was unstable, whereas FLAG-TRC8 RING mut was quite stable. After cells were treated with cycloheximide to block *de novo* protein synthesis, the decay of TRC8 proteins was chased in a medium supplemented with either LPDS or FBS. In the absence of lipoprotein, TRC8 protein had a more prolonged half-life (Fig. 7*A*, ~6 h for FBS and ~11 h for LPDS). In contrast, the TRC8 RING mut protein was quite stable for 9 h in the presence or absence of lipoprotein in the culture medium (Fig. 7*B*). When WT4 cells were treated with the proteasome inhibitor ALLN, FLAG-TRC8 became more stable, suggesting that TRC8 protein was at least partly degraded by the proteasome (Fig. 7*C*). Treatment with another proteasome inhibitor, MG132, also stabilized the TRC8 protein (supplementary Fig. S6). These findings suggest the possibility that the SSD in TRC8 functions as a sterol sensor to regulate its own stability.

Moreover, the relationship between ubiquitination and the degradation of TRC8 was examined in these stable cells. Stable cells were transfected with an expression plasmid for HA-ubiquitin and cultured with 50 μM ALLN for the last 3 h. Immunoprecipitates were immunoblotted with anti-FLAG antibody. In WT4 cells, wild type TRC8 was conjugated by ubiquitin, but in MUT24 cells mutant TRC8 lacking the E3 ubiquitin ligase activity was not ubiquitinated (Fig. 7*D*). This suggests that the self-ubiquitination of TRC8 requires its own E3 ubiquitin ligase activity, consistent with a previous finding obtained by *in vitro* ubiquitination assays (24). When WT4 cells were refed with an

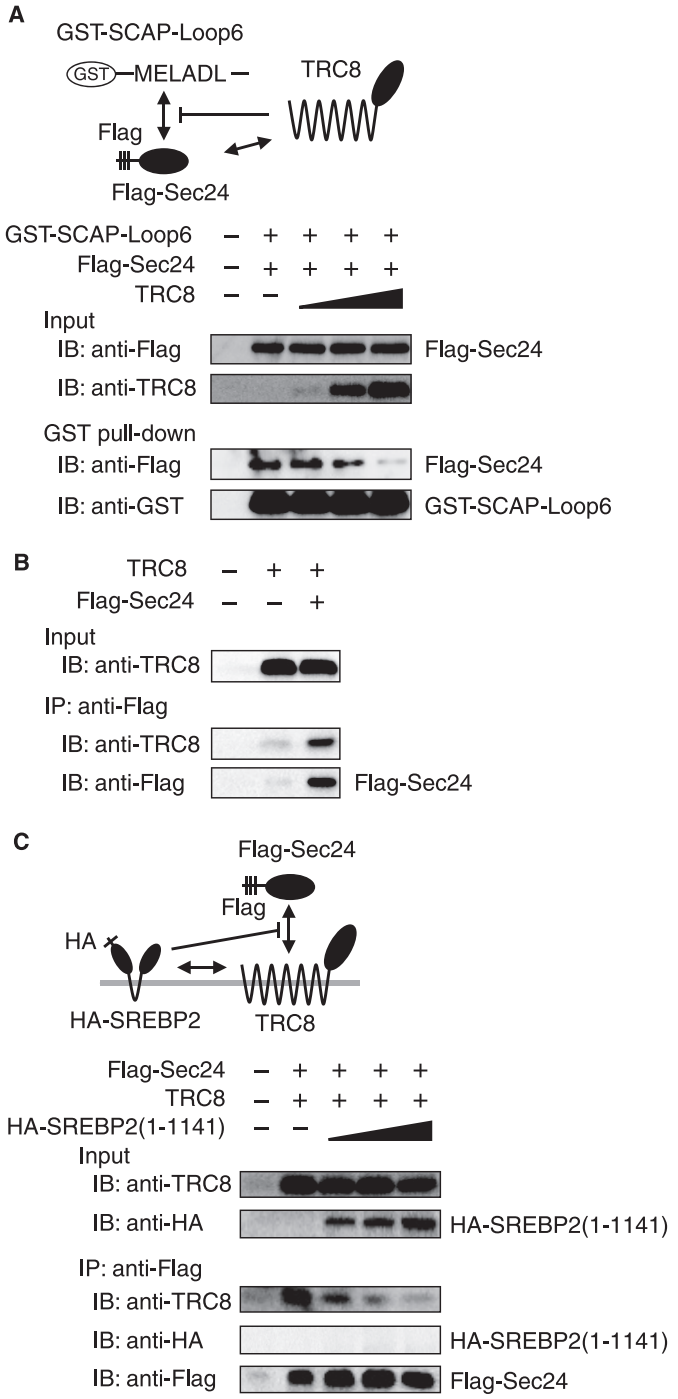

FIGURE 6. **Sec24 interacts with GST-SCAP-Loop6 or TRC8.** *A*, HEK293 cells were transfected with expression plasmids for TRC8, FLAG-Sec24, and GST-SCAP-Loop6. Whole cell lysates were subjected to GST pull-down. Both cell lysates (*Input*) and GST pull-down samples (*GST pull-down*) were subjected to immunoblot (*IB*) analysis with anti-TRC8, anti-FLAG, and anti-GST antibodies. *B*, HEK293 cells were transfected with expression plasmids for TRC8 and FLAG-Sec24. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates (*IP*) were subjected to immunoblot analysis with anti-TRC8 and anti-FLAG antibodies. *C*, HEK293 cells were transfected with expression plasmids for TRC8, FLAG-Sec24, and HA-SREBP2-(1–1141). Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Both cell lysates (*Input*) and immunoprecipitates were subjected to immunoblot analysis with anti-TRC8, anti-HA, and anti-FLAG antibodies. The same results (*A–C*) were obtained in three separate experiments.

LPDS-containing medium, ubiquitination of TRC8 was gradually decreased in a time-dependent manner (Fig. 7*E*). These results reflect the notion that a reduced ubiquitination of TRC8 in response to lipoprotein depletion may contribute to the prolonged half-life.

To understand the physiological significance of the TRC8-dependent inhibition of SREBP-2 processing, we analyzed the time-dependent correlation between SREBP-2 processing and TRC8 stabilization in response to lipoprotein depletion. HepG2 cells cultured with normal serum were refed with a medium containing LPDS, and then the cells were harvested to perform time course analyses of SREBP-2 processing along with TRC8 protein levels. The lipoprotein depletion provoked enhanced SREBP-2 processing in the initial hours, and subsequently the processing slowed down to the initial level at 12 h (supplemental Fig. S7). The elevation of TRC8 protein levels required a relatively long time (~10 h), and in conjunction with this increase, SREBP-2 processing was down-regulated. It is therefore possible that the increased TRC8 in response to lipoprotein deficiency is involved in blocking further activation of SREBP-2 processing at ~10 h.

DISCUSSION

The current study shows that TRC8, localized in the ER along with the SREBP·SCAP complex, functions as a novel inhibitory protein of SREBP processing. We obtained the following findings. 1) Overexpression of TRC8 suppresses the expression of SREBP target genes, including *SQS* and *HMG-CoA synthase*, by hindering SREBP-2 processing. 2) RNA interference-mediated depletion of endogenous TRC8 conversely enhances SREBP-2 target gene expression through an augmentation of SREBP-2 processing. 3) TRC8 interacts with both SREBP-2 and SCAP, thereby inhibiting the binding of SCAP to Sec24 and hampering the ER to Golgi transport of the SREBP·SCAP complex. 4) TRC8 is rapidly degraded by the ubiquitin-proteasome pathway and becomes stable under a lipoprotein-depleted condition. Thus, several lines of evidence show that INSIG-1 and -2 are exclusive regulatory membrane proteins that associate with the SREBP·SCAP complex and retain it in the ER in response to changes in the ER membrane cholesterol content (28, 29). Once the proteolytic processing of SREBP-2 is initiated under sterol-depleted conditions, this processing evidently continues until the SREBP·SCAP·INSIG complex is reconstituted in response to increased cholesterol synthesis. It is reasonable to assume that enhanced SREBP-2 processing is finally hindered by the TRC8 that accumulates under lipoprotein-depleted conditions to avoid prolonged overactivation of SREBP-2 in the absence of INSIG-mediated blockade of SREBP processing.

In the liver of SCAP-null mice SREBPs are not processed without their escort protein and become unstable (7, 8). It is possible that TRC8 might directly interact with SREBPs in the absence of SCAP, thereby enhancing degradation as a result of its E3 ligase activity. However, this does not seem to be the case, because there was no significant change in the level of precursor SREBPs when TRC8 was overexpressed or depleted by siRNA (Fig. 2). Indeed, ubiquitination of endogenous or exogenous SREBP-2 was not observed even when TRC8 was highly expressed in cultured cells (data not shown). It is, therefore,

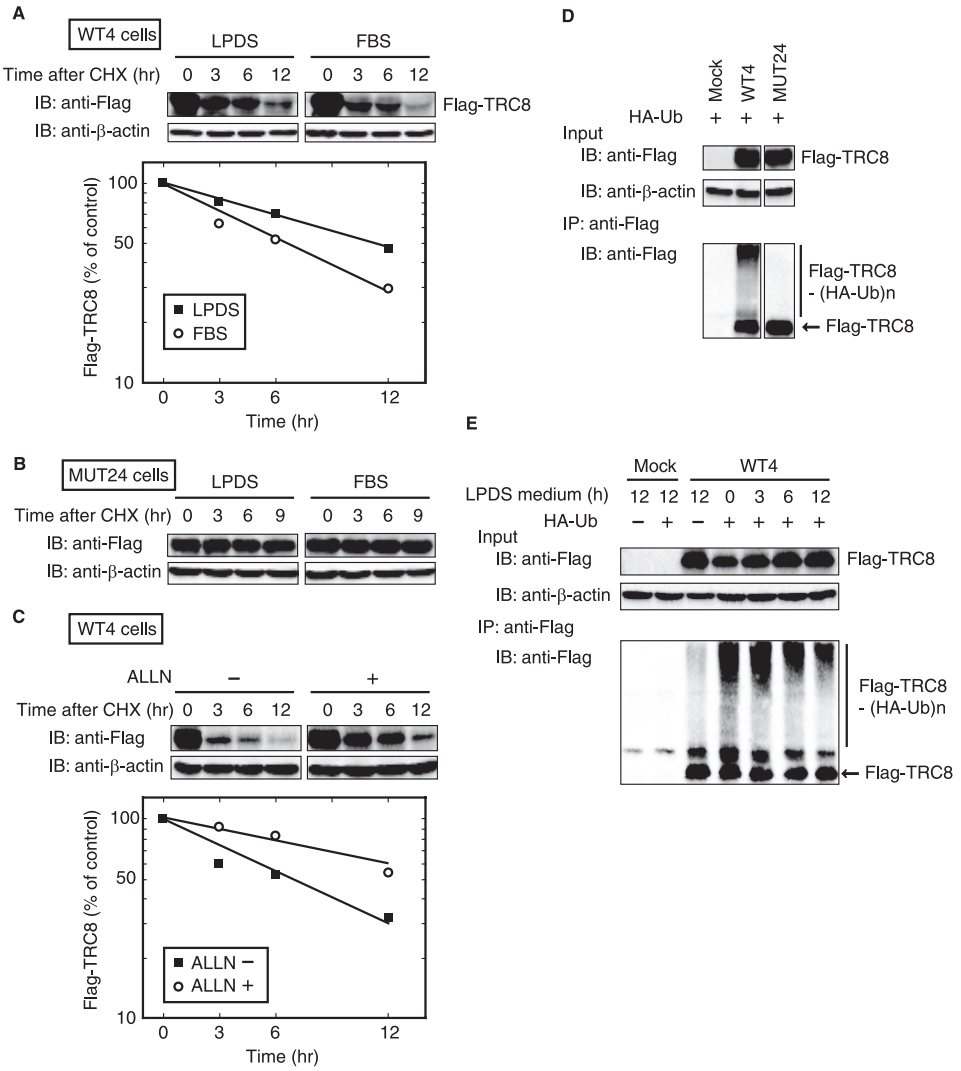

FIGURE 7. **TRC8 is rapidly degraded.** *A* and *B*, stable HEK293 cells (WT4 and MUT24) were cultured with a medium containing 5% LPDS for 12 h. The cells were refed with a medium containing either LPDS or FBS supplemented with 50 μM cycloheximide (*CHX*) at 0 time. The cells were harvested at the indicated times, and whole cell lysates were subjected to immunoblot (*IB*) analysis with anti-FLAG and anti-β-actin antibodies. The signals on the membrane were quantified, and data were plotted as the percentage of FLAG-TRC8 protein remaining. *C*, WT4 cells were cultured as described in *A*. At 0 time, 50 μM ALLN was added to the medium. The signals on the membrane were quantified, and data were plotted as the percentage of FLAG-TRC8 protein remaining. *D*, stable mock, WT4, and MUT24 cells were transfected with an expression plasmid for HA-ubiquitin (*HA-Ub*), and then cultured with an FBS containing medium for 48 h. For the last 3 h, the cells were incubated with 50 μM ALLN to stabilize polyubiquitin-conjugated TRC8. Whole cell lysates (*Input*) and immunoprecipitates (*IP*) with anti-FLAG antibody were subjected to immunoblot analysis with anti-FLAG and anti-β-actin antibodies. *E*, stable mock cells and WT4 cells were transfected with an expression plasmid for HA-Ub, and then cultured with FBS containing medium for 48 h. The cells were refed with LPDS containing medium for the indicated time. For the last 3 h, the cells were incubated with 50 μM ALLN to stabilize polyubiquitin-conjugated TRC8. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody. Whole cell lysates (*Input*) and immunoprecipitates were subjected to immunoblot analysis with anti-FLAG and anti-β-actin antibodies. The same results (*A–E*) were obtained in three separate experiments.

possible that TRC8 in the SCAP-deficient liver retains SREBPs in the ER and assists in their rapid turnover, although it remains unclear how SREBPs free of SCAP become unstable. Under physiological conditions, it has been estimated that the amount of SCAP is a rate-limiting step in SREBP·SCAP complex formation and that only 20–40% of the SREBP precursors are in the complex with SCAP (30). It seems likely that TRC8 might interact with the SREBPs free of SCAP. The preceding interaction between TRC8 and SREBPs might hinder formation of the SREBP·SCAP complex, thereby inhibiting exit from the ER and SREBP processing. This would account for the current findings that RNA interference-mediated depletion of endogenous TRC8 enhances SREBP-2 processing.

The binding site of SCAP for Sec24 has been identified as a hexapeptide sequence, located in the cytoplasmic loop between transmembrane helices 6 and 7 (26, 31). In the current study, we established a novel assay system to estimate the direct interaction between SCAP and Sec24 by means of the GST protein fused to this loop (GST-SCAP-Loop6). This assay revealed that TRC8 interfered with the association between Sec24 and the loop (Fig. 6). Because a direct interaction between TRC8 and the loop was not observed (supplemental Fig. S3), it is possible that TRC8 masked the binding site of Sec24 for the hexapeptide. Indeed, the TRC8 and Sec24 interaction was confirmed (Fig. 6), suggesting that TRC8 moves to the Golgi through the COPII protein-coated vesicles. Next we examined whether TRC8 binds Sec24 even after the SREBP·SCAP·TRC8 complex is formed. The interaction between TRC8 and Sec24 was inhibited by SREBP-2 in a dose-dependent manner (Fig. 6). This demonstrates that the SREBP·SCAP·TRC8 complex tends to remain in the ER as the result of weakening of Sec24 binding to both SCAP and TRC8.

It is noteworthy that TRC8 was determined to be one of the functional sterol-sensing proteins. TRC8 is rapidly degraded through a ubiquitin-proteasome pathway, and becomes stable in the absence of lipoprotein in the culture medium. Another sterol-sensing protein, HMG-CoA reductase, is similarly degraded in response to an increase in intracellular sterols, especially lanosterol, which induces the formation of an HMG-CoA reductase·INSIG complex, including gp78 with its E3 ligase activity (32–35). The degradation of HMG-CoA reductase, therefore, requires two sequential steps, sterol-mediated INSIG binding and recruitment of gp78. On the other hand, TRC8 contains two functional domains, the SSD and RING finger domain, thereby controlling its own expression level by sensing the intracellular sterol levels without the need of any associated proteins. For as long as the examination was performed, the TRC8 protein was more rap-

idly degraded in the presence of lipoprotein, which is taken up by the cell surface low density lipoprotein receptors, rather than in the presence of the cholesterol and 25-hydroxycholesterol supplemented in the culture medium. It is possible that lipoprotein-derived sterols are more accessible to TRC8 via the internalization pathway in the vicinity of the ER and Golgi, which probably leads to the conformational change and ubiquitination of TRC8, thereby accelerating protein turnover. The detailed molecular mechanism of lipoprotein-accelerated TRC8 degradation is now under investigation.

We do not consider the major physiological function of TRC8 to be restricted to the regulation of SREBP processing, because this inhibitory effect does not require its RING domain. We attempted to identify as yet unknown TRC8-associated proteins using stable HEK293 cells expressing the FLAG-TRC8 established in the current study. Co-immunoprecipitation with anti-FLAG antibodies and mass spectrometry analyses revealed a highly specific cytoplasm protein, which was not precipitated with mutant TRC8 lacking the E3 ligase activity (MUT24 cells), and is unlikely to be related to cholesterol metabolism. This protein physically interacted with TRC8 when both proteins were exogenously expressed in culture cells. As long as the protein levels were exogenously elevated or reduced with siRNA, SREBP processing was not affected.[3] It will be of interest to determine whether this protein is related to a putative original function of TRC8 as a tumor suppresser. These findings indicate that the transmembrane region with the SSD and the C-terminal region with the RING domain of TRC8 independently interact with individual associated proteins and exert their respective functions. Further studies are now underway to verify the additional TRC8 biological functions.

In conclusion, evidence is presented that TRC8 is a novel sterol-sensing ER membrane protein. This protein interacts with SREBP-2 and SCAP, thereby inhibiting the association between SCAP and Sec24, which drives the ER to Golgi translocation of the SREBP·SCAP complex via COPII protein-coated vesicles. TRC8 tends to hinder SREBP-2 processing rather than SREBP-1 processing, and affects the transcription of genes related to cholesterol metabolism. The molecular mechanism for this preferentiality should be determined in the near future. The inhibitory effect of TRC8 on cholesterol metabolism reflects its originally proposed function as a tumor suppressor, because tumor cells require large amounts of membrane cholesterol for their rapid growth.

Acknowledgments—We thank Dr. Joseph L. Goldstein for insightful suggestions. We also thank Dr. Ken-ichi Nishida (Daiichi-Sankyo) for expression plasmids and Dr. Norihiko Furuya for helpful discussion. We are grateful to Dr. Kevin Boru of Pacific Edit for review of the manuscript.

REFERENCES

1. Brown, M. S., and Goldstein, J. L. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 11041–11048
2. Goldstein, J. L., DeBose-Boyd, R. A., and Brown, M. S. (2006) *Cell* **124,** 35–46
3. Horton, J. D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 5987–5992
4. Shimano, H., Yahagi, N., Amemiya-Kudo, M., Hasty, A. H., Osuga, J., Tamura, Y., Shionoiri, F., Iizuka, Y., Ohashi, K., Harada, K., Gotoda, T., Ishibashi, S., and Yamada, N. (1999) *J. Biol. Chem.* **274,** 35832–35839
5. Yabe, D., Komuro, R., Liang, G., Goldstein, J. L., and Brown, M. S. (2003) *Proc. Natl. Acad. Sci. U.S.A.* **100,** 3155–3160
6. Yellaturu, C. R., Deng, X., Cagen, L. M., Wilcox, H. G., Mansbach, C. M., 2nd, Siddiqi, S. A., Park, E. A., Raghow, R., and Elam, M. B. (2009) *J. Biol. Chem.* **284,** 7518–7532
7. Rawson, R. B., DeBose-Boyd, R., Goldstein, J. L., and Brown, M. S. (1999) *J. Biol. Chem.* **274,** 28549–28556
8. Matsuda, M., Korn, B. S., Hammer, R. E., Moon, Y. A., Komuro, R., Horton, J. D., Goldstein, J. L., Brown, M. S., and Shimomura, I. (2001) *Genes Dev.* **15,** 1206–1216
9. Engelking, L. J., Kuriyama, H., Hammer, R. E., Horton, J. D., Brown, M. S., Goldstein, J. L., and Liang, G. (2004) *J. Clin. Invest.* **113,** 1168–1175
10. Engelking, L. J., Liang, G., Hammer, R. E., Takaishi, K., Kuriyama, H., Evers, B. M., Li, W. P., Horton, J. D., Goldstein, J. L., and Brown, M. S. (2005) *J. Clin. Invest.* **115,** 2489–2498
11. Gemmill, R. M., West, J. D., Boldog, F., Tanaka, N., Robinson, L. J., Smith, D. I., Li, F., and Drabkin, H. A. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95,** 9572–9577
12. Gemmill, R. M., Bemis, L. T., Lee, J. P., Sozen, M. A., Baron, A., Zeng, C., Erickson, P. F., Hooper, J. E., and Drabkin, H. A. (2002) *Oncogene* **21,** 3507–3516
13. Brauweiler, A., Lorick, K. L., Lee, J. P., Tsai, Y. C., Chan, D., Weissman, A. M., Drabkin, H. A., and Gemmill, R. M. (2007) *Oncogene* **26,** 2263–2271
14. Sato, R., Miyamoto, W., Inoue, J., Terada, T., Imanaka, T., and Maeda, M. (1999) *J. Biol. Chem.* **274,** 24714–24720
15. Sato, R., Inoue, J., Kawabe, Y., Kodama, T., Takano, T., and Maeda, M. (1996) *J. Biol. Chem.* **271,** 26461–26464
16. Inoue, J., Sato, R., and Maeda, M. (1998) *J. Biochem.* **123,** 1191–1198
17. Nadanaka, S., Yoshida, H., Kano, F., Murata, M., and Mori, K. (2004) *Mol. Biol. Cell* **15,** 2537–2548
18. Hirano, Y., Murata, S., Tanaka, K., Shimizu, M., and Sato, R. (2003) *J. Biol. Chem.* **278,** 16809–16819
19. Hirano, Y., Yoshida, M., Shimizu, M., and Sato, R. (2001) *J. Biol. Chem.* **276,** 36431–36437
20. Sakai, J., Rawson, R. B., Espenshade, P. J., Cheng, D., Seegmiller, A. C., Goldstein, J. L., and Brown, M. S. (1998) *Mol. Cell* **2,** 505–514
21. Kanayama, T., Arito, M., So, K., Hachimura, S., Inoue, J., and Sato, R. (2007) *J. Biol. Chem.* **282,** 10290–10298
22. Sato, R., Okamoto, A., Inoue, J., Miyamoto, W., Sakai, Y., Emoto, N., Shimano, H., and Maeda, M. (2000) *J. Biol. Chem.* **275,** 12497–12502
23. Arito, M., Horiba, T., Hachimura, S., Inoue, J., and Sato, R. (2008) *J. Biol. Chem.* **283,** 15224–15231
24. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96,** 11364–11369
25. Okada, T., Haze, K., Nadanaka, S., Yoshida, H., Seidah, N. G., Hirano, Y., Sato, R., Negishi, M., and Mori, K. (2003) *J. Biol. Chem.* **278,** 31024–31032
26. Espenshade, P. J., Li, W. P., and Yabe, D. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 11694–11699
27. Sun, L. P., Li, L., Goldstein, J. L., and Brown, M. S. (2005) *J. Biol. Chem.* **280,** 26483–26490
28. Yang, T., Espenshade, P. J., Wright, M. E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J. L., and Brown, M. S. (2002) *Cell* **110,** 489–500
29. Yabe, D., Xia, Z. P., Adams, C. M., and Rawson, R. B. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 16672–16677
30. Sakai, J., Nohturfft, A., Cheng, D., Ho, Y. K., Brown, M. S., and Goldstein, J. L. (1997) *J. Biol. Chem.* **272,** 20213–20221
31. Sun, L. P., Seemann, J., Goldstein, J. L., and Brown, M. S. (2007) *Proc. Natl. Acad. Sci. U.S.A.* **104,** 6519–6526
32. Sever, N., Yang, T., Brown, M. S., Goldstein, J. L., and DeBose-Boyd, R. A. (2003) *Mol. Cell* **11,** 25–33
33. Song, B. L., and DeBose-Boyd, R. A. (2004) *J. Biol. Chem.* **279,** 28798–28806
34. Song, B. L., Sever, N., and DeBose-Boyd, R. A. (2005) *Mol. Cell* **19,** 829–840
35. Song, B. L., Javitt, N. B., and DeBose-Boyd, R. A. (2005) *Cell Metab.* **1,** 179–189

[3] M. Irisawa, J. Inoue, and R. Sato, unpublished observations.