Ring finger protein 145 (RNF145) is a ubiquitin ligase for sterol-induced degradation of HMG-CoA reductase

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ABSTRACT

Cholesterol biosynthesis is tightly regulated in the cell. For example, high sterol concentrations can stimulate the degradation of the rate-limiting cholesterol biosynthesis enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase, HMGCR). HMGCR is broken down by endoplasmic reticulum (ER) membrane-associated protein complexes consisting of insulin-induced genes (Insigs) and the E3 ubiquitin ligase gp78. Here, we found that HMGCR degradation is partially blunted in Chinese hamster ovary (CHO) cells lacking gp78 (gp78-KO). To identify other ubiquitin ligase(s) that may function together with gp78 in triggering HMGCR degradation, we performed a small-scale shRNA-based screening targeting ER-localized E3s. We found that knockdown of both ring finger protein 145 (Rnf145) and gp78 genes abrogates sterol-induced degradation of HMGCR in CHO cells. We also observed that RNF145 interacts with Insig-1 and -2 proteins and ubiquitinitates HMGCR. Moreover, the tetrapeptide sequence YLYF in the sterol-sensing domain and the C537 residue in the RING finger domain were essential for RNF145 binding to Insigs and RNF145 E3 activity, respectively.

Of note, amino acid substitutions in the YLYF or of Cys-537 completely abolished RNF145-mediated HMGCR degradation. In summary, our study reveals that RNF145, along with gp78, promotes HMGCR degradation in response to elevated sterol levels and identifies residues essential for RNF145 function.

INTRODUCTION

Cholesterol is the most abundant sterol in mammalian cells. It regulates membrane function and serves as the precursor for bile acids and steroid hormones. Cholesterol can either be synthesized through the mevalonate pathway (1) or taken up from diets via Niemann-Pick C1-like 1 (NPC1L1)-mediated absorption (2-5).

HMG-CoA reductase (HMGCR) catalyzes the rate-limiting step in cholesterol biosynthesis in which HMG-CoA is converted to mevalonate. The half-life of HMGCR varies with cellular sterol levels: more than 12 h in sterol-depleted cells and less than 1 h in sterol-overloaded cells (6,7). HMGCR is tightly regulated by sterols at both transcriptional and post-translational levels. High concentrations of cholesterol decrease the transcription of the HMGCR gene by inhibiting the activation of sterol regulatory
element-binding protein 2 (SREBP-2) (8,9). In addition, excess levels of 24, 25-dihydrolanosterol, an intermediate in the mevalonate pathway, promote the ubiquitination and degradation of the HMGCR protein (10-12). Oxysterols can inhibit HMGCR transcription and stimulate HMGCR degradation (13,14). Besides sterols, geranylgeraniol (GGOH), a non-sterol product downstream of mevalonate, acts on the post-ubiquitination step to accelerate sterol-induced HMGCR degradation (7).

The sterol-induced degradation of HMGCR initiates when ER-located proteins Insig-1 and -2 bind to HMGCR and recruit the ubiquitin ligase (E3) gp78 to catalyze ubiquitination (16). The HMGCR protein is eventually degraded in the proteasome. Ufd1 enhances the E3 activity of gp78 and accelerates the degradation of HMGCR (17). Ablation of gp78 in mouse liver increases the stability of HMGCR, Insig-1 and Insig-2 (18,19). The elevated levels of Insigs inhibit the SREBP pathway and decrease cholesterol synthesis (18). These data suggest gp78 is a major E3 essential for HMGCR degradation in hepatocytes.

Besides gp78, TRC8 and MARCH6 are two other ER-located E3s involved in HMGCR degradation (20,21). TRC8 interacts with Insig-1 and -2 and ubiquitinates HMGCR for proteasomal degradation. In addition to sterol-regulated degradation, the basal turnover of HMGCR is mediated by Hrd1, an ER-anchored E3 homologous to gp78 (22,23). Interestingly, sterol-induced HMGCR degradation has been found to persist in gp78-deficient primary mouse embryonic fibroblasts (MEFs) (24), which leads us to speculate that there might be other E3(s) compensating for the function of gp78 in the cultured cells.

In this study, we identified that an ER-anchored E3 named RNF145 catalyzed sterol-induced ubiquitination of HMGCR. Knockout of gp78 or Rnf145 alone had partial or little effect on HMGCR degradation in Chinese hamster ovary (CHO) cells. However, knockout of both genes dramatically blunted sterol-induced degradation of HMGCR. The E3 activity deficient RNF145 (C537A) failed to promote sterol-induced ubiquitination and degradation of HMGCR. Moreover, we found that Insigs were required for RNF145-catalyzed HMGCR degradation, and that RNF145 interacted with Insigs constitutively through its transmembrane domains. We therefore conclude that RNF145 is a new E3 promoting sterol-induced degradation of HMGCR.

RESULTS
Identification of Rnf145 involved in HMGCR degradation
To determine whether gp78 is exclusively responsible for HMGCR degradation, we treated wild-type (WT) CHO and gp78-knockout (gp78-KO) cells with increasing concentrations of 25-hydroxycholesterol (25-HC). Although the degradation of HMGCR was partially impaired when cells were treated with low concentrations of 25-HC (0.03 and 0.1 µg/ml), it could still be completely destroyed when challenged with high concentrations of 25-HC (0.3 and 1 µg/ml) in gp78-KO cells (Fig. 1A and B), suggesting that gp78 is not the only E3 mediating HMGCR degradation. Because previous studies have identified a total of 24 ER-membrane-spanning E3s (25), we next transfected the plasmids expressing shRNAs targeting each E3 together with those expressing HMGCR-T7 and Insig-1-myc into gp78-KO cells and examined sterol-induced degradation of HMGCR (Fig. S1). Compared with negative control (NC) cells where HMGCR degraded rapidly in response to sterols (Fig. 1C, compare lanes 1 and 2), no HMGCR degradation was detected upon Rnf145 deficiency in gp78-KO cells (Fig. 1C, lanes 3-6). These results suggest that RNF145 was involved in HMGCR degradation. Because previous studies have identified a total of 24 ER-membrane-spanning E3s (25), we next transfected the plasmids expressing shRNAs targeting each E3 together with those expressing HMGCR-T7 and Insig-1-myc into gp78-KO cells and examined sterol-induced degradation of HMGCR (Fig. S1). Compared with negative control (NC) cells where HMGCR degraded rapidly in response to sterols (Fig. 1C, compare lanes 1 and 2), no HMGCR degradation was detected upon Rnf145 deficiency in gp78-KO cells (Fig. 1C, lanes 3-6). These results suggest that RNF145 was involved in HMGCR degradation. We further generated Rnf145-knockout (Rnf145-KO) and gp78 plus Rnf145 knockout (Double-KO) CHO cells using CRISPR/Cas9 technique (26). Knockout of gp78 slightly affected HMGCR degradation relative to WT cells (Fig. 1D, lanes 4-6), and knockout of Rnf145 alone had little influence on HMGCR...
degradation (Fig. 1D, lanes 7-9). However, HMGCR degradation was largely blunted in the Double-KO cells (Fig. 1D, lanes 10-12). Next we measured sterol-induced ubiquitination of HMGCR in the cells lacking either E3 or both. Knockout of gp78, Rnf145, or both genes decreased the sterol-induced ubiquitination of HMGCR (Fig. 1E). These results suggest that RNF145 plays a critical role in the sterol-induced degradation of HMGCR.

**RNF145 is an ER-localized ubiquitin ligase mediating HMGCR degradation**

RNF145 is a putative transmembrane ER protein (Fig. 2A) (25). To confirm the subcellular location of RNF145, we performed the immunofluorescence experiments by co-staining transfected RNF145 together with the endogenous ER marker Calnexin. RNF145-FLAG was largely co-localized with Calnexin, indicating that RNF145 is indeed an ER-localized protein (Fig. 2B). Next, we purified the recombinant cytosolic domain (a.a. 511-663) of RNF145 and performed the *in vitro* ubiquitination assay. The recombinant cytosolic domain of gp78 (309-643) was used as a positive control. We found that RNF145 (511-663) could efficiently catalyze the formation of poly-ubiquitin chain in the presence of E1, E2, FLAG-Ubiquitin (FLAG-Ub) and ATP (Fig. 2C). Replacement of the conserved Cys537 residue with alanine (C537A) in the RING finger domain of RNF145 (27), however, completely abolished the E3 ligase activity of RNF145 (Fig. 2D). These results indicate that RNF145 is an ER-localized ubiquitin ligase and the C537 residue is required for its E3 activity.

We then sought to determine whether C537 is required for sterol-induced HMGCR ubiquitination. As shown in Figure 3A, sterols substantially increased the ubiquitination of HMGCR, as evidenced by the high-molecular-weight smears of the immunoprecipitates (lanes 1 and 2). Addition of RNF145 (C537A) completely blocked the ubiquitination of HMGCR induced by sterols (lane 3). Consistent with ubiquitination results, the WT form of RNF145 accelerated HMGCR degradation (Fig. 3B, lanes 1-4), whereas the C537A mutant completely abrogated HMGCR degradation (Fig. 3B, lanes 1, 2 and 5-10). These results suggest that RNF145 acts as a ubiquitin ligase in promoting HMGCR degradation.

**Insigs are required for RNF145-mediated HMGCR degradation**

Insigs are indispensable for sterol-induced degradation of HMGCR mediated by gp78 (7,10,16). Indeed, SRD15 cells, a cell line lacking both Insig-1 and Insig-2 (28), failed to undergo sterol-regulated degradation of HMGCR (Fig. 4A, lanes 4-6). To test whether RNF145-mediated degradation of HMGCR requires Insigs, we generated the SRD15 cell line deficient in *Rnf145* and *gp78* (4KO) (Fig. 4B) that are also insensitive to sterol treatment (Fig. 4A, lanes 7-9). Re-expression of Insig-1, RNF145 alone in 4KO cells did not restore sterol-induced degradation of HMGCR (Fig. 4C, lanes 3-6). Interestingly, co-expression of Insig-1 and RNF145 triggered HMGCR degradation in 4KO cells exposed to sterols (Fig. 4C, lanes 7-8). It was noteworthy that sterol-regulated HMGCR degradation was also detected in 4KO cells co-expressing Insig-1 and gp78 (Fig. 4D, lanes 7-8), suggesting that gp78 and RNF145 may function redundantly in mediating HMGCR degradation. In contrast to these findings, RNF145 (C537A) did not induce the degradation of HMGCR in the reconstitution system even with the presence of Insig-1 (Fig. 4E, lanes 5-8).

We then tested whether RNF145 could interact with Insigs. Figure 5A showed that RNF145 co-immunoprecipitated with both Insig-1 and Insig-2 regardless of sterol levels. Specifically, it was the transmembrane domain (a.a. 1-510) but not the cytosolic domain (a.a. 511-663) of RNF145 that bound to Insig-1 (Fig. 5B). Further, overexpression of the transmembrane domain (a.a. 1-510) of RNF145 effectively blunted sterol-regulated HMGCR degradation (Fig. 5C).

**The sterol-sensing domain of RNF145 is**
crucial for HMGCR degradation
It is known that SCAP and HMGCR harbor the sterol-sensing domain (SSD) for Insig binding and the tetrapeptide YIYF is highly conserved within the SSD (7,15). There is a putative SSD in RNF145 (Fig. 2A). Sequence alignment revealed a YLYF tetrapeptide in RNF145 corresponding to the YIYF found in HMGCR and SCAP (Fig. 6A). To test whether this motif was essential for RNF145-mediated degradation of HMGCR, we mutated YLYF to AAAA and evaluated its function in 4KO cells. Unlike WT RNF145, the RNF145 (YLYF-AAAA) mutant could not elicit the degradation of HMGCR following sterol treatment (Fig. 6B). We next combined the YLYF-AAAA mutation with E3 activity deficiency together. As shown in Figure 6C, RNF145 (C537A & YLYF-AAAA) no longer blocked sterol-regulated degradation of HMGCR. Moreover, the RNF145 (YLYF-AAAA) mutant could not be co-immunoprecipitated with Insig-1 (Fig. 6D). Collectively, these results suggest that the YLYF motif is required for Insig binding as well as RNF145-mediated HMGCR degradation.

DISCUSSION
In the present study, we identified RNF145 as another E3 mediating the degradation of HMGCR through unbiased shRNA screening. RNF145 resides on the ER with putative 14 transmembrane segments, 1 to 5 of which constitutes a SSD that is also present in SCAP, HMGCR, NPC1, NPC1L1 and TRC8 (29-31). The RING finger domain located in the cytosolic C-terminus confers RNF145 E3 activity. RNF145 and TRC8 display similar structures and sequences.

In CHO cells, knockout gp78 partially delayed the turnover of HMGCR in response to low concentrations of sterols and ablation of Rnf145 alone also had little effect (Fig. 1). Notably, knockout of both genes largely abolished sterol-induced degradation of HMGCR (Fig. 1D). Similar to gp78 and TRC8, Insigs are required by RNF145 for HMGCR ubiquitination. The binding between Insigs and HMGCR is regulated by sterol levels, whereas the Insig and E3 (gp78, TRC8 and RNF145) interaction is constitutive. The inactivated mutation (C537A) of RNF145 had a dominant negative effect on HMGCR degradation, a similar mutation was found in gp78 (C356G) (16). Interestingly, HMGCR, SCAP and RNF145 all bind Insigs through the SSD and require the conserved YI(L)YF tetrapeptide.

The question why multiple E3s are involved in the sterol-induced degradation of HMGCR is intriguing. One possibility is that different cells express different levels of E3s in response to distinct signals. In fact, recent studies have identified Rnf145 as an LXR target gene (32,33). We hypothesize that activation of LXR might elevate RNF145 level and subsequently downregulate cholesterol biosynthesis through degrading HMGCR. Another possibility is that the existence of multiple E3s for HMGCR degradation prevents the saturation of specific E3(s) and ensures ER-associated degradation (ERAD) functions properly when HMGCR is degraded.

The protein machineries involved in HMGCR degradation may also participate in other cholesterol-regulating processes. gp78 is the first characterized E3 catalyzing HMGCR ubiquitination (16). It is highly expressed in the liver. Knockout of gp78 in hepatocytes largely blunted the degradation of HMGCR (18). However, gp78 deficiency also stabilizes Insigs (especially Insig-2), resulting in suppressed processing of SREBP and subsequently decreased expression of Hmgcr and other genes in the mevalonate pathway (18). As the protein levels of Insigs were dramatically increased in gp78-deficient cells (18) (Fig. S2), the effects of other E3s such as TRC8 and RNF145 might be boosted, which may explain why HMGCR is still degraded in gp78-KO cells (24). gp78 can also catalyze ubiquitination of acyl-CoA: cholesterol acyltransferase (ACAT) on a cysteine residue (34). In addition to degrading HMGCR, RNF145 triggers the ubiquitination of SCAP and interferes with SCAP binding to COPII, thus inhibiting SREBP-2 maturation.
In summary, we hereby identify that RNF145 is an E3 governing sterol-regulated degradation of HMGCR. Together with previous findings that RNF145 is an LXR-regulated gene and that RNF145 inhibits the SREBP pathway through ubiquitinating SCAP, RNF145 serves as an important negative regulator of cholesterol biosynthesis. Activation of RNF145 may be effective for treating hypercholesterolemia through inhibiting endogenous cholesterol synthesis.

EXPERIMENTAL PROCEDURES

Reagents

We obtained lovastatin, mevalonate and 25-hydroxycholesterol from Sigma; MG132 from Calbiochem; FLAG-Ubiquitin from Boston Biochem; linear polyethylenimine (LPEI) from Polysciences; lipoprotein-deficient serum (LPDS) was prepared from newborn calf serum as described before (35).

Antibodies

Primary antibodies used for immunoblotting were as follows: mouse monoclonal antibody against T7-tag (Novagen); mouse monoclonal antibody P4D1 against ubiquitin and goat polyclonal antibody against Calnexin (Santa Cruz); mouse monoclonal antibody (clone 16B12) against HA-tag (Biolegend); mouse monoclonal antibody (clone AC-15) against β-actin and monoclonal antibody (clone M2) against FLAG-tag (Sigma); mouse monoclonal antibody (clone 10E2) against His-Tag (Abmart); mouse monoclonal antibodies (clone 9E10) against Myc-tag and (clone A9) against HMGCR were prepared from hybridomas (ATCC); Rabbit polyclonal antibodies against gp78 and HMGCR were prepared as described before (18); Rabbit polyclonal antibody against RNF145 (a.a. 511-663) was generated by immunizing rabbits followed by affinity purification with antigens.

Plasmids

The following plasmids were described in the indicated references or constructed by standard molecular cloning techniques: pCMV-HMGCR-T7 encodes full-length hamster HMGCR followed by T7 tag epitope, pCMV-Insig-1-Myc encodes human Insig-1 followed by Myc epitope and pCMV-Insig-2-Myc encodes human Insig-2 followed by Myc epitope, pEF-HA-Ubiquitin encodes human ubiquitin preceded by HA tag epitope (7); pCMV-gp78-FLAG encodes human gp78 followed by 3 × FLAG epitope; pCMV-RNF145-FLAG encodes mouse RNF145 followed by 3 × FLAG epitope, pCMV-RNF145 (1-510)-FLAG encodes the N terminal mouse RNF145 (a.a. 1-510) followed by 3 × FLAG epitope, pCMV-RNF145 (511-663)-FLAG encodes the C terminal mouse RNF145 (a.a. 511-663) followed by 3 × FLAG epitope, pCMV-RNF145 (C537A)-FLAG in which the cysteine-537 was mutated into alanine, pCMV-RNF145 (YLYF-AAAA)-FLAG in which the Y81LYF was mutated into AAAA, pCMV-RNF145 (C537A & YLYF-AAAA)-FLAG in which the cysteine-537 was mutated into alanine and the Y81LYF was mutated into AAAA.

Cell Culture

Medium A contains a 1:1 mixture of Dulbecco’s modified Eagle medium (DMEM) and Ham’s F-12 medium containing 100 units/ml penicillin and 100 μg/ml streptomycin sulfate. Medium B contains Medium A supplemented with 5% FBS. Medium C contains Medium A supplemented with 5% lipoprotein-deficient serum (LPDS), 1 μM lovastatin and 50 μM mevalonate. All cells were kept in Medium B at 37 °C and 5% CO2 and treated as indicated in the figure legends.

Transient Transfection of CHO cells

Cells were set up on day 0 at 8 × 10^5 cells per 60-mm dishes; on day 1, a total of 3 μg DNA and 6 μg LPEI were mixed and transfected to each dish. Medium was refreshed 6 h after transfection.

Generating of knockout cells

CHO cells deficient in gp78 were generated by TALEN technology as described before (20); CHO cells deficient in Rnf145 and
**Immunoblot Analysis**

Cells were lysed with 200 μL RIPA buffer and mixed with loading buffer (final concentration: 23.4 mM Tris-HCl, 5.625 % SDS, 1M urea, 3.75% glycerol, 37.5mM DTT) and incubated at 37 °C for 30 min. Protein concentration of each lysate was quantified by Pierce BCA Protein Assay, and equal amount of total proteins were loaded for SDS-PAGE gels and transferred to PVDF membranes. Membranes were blocked by TBS-Tween (1‰) supplemented with 5% skim milk for 1 h at RT and then incubated with indicated primary antibodies overnight at 4 °C. Membranes were washed 3 times with TBS-Tween and incubated with secondary antibodies (1:5000) diluted in TBS-Tween (1‰) supplemented with 5% skim milk for 1 h at RT followed by at least 3 times wash with TBS-Tween. Quantification of Immunoblot was performed with Image J.

**Immunoprecipitation**

Cells were harvested and lysed in 1 ml of IP buffer (1 × PBS, 0.5% NP-40, 5mM EDTA, 5mM EGTA, 0.1 mM Leupeptin, protease inhibitors, 10 μM MG132 and 10 mM N-ethylmaleimide (NEM)). Lystes were first immunoprecipitated at 4 °C with 3 μg polyclonal antibody against GFP plus 40 μL Protein A/G agarose beads for 1 h, beads were removed by centrifugation, supernatants were then immunoprecipitated with 40 μL anti-FLAG agarose beads at 4 °C for 5 h. After incubation, beads were washed 3 times with the HMG-IP buffer at 4 °C and boiled at 95 °C for 10 min. Aliquots were subjected to immunoblot analysis.

For detecting ubiquitination of endogenous HMGCR, CHO cells were set up on day 0 at 2.5×10^6 per 100-mm dish. Duplicate dishes were treated as described in figure legends. 6 μg polyclonal antibody against HMGCR plus 100 μL Protein A/G agarose beads were used to precipitate HMGCR. Aliquots were subjected to immunoblot analysis.

**In vitro ubiquitination**

Ubiquitination experiments were carried out with 60 nM E1, 50 nM Ubc7, 10 μM FLAG-tagged ubiquitin and 1 μM RNF145 (511-663), 1 μM RNF145-511-663 (C537A) or 300 nM gp78 (309-643) at 37 °C in buffers containing 25mM Tris-HCl, pH 7.4, 2 mM magnesium/ATP, and 0.1 mM DTT. Reactions were stopped by directly adding SDS loading buffer and incubated at 95 °C for 10 min.

**Immunostaining**

Cells were fixed with 4% PFA for 15 min, and then permeabilized with 1 × PBS containing 0.1% triton X-100 for 5 min. Permeabilized cells were blocked with 1% BSA for 30 min, followed by incubation with primary and secondary antibody diluted in 1% BSA for 1h at room temperature (36). Cells were analyzed by Leica confocal scanning laser (Leica SP8) microscope with a 63 × oil immersive objective.

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Author contributions
B.L.S. conceived the research. L.Y.J., W.J., J.W., J.L., X.J.S. and B.L.S. designed the experiments. L.Y.J. and W.J. performed the main experiments and analyzed data. N.T., Y.N.X. and J.L. contributed to the screening experiments. K.Y.W. contributed to the *in vitro* ubiquitination assay. The manuscript was written by L.Y.J. J.L., and B.L.S. with input from all the other authors.

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FIGURE LEGENDS

FIGURE 1. RNF145 is involved in sterol-regulated HMGCR degradation

(A) Wild-type (WT) CHO and gp78-knockout (gp78-KO) CHO cells were depleted of sterol in Medium C for 16 h. Cells were then treated with Medium C supplemented with the indicated concentrations of 25-HC plus 10 mM mevalonate for 5 h. Cells were harvested and subjected to SDS-PAGE followed by immunoblot analysis.

(B) Quantification of the HMGCR protein in (A).

(C) The gp78-KO CHO cells were transfected with plasmids encoding HMGCR-T7, Insig-1-Myc and the shRNA targeting RNF145. After 48 h, cells were depleted of sterol and then treated with or without 0.3 μg/ml 25-HC plus 10 mM mevalonate for 5 h as described in (A). Cells were harvested and subjected to SDS-PAGE followed by immunoblot analysis. Results shown are representative of three independent experiments.

(D) The WT, gp78-KO, Rnf145-KO and Double-KO CHO cells were depleted of sterol and then treated with indicated concentration of 25-HC plus 10 mM mevalonate for 5 h as described in (A). Cells were harvested and subjected to SDS-PAGE followed by immunoblot analysis. Asterisk indicates non-specific bands. Results shown are representative of two independent experiments.

(E) The WT, gp78-KO, Rnf145-KO and Double-KO CHO cells were depleted of sterol and then treated with 10 μM MG132 in the presence or absence of 1 μg/ml 25-HC and 10 mM mevalonate for 2 h. Cells were harvested and lysates were immunoprecipitated with anti-HMGCR antibody plus protein A/G beads. Pellet fractions were immunoblotted with anti-ubiquitin (P4D1) and polyclonal anti-HMGCR antibodies.

FIGURE 2. RNF145 is an ER-localized ubiquitin ligase

(A) Predicted topology of RNF145. SSD, sterol-sensing domain; YLYF, the amino acids from 81 to 84 of RNF145.

(B) Subcellular localization of RNF145. HeLa cells were transfected with the plasmid encoding RNF145-FLAG and stained with the anti-FLAG and anti-calnexin antibodies. Scale bar, 10 μm.

(C) In vitro ubiquitination assay showing that RNF145 (511-663) possesses E3 activity. Recombinant proteins including E1, E2 (Ubc7), FLAG-ubiquitin, RNF145 (511-663) and gp78 (309-643) were added into the reaction system as indicated. After incubation at 37 °C for 15 min, samples were subjected to SDS-PAGE followed by immunoblot analysis.

(D) In vitro ubiquitination assay comparing RNF145 (511-663) and RNF145 (511-663) (C537A). Experiments were carried out as described in (C).
FIGURE 3. The E3 activity deficient RNF145 blocks sterol-induced ubiquitination and degradation of HMGCR

(A) CHO cells were transfected with indicated plasmids, depleted of sterol and treated with 10 μM MG132 in the presence or absence of 1 μg/ml 25-HC and 10 mM mevalonate for 3 h. Cells were harvested and lysates were immunoprecipitated with anti-FLAG beads. Input and pellet fractions were immunoblotted with anti-HA, anti-FLAG and polyclonal anti-RNF145 antibodies. Results shown are representative of two independent experiments.

(B) CHO cells were transfected with indicated plasmids, depleted of sterol and treated with or without 1 μg/ml 25-HC plus 10 mM Mevalonate for 5 h. Cells were harvested and subjected to SDS-PAGE and immunoblot analysis. Results shown are representative of two independent experiments.

FIGURE 4. Insig is required for RNF145-mediated degradation of HMGCR

(A) The CHO, SRD15 (deficient in Insig-1 and -2) and 4KO (deficient in Insig-1, Insig-2, gp78 and RNF145) cells were incubated in the indicated media for 16 h. Cells were harvested and subjected to SDS-PAGE followed by immunoblot analysis. LPDS, lipoprotein-deficient serum.

(B) Sequence alignment of the gRNA targeting region showing that Rnf145 is knocked out. CDS: coding sequence.

(C-E) The 4KO cells were transfected with indicated plasmids, depleted of sterol and treated with or without 1 μg/mL 25-HC plus 10 mM Mevalonate for 5 h. Cells were harvested and subjected to SDS-PAGE and immunoblot analysis.

FIGURE 5. RNF145 interacts with Insigs through its transmembrane domain

(A) CHO cells were transfected with indicated plasmids, depleted of sterol and treated with or without 1 μg/ml 25-HC plus 10 mM Mevalonate for 2 h. The cell lysates were immunoprecipitated with anti-Myc beads. Pellets and input were blotted with indicated antibodies. Results shown are representative of two independent experiments.

(B) CHO cells were transfected with indicated plasmids and cultured in medium B. 48 h later, cells were harvested and lysates were immunoprecipitated with anti-Myc beads. Pellets and input were blotted with indicated antibodies.

(C) CHO cells were transfected with indicated plasmids, depleted of sterol and treated with or without 1 μg/ml 25-HC plus 10 mM Mevalonate for 5 h. Cells were harvested and subjected to SDS-PAGE followed by immunoblot analysis. Results shown are representative of two independent experiments.

FIGURE 6. The sterol-sensing domain of RNF145 is essential for Insig binding

(A) Sequence alignment of human RNF145, HMGCR and SCAP. Invariant amino acids are shaded in grey. The conserved motif Yl(L)YF was boxed.

(B, C) The 4KO cells (B) and CHO cells (C) were transfected with indicated plasmids, depleted of sterol and treated with or without 1 μg/ml 25-HC plus 10 mM Mevalonate for 5 h. Cells were harvested and subjected to SDS-PAGE followed by immunoblot analysis.

(D) CHO cells were transfected with indicated plasmids and cultured in medium B. 48 h later, cells were harvested and lysates were immunoprecipitated with anti-Myc beads. Pellets and input were blotted with indicated antibodies.
Figure 1

**A**

| Mevalonate (10 mM) | WT | gp78-KO |
|-------------------|----|---------|
| 25-HC (μg/ml)     | 1  | +       |
|                   | -  | +       |
|                   | -  | -       |
|                   | -  | +       |
|                   | -  | -       |
|                   | -  | -       |
|                   | -  | +       |
|                   | -  | -       |
|                   | -  | -       |
|                   | -  | +       |
|                   | -  | -       |
|                   | -  | -       |

**B**

Relative HMGCR abundance

WT
gp78-KO

25-HC (μg/ml)

0 0.03 0.1 0.3 1

0.0 0.5 1.0

**C**

| pCMV-HMGCR-T7 | gp78-KO |
|----------------|---------|
| +              | +       |
| -              | -       |

| pCMV-Insig-1-Myc |
|------------------|
| +                |
| -                |

| pU6-shRNA       |
|------------------|
| NC 0.5 μg       |
| RNF145 0.2 μg   |
| RNF145 0.5 μg   |

25-HC + Mevalonate

| 25-HC (μg/ml) |
|---------------|
| 1 2 3 4 5 6 7 8 9 10 11 12 |

**D**

| Mevalonate (10 mM) | WT | gp78-KO | Rnf145-KO | Double-KO |
|--------------------|----|---------|-----------|-----------|
| 25-HC (μg/ml)      | -  | +       | -         | +         |
|                    | +  | -       | +         | -         |
|                    | -  | +       | -         | +         |
|                    | -  | -       | +         | -         |
|                    | -  | -       | -         | +         |
|                    | -  | +       | -         | +         |
|                    | -  | -       | -         | +         |
|                    | -  | -       | +         | -         |
|                    | -  | -       | -         | +         |
|                    | -  | +       | -         | +         |
|                    | -  | -       | -         | +         |
|                    | -  | -       | +         | -         |

**E**

| 25-HC + Mevalonate |
|--------------------|
| -                  |
| -                  |
| +                  |
| -                  |
| -                  |
| +                  |
| -                  |
| -                  |
| -                  |
| -                  |

IP: HMGCR

Ubiquitin (IB: P4D1)

HMGCR (IB: HMGCR)
Figure 2

(A) Diagram showing the localization of the RING finger domain (SSD) within the Cytosol and ER Lumen, specifically highlighting the region 511-663 with amino acids YLYF.

(B) Immunoblot showing Calnexin Merge with bands for RNF145-C537A-FLAG and Coomassie blue.

(C) Table summarizing the effects of different components on ubiquitination.

|                  | E1 | E2 | FLAG-Ub | ATP | His-RNF145 (511-663) | His-gp78 (309-643) | Time (min) |
|------------------|----|----|---------|-----|----------------------|-------------------|------------|
|                  | +  | +  | +       | +   | −                    | +                 | 0 15 0 15 15 15 15 0 15 |
|                  | +  | +  | +       | +   | +                    | −                 | 1 2 3 4 5 6 8 9 10 |
|                  | +  | +  | +       | +   | −                    | −                 | 12345 |
|                  | +  | +  | +       | +   | −                    | +                 | 12345 |

(D) Table showing the effects of His-RNF145 on ubiquitination over time (min).

|                  | His-RNF145 (511-663) | WT | CS37A |
|------------------|-----------------------|----|-------|
| Time (min)       | 0 3 30                | 0 3 30 |
| IB: FLAG         | poly-Ubs              | E2-Ub | di-Ubs | mono-Ub |
| IB: His          | His-RNF145 (511-663)  | 20 25 30 50 100 | -15 | -20 | -25 | -30 | -35 | -40 | -45 | -50 | -55 | -60 | -65 | -70 | -75 | -80 | -85 | -90 | -95 | -100 |
Figure 3

A

|                | 1  | 2  | 3   |
|----------------|----|----|-----|
| pCMV-HMGCR-FLAG| +  |    |     |
| pCMV-Insig-1-Myc| +  |    |     |
| pEF-HA-Ubiquitin| +  |    |     |
| pCMV-RNF145 (C537A) |−− + | +  |     |
| 25-HC + Mevalonate |−− + | +  |     |

B

|                | 1  | 2  | 3   |
|----------------|----|----|-----|
| pCMV-HMGCR-T7  | +  |    |     |
| pCMV-Insig-1-Myc| +  |    |     |
| pCMV-RNF145-FLAG (ng) |−  100  |−  |−   |
| pCMV-RNF145 (C537A)-FLAG (ng) |−  30  |100  |300  |
| 25-HC + Mevalonate |−  +  |−  +  |−   |

- IP: FLAG
- Pellet
- Ubiquitin (IB: HA)
- HMGCR (IB: T7)
- RNF145 (IB: FLAG)
- Actin (IB: Actin)
**Figure 4**

### A

|                | CHO | SRD15 | 4KO |
|----------------|-----|-------|-----|
| FBS (5%)       | +   | −     | −   |
| LPDS (5%)      | −   | +     | +   |
| Lovastatin (1 μM) | −   | +     | +   |
| 25-HC + Mevalonate | −   | −     | −   |

### B

**RNF145 CDS:**

- **WT:** GATGTCCTCTACAGATGGGACG
- **4KO:** GATGTCCTCT..AGATGGGACG

### C

|                | pCMV-HMGCR-T7 | + |
|----------------|---------------|---|
| pCMV-Insig-1-Myc | −            | + |
| pCMV-RNF145-FLAG | −            | + |
| 25-HC + Mevalonate | −            | + |

### D

|                | pCMV-HMGCR-T7 | + |
|----------------|---------------|---|
| pCMV-Insig-1-Myc | −            | + |
| pCMV-gp78-FLAG   | −            | + |
| 25-HC + Mevalonate | −            | + |

### E

|                | pCMV-HMGCR-T7 | + |
|----------------|---------------|---|
| pCMV-Insig-1-Myc | −            | + |
| pCMV-RNF145-FLAG (ng) | −       | 50 |
| pCMV-RNF145 (C537A)-FLAG (ng) | −       | 15 |
| 25-HC + Mevalonate | −            | + |

### Notes

- **CHO** represents CHO cell line.
- **SRD15** and **4KO** represent different conditions or treatments.
- **FBS (5%)** and **LPDS (5%)** indicate the presence of fetal bovine serum and lipoprotein-deficient serum, respectively.
- **Lovastatin (1 μM)** indicates the addition ofLovastatin to the culture medium.
- **25-HC + Mevalonate** refers to the presence of 25-hydroxycholesterol and mevalonate.

### Diagrams

- **HMGCR (IB: HMGCR)**
- **gp78 (IB: gp78)**
- **Actin (IB: Actin)**
- **RNF145 (IB: FLAG)**
- **Insig-1 (IB: Myc)**
- **HMGCR (IB: T7)**
- **Actin (IB: Actin)**

**KDa:**

- HMGCR: 100 kDa
- HMGCR (IB: T7): 37 kDa
- Insig-1 (IB: Myc): 37 kDa
- RNF145 (IB: FLAG): 75 kDa
- Actin (IB: Actin): 37 kDa
Figure 6

A

RNF145 VLTLPRQHLVQLLYFLLGFLYTHLGYAGHQIS 68-97
HMGCR IILTIT-RCIAILYIYIQFONLRLGSKYI 63-91
SCAP IPLVTT-YIILFAVYIYSTRKIDMVKS 286-314

B

|                       | pCMV-HMGCR-T7 | pCMV-Insig-1-Myc |
|-----------------------|---------------|------------------|
| pCMV-RNF145-FLAG (ng) | -  20         | 50               |
| pCMV-RNF145 (YLYF-AAAA)-FLAG (ng) | - - - - - 50 | 100              |
| 25-HC + Mevalonate    | - + + + + + + |

C

|                       | pCMV-HMGCR-T7 | pCMV-Insig-1-Myc |
|-----------------------|---------------|------------------|
| pCMV-RNF145 (C537A)-FLAG | - + -       |
| pCMV-RNF145 (C537A & YLYF-AAAA)-FLAG | - - +    |
| 25-HC + Mevalonate    | - + + + + +   |

D

|                      | pCMV-RNF145 (C537A)-FLAG |
|----------------------|--------------------------|
|                      | WT | YLYF-AAAA |
| pCMV-Insig-1-Myc (ng) | -  | 15 50 150 |
|                       | -  | 30 100 300|

|          | IP: Myc | Pellet |
|----------|---------|--------|
| RNF145 (IB: FLAG) | -75     |
| Insig-1 (IB: Myc)  | -37     |

|          | IP: Myc | Pellet |
|----------|---------|--------|
| RNF145 (IB: FLAG) | -75     |
| Insig-1 (IB: Myc)  | -37     |

|          | Input |
|----------|-------|
| RNF145 (IB: FLAG) | -75    |
| Insig-1 (IB: Myc)  | -37    |
Ring finger protein 145 (RNF145) is a ubiquitin ligase for sterol-induced degradation of HMG-CoA reductase

Lu-Yi Jiang, Wei Jiang, Na Tian, Yan-Ni Xiong, Jie Liu, Jian Wei, Kai-Yue Wu, Jie Luo, Xiong-Jie Shi and Bao-Liang Song

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