

**Vitamin D metabolite, 25-Hydroxyvitamin D, regulates lipid metabolism  
by inducing degradation of SREBP/SCAP**

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## Summary

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that control lipid homeostasis. SREBP activation is regulated by a negative feedback loop in which sterols bind to SREBP cleavage-activating protein (SCAP), an escort protein essential for SREBP activation, or to Insigs (ER anchor proteins), sequestering the SREBP-SCAP-Insig complex in the ER. We screened a chemical library of endogenous molecules and identified 25-hydroxyvitamin D (25OHD) as an inhibitor of SREBP activation. Unlike sterols and other SREBP inhibitors, 25OHD impairs SREBP activation by inducing proteolytic processing and ubiquitin-mediated degradation of SCAP, thereby decreasing SREBP levels independently of the vitamin D receptor. Vitamin D supplementation has been proposed to reduce the risk of metabolic diseases, but the mechanisms are unknown. The present results suggest a previously unrecognized molecular mechanism of vitamin D-mediated lipid control that might be useful in the treatment of metabolic diseases.

## Introduction

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that control lipid metabolism by stimulating expression of lipogenic genes (Brown and Goldstein, 1997; Goldstein et al., 2006). Newly synthesized SREBPs are localized on the endoplasmic reticulum (ER) membrane, where they bind to SREBP cleavage-activating protein (SCAP), an escort protein of SREBPs. When cellular sterol levels are low, the SREBP-SCAP complex is transported from the ER to the Golgi apparatus, where SREBPs are cleaved sequentially by site-1 protease (S1P) and site-2 protease (S2P), liberating the NH<sub>2</sub>-terminal transcription factor domain of SREBPs. The mature forms translocate to the nucleus, where they activate lipogenic genes. The activation of SREBP is tightly regulated by a negative feedback loop in which 25-hydroxycholesterol (25-HC) and cholesterol bind to SCAP and Insigs (ER anchor proteins), respectively, inducing the SCAP-Insig interaction and resulting in retention of the SREBP-SCAP complex in the ER (Radhakrishnan et al., 2007). In the present study, we screened a chemical library to identify secosteroid molecules that inhibit SREBP activation. The results

showed that 25-hydroxyvitamin D (25OHD) inhibits SREBP activation independently of the vitamin D receptor (VDR).

## RESULTS

### Screening for endogenous inhibitors of SREBP

We screened a chemical library of 269 endogenous lipid-related molecules, using an SREBP-responsive luciferase reporter construct and a constitutively active  $\beta$ -galactosidase ( $\beta$ -gal) reporter gene, co-transfected into Chinese hamster ovary (CHO)-K1 cells (Kamisuki et al., 2009). Eleven of the screened molecules decreased the lipid-free serum-induced expression of the reporter gene by more than 40% at 1 or 10  $\mu$ M. These molecules were further evaluated using a secreted alkaline phosphatase, fused with an SREBP-2 fragment lacking the NH<sub>2</sub>-terminal DNA-binding domain (PLAP-BP2<sub>513-1141</sub>), to monitor the ER-Golgi translocation and proteolytic activation process of SREBP through changes in the chemiluminescence of a phosphatase substrate (Sakai et al., 1998). When cells were co-transfected with plasmids encoding PLAP-BP2<sub>513-1141</sub> and SCAP, elimination of lipids from the serum induced secretion of PLAP phosphatase and generated chemiluminescence signals. Decreased PLAP secretion at levels comparable to that induced by a mixture of 25-HC and cholesterol was observed for three hydroxylated vitamin D metabolites: 25-hydroxyvitamin D [25OHD], 1 $\alpha$ ,25-dihydroxyvitamin D [1,25(OH)<sub>2</sub>D], and 24R,25-dihydroxyvitamin D [24,25(OH)<sub>2</sub>D] (**Figures 1A and 1B**). Results of the luciferase reporter assays showed that 25OHD was the most potent, with an IC<sub>50</sub> value of  $\sim$ 1  $\mu$ M (**Figure 1C**).

### 25OHD downregulates SREBP-target genes via a unique mechanism

Quantitative RT-PCR (qPCR) experiments showed that 25OHD decreased the mRNA levels of SREBP target genes (Brown and Goldstein, 1997) in CHO-K1 cells (**Figure 1D**). Metabolic analysis confirmed that 25OHD decreased cellular levels of palmitic acid, oleic acid, and stearic acid, consistent with SREBP suppression (**Figure 1E**). The 25OHD-mediated inhibition of SREBP activation was further confirmed by western blot analysis (**Figure 1F**). It was previously shown that 25-HC and cholesterol caused the accumulation of the precursor form

of SREBP-2, with a concomitant decrease in the mature form of SREBP-2 (Adams et al., 2004). In contrast, treatment with the hydroxylated vitamin D metabolites diminished protein levels of both the mature and precursor forms, suggesting that they inhibit SREBPs through a different mechanism. 25OHD was most effective in decreasing SREBP levels, consistent with the results of the luciferase reporter assays. 25OHD is the most abundant endogenous vitamin D metabolite, and is produced in the liver, a primary site of lipogenesis (Ponchon and DeLuca, 1969). Therefore, we selected 25OHD for further analysis.

### **25OHD induces degradation of SCAP**

The stability of SREBPs depends on the escort protein, SCAP, which is necessary for activation of all three SREBP isoforms (Brown et al., 2002; Radhakrishnan et al., 2004). SREBP levels are essentially undetectable in SCAP-deficient CHO-K1 cells and in hepatic SCAP-deficient mice (Matsuda et al., 2001; Moon et al., 2012; Rawson et al., 1999). Results of western blot analysis indicated that treatment with 25OHD reduced the levels of both endogenous SCAP and SREBP-2 in a time-dependent manner (**Figure 2A**), while qPCR analysis showed that levels of SCAP mRNA were unchanged (**Figure 1D**). Treatment of CHO-K1 cells with 25OHD for 12 h negated CMV promoter-driven overexpression of Flag-tagged SCAP(1-767), a truncated version of SCAP often used in studies with CHO-K1 cells (Brown et al., 2002; Radhakrishnan et al., 2004), whereas treatment with 25-HC had no detectable effect (**Figure 2B**). Remarkably, non-hydroxylated vitamin D had no effects on SCAP levels, indicating that the hydroxylation is required. The decrease in SCAP protein levels was accelerated after protein synthesis was blocked by cycloheximide, suggesting that 25OHD promotes protein degradation of SCAP (**Figure 2C**). The protein levels of other ER membrane-bound proteins – calnexin and activating transcription factor 6 (ATF6) – were unchanged (**Figure S1**). These results collectively indicate that 25OHD promotes selective degradation of the SREBP-SCAP complex.

Pharmacological inhibition of site-1 protease has been reported to result in lysosomal degradation of SCAP (Shao and Espenshade, 2014). Treatment with ammonium chloride, a general lysosome inhibitor, had no detectable effects on the 25OHD-induced decrease in SCAP levels (**Figure 2D**). In contrast, addition of MG-132, a general proteasome inhibitor,

counteracted the 25OHD-induced decrease in the protein levels of Flag-tagged SCAP(1-767) (**Figure 2E**). Treatment of cells with 25OHD in the presence of MG-132 resulted in polyubiquitination of Myc-tagged SCAP(1-767), in contrast to treatment with 25-HC (**Figure 2F**). These results suggest that 25OHD stimulates the ubiquitin-proteasome degradation of SCAP.

The ER membrane protein, 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, is susceptible to ubiquitin-proteasome degradation, mediated by its sterols-dependent interaction with Insig-1 or -2 (Jo et al., 2011; Sever et al., 2003; Song et al., 2005). We performed siRNA knockdown experiments to examine whether Insigs are involved in the 25OHD-induced degradation of SCAP. Knockdown of Insig-1 or -2, and of the Insig-binding E3 ubiquitin ligases, gp78 and TRC8, failed to restore protein levels of Flag-tagged SCAP(1-767) (**Figure S2A and S2B**). Furthermore, 25OHD retained its degradation-inducing activity in SCAP point and deletion mutants (Yabe et al., 2002; Yang et al., 2002; Yang et al., 2000) that lacked Insig-binding ability (**Figure S2C and S2D**). Although an E3 ligase for SCAP degradation remains unknown, these results indicate that Insigs are not required for 25OHD-induced degradation of SCAP.

## **25OHD induces proteolytic cleavage of SCAP prior to its proteasomal degradation**

We next examined the effects of the proteasome inhibitor, MG-132, on the 25OHD-induced degradation of endogenous, full-length SCAP. Treatment with MG-132 restored SCAP levels in cells exposed to 25OHD. However, instead of the full-length SCAP, we observed accumulation of a ~100-KDa SCAP fragment in the presence of 25OHD (**Figure 3A**) whereas no such fragments were detected in the presence of 25-HC (**Figure S3A**). Pulse-chase experiments using HaloTag technology, which permits specific, pulse labeling of SCAP protein with a fluorescent molecule in cultured cells, confirmed that the SCAP fragment is a cleavage product of full length SCAP (**Figure 3B**).

Addition of a protease inhibitor cocktail and MG-132 impaired the 25OHD-induced degradation and cleavage of endogenous SCAP (**Figure 3C**). Remarkably, treatment of cells with the protease inhibitor cocktail alone restored the full-length SCAP, suggesting that

25OHD induces proteolytic processing of SCAP prior to its proteasomal degradation (**Figure 3C and S3A**). When the cells were treated with each protease inhibitor contained in the cocktail, AEBSF, a serine protease inhibitor, most potently impaired the proteolytic cleavage of endogenous SCAP (**Figure 3D**), suggesting that a serine protease is responsible for the 25OHD-induced proteolysis of SCAP.

To gain insight into the cleavage site(s), we transfected CHO-K1 cells with an expression vector encoding full-length SCAP Flag-tagged at the NH<sub>2</sub> terminus, and treated them with MG-132. Western blot analysis with a Flag antibody showed a band at ~100 KDa. No such bands were detected when we used the cells transfected with a vector encoding full-length SCAP Flag-tagged at the COOH terminus (**Figure 3E**). These results collectively suggest that 25OHD induces proteolytic cleavage of the ~40 KDa COOH-terminus of SCAP, which is required for its subsequent proteosomal degradation.

To examine the effects of inhibiting the SCAP processing on degradation of SREBP-SCAP, we initially constructed 11 expression vectors each encoding a distinct SCAP deletion mutant (**D1-11; Figure S4A**). Their transfection into CHO-K1 cells and western blot analysis revealed that two mutants, **D4** and **D5**, were resistant to 25OHD-mediated cleavage, suggesting that the processing site is located in the segment encompassing amino acids 860-879 (**Figure S4B**). To further narrow down the processing site, we constructed additional five vectors each encoding deletion mutant that lacks a 6-aa peptide segment from amino acids 860-879 (**D12-16; Figure S4C**). Evident resistance to the 25OHD-mediated cleavage was observed in two mutants, **D13** and **D14**, whose deleted peptide sequences encompasses amino acids 864-872, ALFGDQPDL (**Figure S4D**). Stable expression of a resistant mutant (**D14; SCAP $\Delta$ 867-872**) restored protein levels of SCAP and SREBP, whereas stable expression of wild type SCAP or a non-resistant mutant (**D12; SCAP $\Delta$ 864-869**) had no detectable effects on the degradation (**Figure 4**). These results suggest that the COOH-terminal SCAP processing is required for the 25OHD-mediated degradation of SREBP-SCAP.

## Potential molecular targets of 25OHD

Vitamin D receptor (VDR), a canonical receptor of vitamin D metabolites (McDonnell et al., 1987), is unlikely to be a direct molecular target of SREBP-SCAP degradation. The most potent endogenous VDR ligand is 1,25(OH)<sub>2</sub>D, which has a nM affinity for VDR, ~500-fold lower than the affinity of 25OHD or 24,25(OH)<sub>2</sub>D (Lou et al., 2010). The two latter compounds have been considered as a precursor of 1,25(OH)<sub>2</sub>D or a metabolite of 25OHD, respectively. However, 25OHD-treated cells exhibited a higher degree of SREBP degradation than cells treated with 1,25(OH)<sub>2</sub>D or 24,25(OH)<sub>2</sub>D (**Figure 1F**), providing evidence that VDR is an unlikely target. In fact, siRNA knockdown of VDR in human embryonic kidney (HEK) 293 cells failed to rescue Flag-tagged SCAP(1-767) from 25OHD-induced degradation (**Figure S5**).

To determine whether 25OHD interacts directly with SCAP, we prepared a photo-affinity probe analog of 25OHD. Structure-activity relationship studies showed that a synthetic 25OHD analog, modified at the C-1 position with an alpha configuration (1 $\alpha$ -NHBoc-25OHD; **Figure 5A**), maintained the ability to inhibit SREBP activation. Therefore, we designed affinity probes in which the C-1 position of 25OHD was conjugated with biotin and photoreactive diazirine groups through a polyethyleneglycol linker (diastereomers, molecules **1** and **1'** in **Figure 5B**). Molecules **1** and **1'**, and a negative control (molecule **2**), were incubated with cell lysates of CHO-K1 cells expressing both Flag-tagged SCAP and Insig, followed by ultraviolet (UV) irradiation. Biotinylated proteins were then purified with avidin-conjugated agarose. Western blot analysis showed biotinylation of Flag-tagged SCAP in the presence of molecules **1** and **1'** (10  $\mu$ M), but not molecule **2** (**Figure 5C**). None of the three molecules caused biotinylation of Flag-tagged Insig. The biotinylation of Flag-tagged SCAP by molecule **1** was dose-dependent and was competitively reduced by excess amounts of 25OHD (**Figure 5D**). Although we cannot exclude the existence of other molecular targets, these results suggest that 25OHD interacts physically with SCAP.

To gain insights into the binding site of 25OHD, we also performed competition assays with cholesterol, which has been reported to interact with the sterol-sensing domain of SCAP (transmembrane domains 2-5) (Brown and Goldstein, 2009). Biotinylation of Flag-tagged SCAP by molecule **1** was partially reduced by excess amounts of cholesterol. The extent of its

competition was less than that of 25OHD itself, possibly due to partially overlapped binding sites of the two molecules or a relatively low binding affinity of cholesterol (**Figure S3B**).

## DISCUSSION

Vitamin D deficiency is increasing worldwide prevalence and has been implicated in the pathogenesis of various diseases, including metabolic syndromes and bone diseases (Holick, 2007). The relationship between 25OHD status and lipid levels has been known in humans for more than 20 years (Auwerx et al., 1992). Vitamin D supplementation was also found to increase the serum level of 25OHD, which is inversely correlated with both the severity of metabolic syndromes (Botella-Carretero et al., 2007; Ford et al., 2005) and body mass index (Bouillon et al., 2014). These epidemiological observations of 25OHD deficiency have been attributed to low concentrations of its metabolite, 1,25(OH)<sub>2</sub>D, a VDR agonist. VDR knockout mice exhibited spontaneous liver fibrosis (Ding et al., 2013), and administration of 1,25(OH)<sub>2</sub>D attenuated hepatic steatosis induced in rats by a high fat diet (Yin et al., 2012). On the other hand, 25OHD, a non-VDR ligand, has generally been considered as a biologically inactive, biosynthetic intermediate of 1,25(OH)<sub>2</sub>D, although its circulating concentrations are much higher than that of 1,25(OH)<sub>2</sub>D. The present study identified 25OHD as an inhibitor of SREBP activation, suggesting that 25OHD and 1,25(OH)<sub>2</sub>D act together to control lipogenesis. It is noteworthy that the 25OHD-mediated degradation of SREBP-SCAP is non-genomic and independent from VDR as supported by the VDR silencing studies and the selectivity (25OHD>1,25(OH)<sub>2</sub>D).

We propose a mechanism of action of the 25OHD-induced degradation of SREBP-SCAP, based on results of the present study. In this model (**Figure 6**), the physical association of 25OHD with SCAP initially promotes proteolytic removal of the COOH-terminal segment of SCAP by a serine protease. Identity of the protease and exact cleavage sites remain unknown. The removed segment of SCAP includes the SREBP-binding domain, and its removal promotes 25OHD-dependent polyubiquitination of SCAP, leading to its proteasomal degradation. It remains unclear why this occurs. The release of SCAP from SREBP, or the conformational alteration of SCAP, might potentiate the ubiquitination activity of 25OHD by

exposing ubiquitination sites or recruiting a yet-to-be-identified ubiquitin ligase. The proteasome-mediated degradation of SCAP destabilizes SREBP and ultimately downregulates SREBP-responsive lipogenic genes.

Several other SCAP-binding SREBP inhibitors have been reported. Cholesterol and two non-endogenous molecules, fatostatin and betulin, stabilize the SREBP-SCAP complex in the ER by interacting with SCAP (Kamisuki et al., 2009; Radhakrishnan et al., 2004; Tang et al., 2011). In contrast, 25OHD impairs SREBP through a previously unknown mechanism, suggesting a new opportunity for pharmacological control of SREBP. SREBP has increasingly been recognized as a potential target for intervention of cancers and metabolic disorders. Thus, 25OHD derivatives that lack VDR activity, but inhibit SREBPs, might provide new approaches for drug development.

The physiological impacts of 25OHD-induced inhibition of SREBP on whole-body lipogenesis remain unclear. 25OHD levels in the body are measured as concentrations circulating in the serum or plasma. Although the optimal 25OHD serum concentration is under debate (Hollis, 2005), a desired level is usually considered to be 50-100 nM (Fuleihan et al., 2015), which is lower than the IC<sub>50</sub> value observed in the SREBP reporter gene assay (~1 μM). Moreover, interpretation of these cell culture experiments may need caution because human serum contains vitamin D-binding protein (DBP) that limits availability of free 25OHD (Nielson et al., 2016a; Nielson et al., 2016b). Nonetheless, the dose-responsive curves in the presence of 5% fetal bovine serum (**Figure 1C**) suggests moderate but detectable impacts of 25OHD on SREBP-mediated gene expression at a physiological concentration (100 nM).

One possibility is that 25OHD might work together with cholesterol and 25-HC. In fact, 25OHD remained capable of inducing the degradation of SCAP and SREBP even when SREBP activation is impaired by 25-HC or cholesterol (**Figure S3C**). Another possibility is that 25OHD might be accumulated in the liver, a major site of SREBP expression and lipid synthesis. In the liver, vitamin D from the diet or generated in the skin by exposure to sunlight is hydroxylated at the C-25 position, forming 25OHD (Bouillon et al., 1995; Brommage and DeLuca, 1985). Localized hepatic 25OHD might be responsible for phenotypes similar to those associated with liver-specific deletion of SCAP, which has been reported to prevent diabetic fatty livers and steatosis in mice (Moon et al., 2012). When examined in Hepa1-6, a mouse

hepatocyte cell line (**Figure S6**), 25OHD did decrease the levels of SREBP, suggesting that 25OHD is capable of inhibiting the SREBP activation in hepatocytes. Nevertheless, 25OHD had limited effects on the SCAP protein levels. Perhaps, response of total SCAP levels to 25OHD might depend on cell types and cell lines, which may have varied expression patterns of SCAP and availability of SCAP-processing proteases. A limitation of this study is that the potential mechanism of 25OHD was not investigated in various types of cells involved in the regulation of lipid metabolism. Such further studies are currently underway.

25OHD status has been proposed to be associated with liver physiology. Severe non-alcoholic fatty liver disease (NAFLD) is often associated with vitamin D deficiency (Targher et al., 2007). Furthermore, a recent genome-wide association study of NAFLD patients identified four major single-nucleotide polymorphisms, including one in the gene that encodes vitamin D-binding protein, the main carrier protein for 25OHD (Adams et al., 2013). Two members of the cytochrome P450 family, CYP2R1 and CYP27A1, are considered to be the major 25-hydroxylating enzymes of vitamin D in the liver. Double-knockout of these enzymes in mice resulted in enlarged livers (Barchetta et al., 2012), suggesting their relationship with hepatic lipogenesis. However, CYP27A1 is also involved in bile acid synthesis, which downregulates triacylglycerol levels, so the liver phenotype might not be solely due to a lack of production of 25OHD. While in-vivo studies are needed to confirm the physiological impacts of the 25OH-mediated SREBP inhibition, results of the present study provide evidence of a previously unknown molecular mechanism of vitamin D-mediated lipid control.

## Significance

**To our knowledge, this is the first demonstration that 25OHD inhibits SREBP, a master regulator of lipogenesis. The hydroxylated vitamin D metabolite impairs SREBP-SCAP through a previously unknown mechanism: interaction of 25OHD with SCAP induces processing of SCAP, leading to its ubiquitin-mediated degradation. The results suggest a new opportunity for pharmacological control of SREBP-SCAP, an increasingly recognized potential drug target for cancers and metabolic disorders, by synthetic 25OHD analogs.**

## **Experimental procedure**

### **Cell culture**

CHO-K1 cells were maintained in a medium A (1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle medium, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin sulfate, and 5% (v/v) fetal bovine serum) at 37 °C in a humidified 5% CO<sup>2</sup> incubator. HEK293 cells were maintained in a medium B (Dulbecco's modified Eagle medium, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin sulfate, and 10% (v/v) fetal bovine serum) at 37 °C in a humidified 5% CO<sup>2</sup> incubator.

### **Luciferase reporter assay**

On day 0, CHO-K1 cells were added to 96-well plates at  $1.0 \times 10^4$  cells per well in medium A. On day 1, the cells were co-transfected with an SRE-1-driven luciferase reporter plasmid (pSRE-Luc) and a β-gal reporter plasmid, in which the expression of β-galactosidase is controlled by an actin promote (pAc-β-gal) at a 20/1 ratio, using FuGENE® HD transfection reagent (Promega), according to the manufacturer's protocol. After 8 h incubation, the cells were washed with PBS, and then incubated in medium C (1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle medium, supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin sulfate, 5% (v/v) lipid-depleted serum, 50 µM compactin, and 50 µM lithium mevalonate) containing the specific test compounds. Stock solutions of each compound in DMSO were prepared and added to medium B to give a 200-fold (v/v) dilution (0.5% DMSO). After 20–24 h of incubation, the cells in each well were lysed by freeze-thaw with Reporter Lysis Buffer (Promega), and aliquots were used to measure luciferase and β-galactosidase activities. Luciferase activity was measured using the Steady-Glo® Luciferase Assay System (Promega), and β-galactosidase activity was measured using the β-galactosidase Enzyme Assay System (Promega). Luciferase activity was normalized to β-galactosidase activity.

### **PLAP-BP2 cleavage assay**

On day 0, CHO-K1 cells were added to 96-well plates at  $2.0 \times 10^4$  cells per well in medium A. On day 1, the cells were co-transfected with pCMV-PLAP-BP2513–1141 (0.1 µg/well), pCMV-SCAP (0–2.0 µg/well), and pAc-β-gal (0.1 µg/well) using FuGENE® HD transfection reagent (Promega), according to the manufacturer's protocol. After 5 h incubation, the cells were washed with PBS and then incubated in medium C containing DMSO, vitamin D derivatives (10 µM), or sterols (10 µg/mL of cholesterol and 1.0 µg/mL of 25-HC). After 20 h of incubation, an aliquot of the medium was assayed for secreted alkaline phosphatase activity. The cells in each well were lysed and used for measurement of β-galactosidase activities. The alkaline phosphatase activity was normalized to β-galactosidase activity.

### **Quantitative real-time PCR**

Total RNA from cultured cells was prepared using ISOGEN (Nippon Gene Co., Ltd.), according to the manufacturer's protocol. Triplicate samples of first strand cDNAs were synthesized using Prime-Script 1st Strand cDNA Synthesis Kit (Takara Bio Inc.), according to the manufacturer's protocol. The cDNAs were subjected to real-time PCR quantification using forward and reverse primers for the indicated mRNA and SYBR green with a 7500 Fast real time PCR system (Applied Biosystems®), according to the manufacture's protocol. Relative amounts of mRNAs were calculated using the comparative  $\Delta\Delta CT$  method. Hamster cyclophilin or human actin was used as invariant controls, and RNA from cells treated with DMSO alone was used as a calibrator. Sequences of forward and reverse primers (5' to 3') were as follows: for hamster, GCTGGTAAATGGTGTGATTG and CGGCCCAA AACTGATAAAC for SREBP-2; CATTTCATCGCAGCTGGGTCT and CTTGAACTTGGGCCCCGT for low density lipoprotein receptor (LDLR); CCCAGATTTCTTCTATATTCG and CCCATAGCTAACTGTCGTCC for Insig1; CTGCTGCTACCCTCTGCTGAAG and CTTGTTTGTGGTCAGAGTC for SCAP; AGTCCTTGTCAGGTTTCGTG and CCACCTAAGCCACCAGTGAT for fatty acid synthase (FAS); TGACACCATGTTGGGAGTTG and TGTGAGCAGGAAGGACTTGA for acetyl-CoA carboxylase  $\alpha$  (ACAC); ATTCATGTGCCAGGGTGGTGACT and TCAGTCTTGGCGGTGCAGAT for cyclophilin; for human,

AGCGGAAGGCACTATTCA and CATCATGCCGATGTCCACACA for VDR;  
CCCAGATTTCTCTATATTCGTTCTT and CACCCATAGCTAACTGTCGTCCTA for  
Insig1; TGTCTCTCACACTGGCTGCACTA and CTCCAAGGCCAAAACCACTTC for  
Insig2; TGGCAAATGAAACTGATTCC and ACAGTTAGTGTAGAATCGCACCC for  
Trc8; GGTGCAGCGTAAGGACGAA and GCATCATCTTCAGAACTTTTGTTC for  
gp78; AGCGAGCATCCCCCAAAGTT and GGGCACGAAGGCTCATCATT for actin.

### **Metabolic analysis**

On day 0, CHO-K1 cells were added to 10 cm dishes at  $1.5 \times 10^6$  cells in medium A. On day 1, the cells were washed with PBS buffer, and then incubated in medium C containing 5  $\mu$ M each compound. After 24 h incubation, the culture medium was aspirated from a dish, and the cells were washed twice by a 5% (w/w) mannitol solution (10 mL first and then 2 mL). The cells were treated with 1.3 mL ethanol and scraped by a cell scraper. To the cell extract (1.0 mL) were added 200  $\mu$ L of Milli-Q water containing internal standards (50  $\mu$ M, H3304-1002, Human Metabolome Technologies (HMT), Tsuruoka, Yamagata, Japan) and 800  $\mu$ L of pure Milli-Q water, and the mixture was stirred and centrifuged at 5,000  $\times$ g and 4  $^{\circ}$ C for 5 min. The supernatant was collected and dried under nitrogen purge and re-suspended in 200  $\mu$ L of 50% (v/v) isopropanol for metabolic analysis at HMT. Metabolic analysis was conducted by Dual Scan package of HMT using liquid chromatography time-of-flight mass spectrometry (LC-TOFMS) based on the methods described previously (Ooga et al., 2011). Briefly, LC-TOFMS analysis was carried out by Agilent 1200 series RRLC system SL with an Agilent 6210 LC/MSD TOF mass spectrometer (Agilent Technologies). The system was controlled by MassHunter Workstation (Agilent Technologies). The spectrometer was scanned from m/z 50 to 1,000 and peaks were extracted using MasterHands, automatic integration software (Keio University, Tsuruoka, Yamagata, Japan) in order to obtain peak information including m/z, peak area, and migration time (MT) (Sugimoto et al., 2010). Signal peaks corresponding to isotopomers, adduct ions, and other product ions of known metabolites were excluded, and remaining peaks were annotated according to the HMT metabolite database based on their m/z values with the MTs. Areas of the annotated peaks were then normalized based on internal standard levels and sample amounts in order to obtain relative levels of each

metabolite. Hierarchical cluster analysis (HCA) and principal component analysis (PCA) were performed by HMT's proprietary software, PeakStat and SampleStat, respectively. Detected metabolites were plotted on metabolic pathway maps using VANTED (Junker et al., 2006).

### **Transfection and western-blot analysis**

Transfection of cells were performed using FuGENE® HD transfection reagent (Promega), according to the manufacturer's protocol. Conditions of drug-incubations are described in figure captions. At the end of incubation, the cells were washed three times with cold PBS, and lysed with a buffer A (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 8 M urea, and protease inhibitor cocktail (Nacalai Tesque, Inc.)). The cell lysates were passed 16 times through a 25G needle and centrifuged at 7,000 g at 4 °C for 10 min. The supernatants were transferred to new tubes, and the pellets were extracted with a buffer A. The resulting buffer was centrifuged at 7,000 g at 4 °C for 10 min, and the supernatants were combined to respective original supernatants. The resulting lysate was mixed with 0.20 volume of 6× SDS sample buffer (Nacalai Tesque, Inc.) and incubated at room temperature for 30 min. The samples were separated on a 6, 8, or 10% SDS-PAGE gel and blotted, using specific antibodies. The specific bands were visualized using enhanced chemiluminescence (ECL Prime Western Blotting Detection Reagent, GE Healthcare) on an ImageQuant LAS 500 (GE Healthcare).

### **RNA interference**

Duplexes of small-interfering RNA (siRNA) were synthesized at Hokkaido System Science (Sapporo, Japan). SiRNA sequences targeting human gp78, Trc8, Insig-1, Insig-2, VDR, and GFP (an invariant control) were previously reported (Adams et al., 2004; Costa et al., 2009; Lee et al., 2006). On day 0, HEK293 cells were added to 6-well plates at  $4.0 \times 10^5$  cells per well in medium B without antibiotics. On day 1, the cells were transfected with 60 pmol of siRNAs using Lipofectamine RNAiMAX reagent (Invitrogen), according to the manufacture's protocol. On day 2, the cells were treated as described in the figure captions, and harvested for analysis.

## Ubiquitination of SCAP

Ubiquitination of SCAP was carried out as previously described (Gong et al., 2006). On Day 0, CHO-K1 cells were added to medium A in 10 cm dishes at  $1.0 \times 10^6$  cells per dish. On Day 1, the cells were co-transfected with plasmids encoding Myc-tagged SCAP, Flag-tagged Insig-1, and ubiquitin in medium A. On Day 2, the cells were washed with PBS and incubated in medium E. After treatment of the cells with each molecule, as indicated in **Figure 2F**, the cells were lysed with buffer B. The cell lysates were passed 16 times through a 25G needle and centrifuged. The supernatants were transferred to new tubes, and the pellets were extracted with a buffer B. The resulting buffer was centrifuged, and the respective supernatants were combined. The resulting lysate was diluted with buffer to decrease the urea concentration below 2 M, and subjected to immunoprecipitation (24 h at 4°C) with monoclonal anti-c-Myc IgG-coupled agarose beads (Nacalai Tesque, Inc.). After centrifugation, the pelleted beads were washed three times with buffer C and extracted with 1× SDS sample buffer containing 8 M urea at room temperature for 30 min.

## Photo-affinity labeling assays

The photo-affinity labeling assays were carried out as previously described (Kamisuki et al., 2009; Tang et al., 2011). On Day 0, CHO-K1 cells were added to medium in A 10 cm dishes at  $1.0 \times 10^6$  cells per dish. On Day 1, the cells were co-transfected with plasmids encoding Flag-tagged SCAP and Flag-tagged Insig-1 in medium A. On Day 2, the cells were washed with PBS and incubated in medium C. After 12 h of incubation, the cells were harvested and lysed to remove cytosolic proteins using Cell Wash Solution and Permeabilization (Pierce Biotechnology), according to the manufacture's protocol. The pellet with the membrane proteins was re-suspended in PBS containing 0.1% FOS-Choline®-13 (Affymetrix, Inc.) and protease inhibitor cocktail. The membrane fractions were incubated with each molecule, as shown in **Figure 5**, at 4 °C for 2 h, then exposed to UV at 365 nm at a distance of 5 cm, using a VL-215.L (Vilber Lourmat), for 1 h on ice. For the competition assay, 25OHD or cholesterol were added to the membrane fractions and incubated at 4 °C for 2 h prior to addition of the

probe molecules. After UV irradiation, the mixture was homogenized with buffer D and incubated with NeutrAvidin® agarose resin (Thermo Scientific) at 4 °C for 8 h. The resin was washed three times with buffer D and extracted with 1× SDS sample buffer containing 8 M urea at room temperature for 30 min.

### Protease inhibitor treatment

On Day 0, CHO-K1 cells were added to medium A in a 6-well plate at  $4.0 \times 10^5$  cells per well. On Day 1, the culture medium was changed to medium C. After 9 h, cells were treated with 25OHD or 25-HC and 10 µM MG-132, and each protease inhibitor was added as follows: 1% (v/v) protease inhibitor cocktail, 1.5µM pepstatin A, 50 µM Leupeptin, 1mM AEBSF, 1 µM Bedtatin, 1 µM PF429242, 60 µM E-64d, 1 µM Bestatin, or 0.5µM Aprotinin. After 4 h of incubation, the cells were washed three times with cold PBS, and lysed with buffer A. Immunoblots were performed with anti-SCAP antibodies.

### References

- Adams, C.M., Reitz, J., De Brabander, J.K., Feramisco, J.D., Li, L., Brown, M.S., and Goldstein, J.L. (2004). Cholesterol and 25-hydroxycholesterol inhibit activation of SREBPs by different mechanisms, both involving SCAP and Insigs. *The Journal of biological chemistry* 279, 52772-52780.
- Adams, L.A., White, S.W., Marsh, J.A., Lye, S.J., Connor, K.L., Maganga, R., Ayonrinde, O.T., Olynyk, J.K., Mori, T.A., Beilin, L.J., *et al.* (2013). Association between liver-specific gene polymorphisms and their expression levels with nonalcoholic fatty liver disease. *Hepatology* 57, 590-600.
- Auwerx, J., Bouillon, R., and Kesteloot, H. (1992). Relation between 25-Hydroxyvitamin-D3, Apolipoprotein-a-I, and High-Density-Lipoprotein Cholesterol. *Arterioscler Thromb* 12, 671-674.
- Barchetta, I., Carotti, S., Labbadia, G., Gentilucci, U.V., Muda, A.O., Angelico, F., Silecchia, G., Leonetti, F., Fraioli, A., Picardi, A., *et al.* (2012). Liver vitamin D receptor, CYP2R1, and CYP27A1 expression: relationship with liver histology and vitamin D3 levels in patients with nonalcoholic steatohepatitis or hepatitis C virus. *Hepatology* 56, 2180-2187.
- Botella-Carretero, J.I., Alvarez-Blasco, F., Villafriela, J.J., Balsa, J.A., Vazquez, C., and Escobar-Morreale, H.F. (2007). Vitamin D deficiency is associated with the metabolic syndrome in morbid obesity. *Clin Nutr* 26, 573-580.
- Bouillon, R., Carmeliet, G., Lieben, L., Watanabe, M., Perino, A., Auwerx, J., Schoonjans, K., and Verstuyf, A. (2014). Vitamin D and energy homeostasis: of mice and men. *Nat Rev Endocrinol* 10, 79-87.
- Bouillon, R., Okamura, W.H., and Norman, A.W. (1995). Structure-function relationships in the vitamin D endocrine system. *Endocr Rev* 16, 200-257.
- Brommage, R., and DeLuca, H.F. (1985). Evidence that 1,25-dihydroxyvitamin D3 is the physiologically active metabolite of vitamin D3. *Endocr Rev* 6, 491-511.

Brown, A.J., Sun, L., Feramisco, J.D., Brown, M.S., and Goldstein, J.L. (2002). Cholesterol addition to ER membranes alters conformation of SCAP, the SREBP escort protein that regulates cholesterol metabolism. *Mol Cell* 10, 237-245.

Brown, M.S., and Goldstein, J.L. (1997). The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. *Cell* 89, 331-340.

Brown, M.S., and Goldstein, J.L. (2009). Cholesterol feedback: from Schoenheimer's bottle to Scap's MELADL. *Journal of lipid research* 50 Suppl, S15-27.

Costa, J.L., Eijk, P.P., van de Wiel, M.A., ten Berge, D., Schmitt, F., Narvaez, C.J., Welsh, J., and Ylstra, B. (2009). Anti-proliferative action of vitamin D in MCF7 is still active after siRNA-VDR knock-down. *Bmc Genomics* 10.

Ding, N., Yu, R.T., Subramaniam, N., Sherman, M.H., Wilson, C., Rao, R., Leblanc, M., Coulter, S., He, M., Scott, C., *et al.* (2013). A vitamin D receptor/SMAD genomic circuit gates hepatic fibrotic response. *Cell* 153, 601-613.

Ford, E.S., Ajani, U.A., McGuire, L.C., and Liu, S. (2005). Concentrations of serum vitamin D and the metabolic syndrome among U.S. adults. *Diabetes Care* 28, 1228-1230.

Fuleihan, G.E., Bouillon, R., Clarke, B., Chakhtoura, M., Cooper, C., McClung, M., and Singh, R.J. (2015). Serum 25-Hydroxyvitamin D Levels: Variability, Knowledge Gaps, and the Concept of a Desirable Range. *J Bone Miner Res* 30, 1119-1133.

Goldstein, J.L., DeBose-Boyd, R.A., and Brown, M.S. (2006). Protein sensors for membrane sterols. *Cell* 124, 35-46.

Gong, Y., Lee, J.N., Lee, P.C., Goldstein, J.L., Brown, M.S., and Ye, J. (2006). Sterol-regulated ubiquitination and degradation of Insig-1 creates a convergent mechanism for feedback control of cholesterol synthesis and uptake. *Cell Metab* 3, 15-24.

Holick, M.F. (2007). Vitamin D deficiency. *N Engl J Med* 357, 266-281.

Hollis, B.W. (2005). Circulating 25-hydroxyvitamin D levels indicative of vitamin D sufficiency: implications for establishing a new effective dietary intake recommendation for vitamin D. *J Nutr* 135, 317-322.

Jo, Y., Lee, P.C., Sguigna, P.V., and DeBose-Boyd, R.A. (2011). Sterol-induced degradation of HMG CoA reductase depends on interplay of two Insigs and two ubiquitin ligases, gp78 and Trc8. *Proc Natl Acad Sci U S A* 108, 20503-20508.

Junker, B.H., Klukas, C., and Schreiber, F. (2006). VANTED: a system for advanced data analysis and visualization in the context of biological networks. *BMC bioinformatics* 7, 109.

Kamisuki, S., Mao, Q., Abu-Elheiga, L., Gu, Z., Kugimiya, A., Kwon, Y., Shinohara, T., Kawazoe, Y., Sato, S., Asakura, K., *et al.* (2009). A small molecule that blocks fat synthesis by inhibiting the activation of SREBP. *Chem Biol* 16, 882-892.

Lee, J.N., Song, B., DeBose-Boyd, R.A., and Ye, J. (2006). Sterol-regulated degradation of Insig-1 mediated by the membrane-bound ubiquitin ligase gp78. *The Journal of biological chemistry* 281, 39308-39315.

Lou, Y.R., Molnar, F., Perakyla, M., Qiao, S., Kalueff, A.V., St-Arnaud, R., Carlberg, C., and Tuohimaa, P. (2010). 25-Hydroxyvitamin D(3) is an agonistic vitamin D receptor ligand. *J Steroid Biochem Mol Biol* 118, 162-170.

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). SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation. *Genes Dev* 15, 1206-1216.

McDonnell, D.P., Mangelsdorf, D.J., Pike, J.W., Haussler, M.R., and O'Malley, B.W. (1987). Molecular cloning of complementary DNA encoding the avian receptor for vitamin D. *Science* 235, 1214-1217.

Moon, Y.A., Liang, G., Xie, X., Frank-Kamenetsky, M., Fitzgerald, K., Koteliansky, V., Brown, M.S.,  
 Goldstein, J.L., and Horton, J.D. (2012). The Scap/SREBP pathway is essential for developing diabetic fatty  
 liver and carbohydrate-induced hypertriglyceridemia in animals. *Cell Metab* 15, 240-246.  
 Nielson, C.M., Jones, K.S., Bouillon, R., and MrOS, O.F.M. (2016a). Role of Assay Type in Determining Free  
 25-Hydroxyvitamin D Levels in Diverse Populations. *New Engl J Med* 374, 1695-1696.  
 Nielson, C.M., Jones, K.S., Chun, R.F., Jacobs, J.M., Wang, Y., Hewison, M., Adams, J.S., Swanson, C.M.,  
 Lee, C.G., Vanderschueren, D., *et al.* (2016b). Free 25-Hydroxyvitamin D: Impact of Vitamin D Binding  
 Protein Assays on Racial-Genotypic Associations. *J Clin Endocr Metab* 101, 2226-2234.  
 Ooga, T., Sato, H., Nagashima, A., Sasaki, K., Tomita, M., Soga, T., and Ohashi, Y. (2011). Metabolomic  
 anatomy of an animal model revealing homeostatic imbalances in dyslipidaemia. *Mol Biosyst* 7, 1217-1223.  
 Ponchon, G., and DeLuca, H.F. (1969). The role of the liver in the metabolism of vitamin D. *J Clin Invest* 48,  
 1273-1279.  
 Radhakrishnan, A., Ikeda, Y., Kwon, H.J., Brown, M.S., and Goldstein, J.L. (2007). Sterol-regulated transport  
 of SREBPs from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc Natl Acad*  
*Sci U S A* 104, 6511-6518.  
 Radhakrishnan, A., Sun, L.P., Kwon, H.J., Brown, M.S., and Goldstein, J.L. (2004). Direct binding of  
 cholesterol to the purified membrane region of SCAP: mechanism for a sterol-sensing domain. *Mol Cell* 15,  
 259-268.  
 Rawson, R.B., DeBose-Boyd, R., Goldstein, J.L., and Brown, M.S. (1999). Failure to cleave sterol regulatory  
 element-binding proteins (SREBPs) causes cholesterol auxotrophy in Chinese hamster ovary cells with genetic  
 absence of SREBP cleavage-activating protein. *J Biol Chem* 274, 28549-28556.  
 Sakai, J., Rawson, R.B., Espenshade, P.J., Cheng, D., Seegmiller, A.C., Goldstein, J.L., and Brown, M.S.  
 (1998). Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid  
 composition of animal cells. *Mol Cell* 2, 505-514.  
 Sever, N., Yang, T., Brown, M.S., Goldstein, J.L., and DeBose-Boyd, R.A. (2003). Accelerated degradation of  
 HMG CoA reductase mediated by binding of insig-1 to its sterol-sensing domain. *Mol Cell* 11, 25-33.  
 Shao, W., and Espenshade, P.J. (2014). Sterol regulatory element-binding protein (SREBP) cleavage regulates  
 Golgi-to-endoplasmic reticulum recycling of SREBP cleavage-activating protein (SCAP). *J Biol Chem* 289,  
 7547-7557.  
 Song, B.L., Sever, N., and DeBose-Boyd, R.A. (2005). Gp78, a membrane-anchored ubiquitin ligase, associates  
 with Insig-1 and couples sterol-regulated ubiquitination to degradation of HMG CoA reductase. *Mol Cell* 19,  
 829-840.  
 Sugimoto, M., Wong, D.T., Hirayama, A., Soga, T., and Tomita, M. (2010). Capillary electrophoresis mass  
 spectrometry-based saliva metabolomics identified oral, breast and pancreatic cancer-specific profiles.  
*Metabolomics* 6, 78-95.  
 Tang, J.J., Li, J.G., Qi, W., Qiu, W.W., Li, P.S., Li, B.L., and Song, B.L. (2011). Inhibition of SREBP by a  
 small molecule, betulin, improves hyperlipidemia and insulin resistance and reduces atherosclerotic plaques.  
*Cell Metab* 13, 44-56.  
 Targher, G., Bertolini, L., Scala, L., Cigolini, M., Zenari, L., Falezza, G., and Arcaro, G. (2007). Associations  
 between serum 25-hydroxyvitamin D3 concentrations and liver histology in patients with non-alcoholic fatty  
 liver disease. *Nutr Metab Cardiovasc Dis* 17, 517-524.  
 Yabe, D., Xia, Z.P., Adams, C.M., and Rawson, R.B. (2002). Three mutations in sterol-sensing domain of  
 SCAP block interaction with insig and render SREBP cleavage insensitive to sterols. *Proc Natl Acad Sci U S A*  
 99, 16672-16677.

Yang, T., Espenshade, P.J., Wright, M.E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J.L., and Brown, M.S. (2002). Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* 110, 489-500.

Yang, T., Goldstein, J.L., and Brown, M.S. (2000). Overexpression of membrane domain of SCAP prevents sterols from inhibiting SCAP.SREBP exit from endoplasmic reticulum. *J Biol Chem* 275, 29881-29886.

Yin, Y., Yu, Z., Xia, M., Luo, X., Lu, X., and Ling, W. (2012). Vitamin D attenuates high fat diet-induced hepatic steatosis in rats by modulating lipid metabolism. *Eur J Clin Invest* 42, 1189-1196.

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## Author Contributions

L.A., M.W. and M.U. designed the study; S.S. and J.S. were involved in study design. K.U. and T.Y. synthesized the compounds. L.A., M.W., Y.R., B.K. and M.T. performed the experiments. L. A., M.W. and M.U. wrote the manuscript. K.N. and M.U. supervised all aspects of this work. All authors interpreted and discussed the data.

## Figure Legends

### Figure 1. Structures of vitamin D metabolites and their effects on SREBP activation.

(A) Chemical structures of vitamin D metabolites.

(B) Inhibition of the SREBP proteolytic activation by vitamin D metabolites. The concentration of vitamin D metabolites was set at 10  $\mu$ M. Sterol was a mixture of 10  $\mu$ g/mL cholesterol and 1.0  $\mu$ g/mL 25-HC. PLAP-BP2 remained membrane-bound, unless it was cleaved by S1P in the Golgi apparatus and secreted into the culture medium. When cells were treated with DMSO, the secretion of PLAP increased in proportion to the amount of transfected SCAP. Addition of sterol or vitamin D metabolites suppressed the increase of PLAP signals. 25OHD and 1,25(OH)<sub>2</sub>D inhibited the secretion of PLAP to a greater extent than sterol. Each value represents the average of two independent experiments.

(C) Effects of 25OHD on the ability of endogenous SREBPs to activate transcription of a luciferase reporter gene. CHO-K1 cells were transfected with the reporter gene in which the expression of luciferase was controlled by three repeats of the sterol-responsive element (SRE), and treated with varied concentrations of 25OHD in a lipid-free medium. Values are mean  $\pm$  SD. The same experiment using cholesterol and 25-HC was performed as control.

(D) Expression levels of SREBP-responsive genes and *scap* were analyzed by qPCR. CHO-K1 cells were treated with 5  $\mu$ M 25OHD or 25-HC in a lipid-free medium. Note that both compounds are likely to reach their maximal potency at 5  $\mu$ M. After 18 h of incubation, cells were harvested and subjected to qPCR analysis. Error bars represent the standard deviation of fold changes from three replicates (mean  $\pm$  SD).

(E) Metabolic analysis of the amounts of fatty acids CHO-K1 cells were incubated in medium B containing 5  $\mu$ M of each compound. After 24 h incubation, cells were harvested and subjected to metabolic analysis. Compared with DMSO controls, treatment with 25-HC or 25OHD decreased the amounts of the indicated fatty acids in cells.

(F) Western blot analysis of SREBP-2. Vitamin D metabolites decreased the protein levels of both precursor (P-) and mature (M-) forms of SREBP-2. CHO-K1 cells were treated with 5  $\mu$ M of each molecule in a lipid-free medium for 18 h. Immunoblots were performed with an anti-SREBP-2 antibody.

**Figure 2. Ubiquitin-dependent proteasomal degradation of SCAP induced by 25OHD.**

(A) The time-dependent effects of 25OHD on SREBP-2 and SCAP. On day 0, CHO-K1 cells were added to 6-well plates at  $2.5 \times 10^5$  cells per well in medium A. On day 2, the cells were washed with PBS, and then incubated in medium C in the absence (–) or presence (+) of 5  $\mu$ M 25OHD. After incubation for the indicated period of time, the cells were harvested and lysed, and the levels of precursor (P) and mature (M) forms of SREBP-2 and SCAP were analyzed by western blots.

(B) Western blot analysis of SCAP. CHO-K1 cells expressing Flag-tagged SCAP were treated with 5  $\mu$ M of each compound in a lipid-free medium for 24 h. Immunoblots were performed with an anti-Flag antibody.

(C) Effects of cycloheximide (CHX) on SCAP levels. CHO-K1 cells expressing Flag-tagged SCAP were treated with 50  $\mu$ M CHX in the absence or presence of 5  $\mu$ M 25OHD. Immunoblots were performed with an anti-Flag antibody.

(D) The effects of 25OHD in the absence (–) or presence (+) of  $\text{NH}_4\text{Cl}$  on Flag-SCAP. On day 0, CHO-K1 cells were added to 6-well plates at  $3.0 \times 10^5$  cells per well in medium A. On day 1, the cells were transfected with plasmid encoding Flag-tagged SCAP in medium A. On day 2, the cells were washed with PBS, and then incubated in medium C. After 6 h incubation, the cells were switched to medium C containing vehicle (DMSO), 5  $\mu$ M 25OHD, and 50 mM  $\text{NH}_4\text{Cl}$  as indicated. After 8 h incubation, the cells were harvested and lysed, and the levels of Flag-SCAP were analyzed by western blots. Western blots of actin are shown in the lower panel as a loading control.

(E) Effects of 25OHD or MG-132. CHO-K1 cells expressing Flag-tagged SCAP were treated with vehicle (DMSO), 10  $\mu$ M MG-132, or 5  $\mu$ M 25OHD in a lipid-free medium for 5 h. Immunoblots were performed with an anti-Flag antibody.

(F) Ubiquitination of SCAP. CHO-K1 cells expressing Myc-tagged SCAP and ubiquitin were treated with vehicle (DMSO), 5  $\mu$ M 25OHD, or 5  $\mu$ M 25-HC in a lipid-free medium. After 5 h, 10  $\mu$ M MG-132 was added. After 2 h of incubation, the cells were harvested and lysed. Myc-tagged SCAP was immunoprecipitated with monoclonal anti-c-Myc IgG-coupled agarose

beads. Immunoblots were performed with anti-ubiquitin or anti-Myc antibodies, against ubiquitin or SCAP, respectively.

**Figure 3. 25OHD induces proteolytic processing of SCAP.**

(A) Effects of MG-132 on endogenous SCAP. CHO-K1 cells were pre-incubated in lipid-free medium for 12 h, then treated with 10  $\mu$ M MG-132 for 3 h in the absence or presence of 5  $\mu$ M 25OHD.

(B) Pulse-chase analysis of SCAP processing. Pulse labeling of HaloTag-fused SCAP protein with TMR Ligand was chased at different time points by fluorescence imaging (upper panel) and by western blotting using an antibody against HaloTag (lower panel).

(C and D) Effect of protease inhibitor on endogenous SCAP. CHO-K1 cells were pre-incubated in lipid-free medium for 12 h, then treated with 10  $\mu$ M MG-132 and protease inhibitor cocktail or each protease inhibitor for 3 h in the absence or presence of 5  $\mu$ M 25OHD.

(E) CHO-K1 cells expressing Flag-tagged SCAP were pre-incubated in lipid-free medium, then incubated with 25OHD for 4 h. Cells were then treated with 10 $\mu$ M MG-132 for 3 h. Immunoblots were performed with an anti-Flag antibody.

**Figure 4. Evaluation of D14, a SCAP deletion mutant resistant to 25OHD-induced processing.**

Three cell lines of CHO-K1 cells stably expressing wild type-SCAP (**WT**), a 25OHD-resistant mutant SCAP (**D14**), or a non-resistant mutant SCAP (**D12**) were generated through transient transfection of neomycin plasmid DNA vectors. (A) Western blot analysis of SCAP processing status in the presence of MG-132. Stably expressed SCAP proteins were detected by a FLAG antibody. Each stable line was treated with 10  $\mu$ M MG-132 for 3 h in the absence or presence of 5  $\mu$ M 25OHD. It is evident that **D14** is resistant to 25OHD-induced processing. (B) Western blot analysis of endogenous SREBP-2. The precursor and mature forms of SREBP-2 are shown. Each cell line was incubated in the presence or absence of 5  $\mu$ M of 25OHD for 18 h. (C) Western blot analysis of stably expressed SCAP proteins. Each cell line was incubated in the presence or absence of 5  $\mu$ M of 25OHD for 6 h.

**Figure 5. 25OHD interacts with SCAP.**

(A) The chemical structure of 1 $\alpha$ -NHBoc-25OHD (**23**) and its inhibitory effect on the activation of SREBP in the reporter assay. CHO-K1 cells were transfected with an SRE-Luc and pAc- $\beta$ -gal in medium A, and treated with varied concentrations of **23** in medium C. The data were normalized to  $\beta$ -galactosidase activity. Values are the mean  $\pm$  SD of three independent experiments. Molecule **23** inhibited SREBP in dose-dependent manner, and its IC<sub>50</sub> values were approximately 0.7  $\mu$ M.

(B) Chemical structures of 25OHD-derived photo-affinity probes (**1** and **1'**) and negative control (**2**).

(C-D) CHO-K1 cells were co-transfected with plasmids encoding Flag-tagged SCAP or Flag-tagged Insig. 16 h after transfection, cells were incubated in medium C. After incubation for 8 h, cells were harvested and lysed. The lysate were incubated with the indicated concentrations of each molecules at 4 °C for 2 h. and were then exposed to UV at 365 nm for 1 h on ice. For the competition assay, 25OHD were added to the membrane fractions and incubated at 4 °C for 2 h prior to addition of the probe molecules. After UV irradiation, the mixture were homogenized and incubated with avidin-conjugated agarose at 4 °C for 8 h to pull down 25OHD-targeting protein. Immunoblots were performed with anti-Flag antibodies.

**Figure 6. Schematic model of SREBP inhibition mechanism by 25OHD.**

25OHD binding to SCAP mediates processing of SCAP at its COOH terminus. The processed SCAP cannot interact with SREBP and undergoes ubiquitin-mediated degradation. SREBP becomes unstable because of loss of binding partner leading to its degradation.



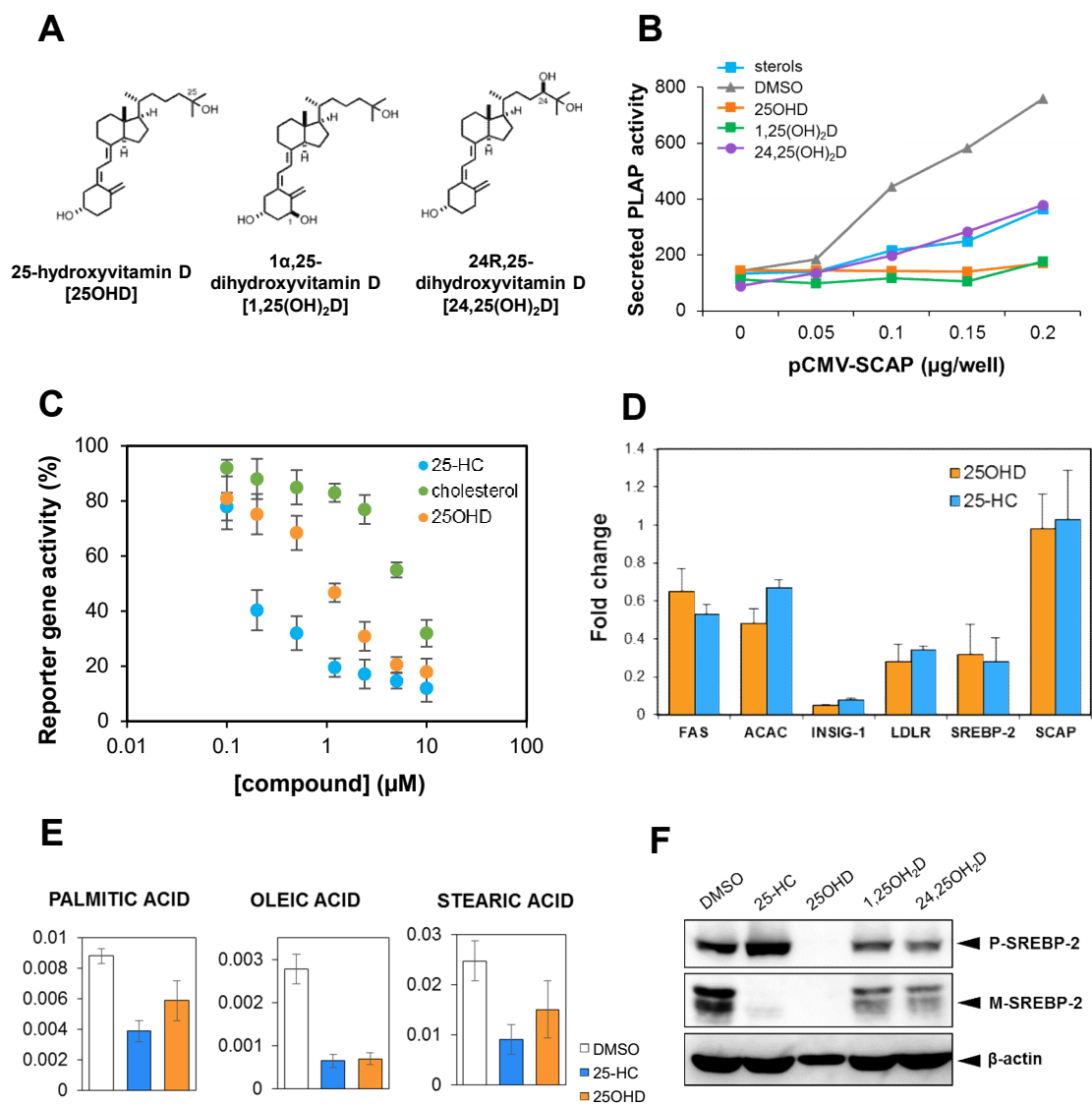
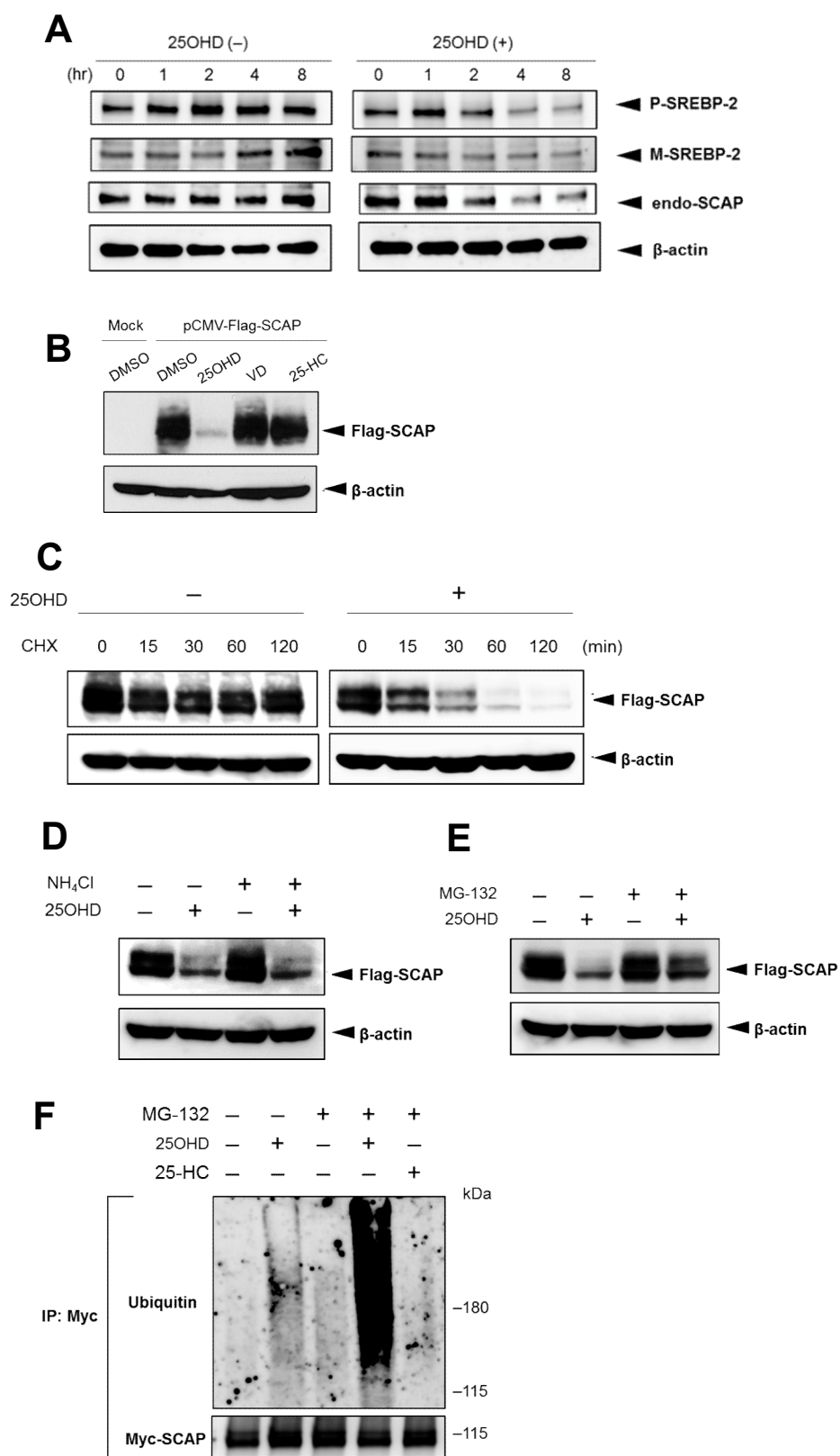


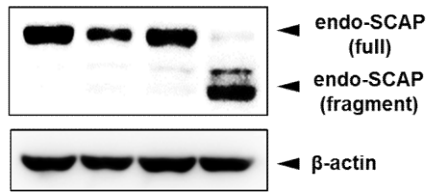
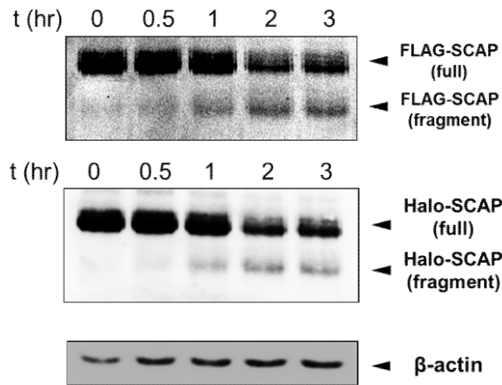
Figure 1



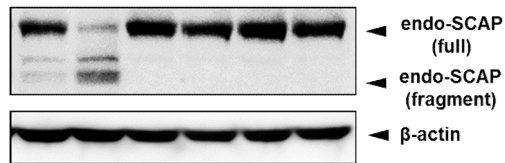
**Figure 2**

**A**

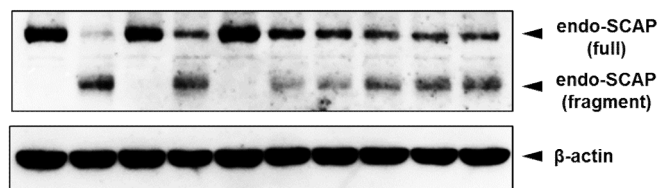
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|---------|---|---|---|---|
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| 25(OH)D | - | + | - | + |

**B****C**

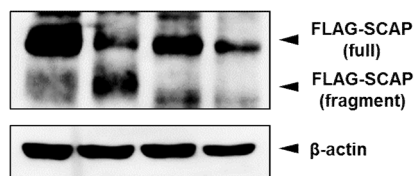
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|-----------------------------|---|---|---|---|---|---|
| Protease inhibitor cocktail | - | - | + | + | + | + |
| MG-132                      | + | + | - | - | + | + |
| 25(OH)D                     | - | + | - | + | - | + |

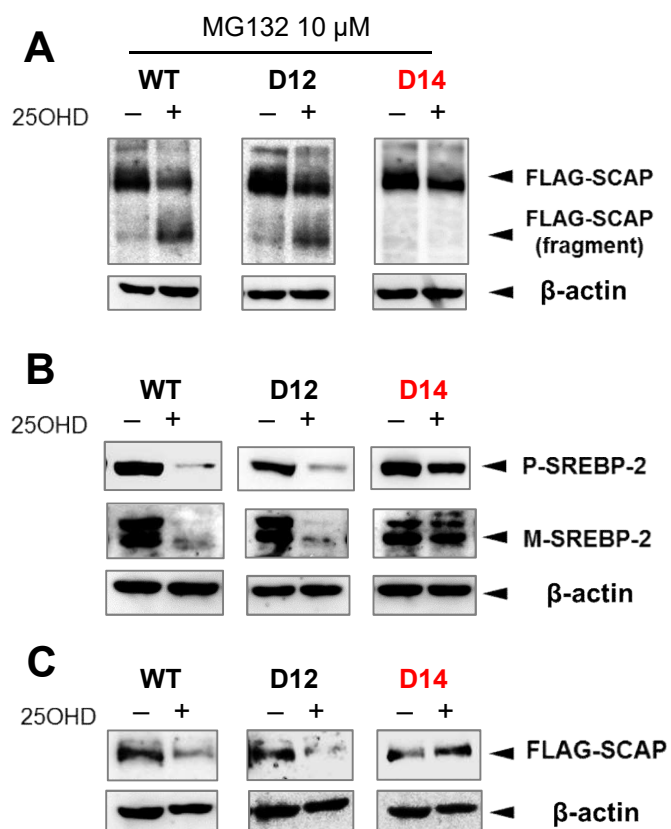
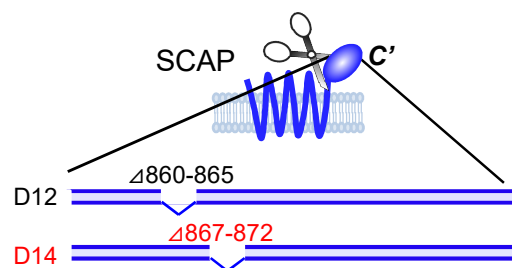
**D**

|                    |   |   |          |            |       |          |               |       |             |           |
|--------------------|---|---|----------|------------|-------|----------|---------------|-------|-------------|-----------|
| Protease inhibitor | - | - | cocktail | leupeptine | AEBSF | bestatin | S1P inhibitor | E-64d | pepstatin A | aprotinin |
| 25(OH)D            | - | + | +        | +          | +     | +        | +             | +     | +           | +         |

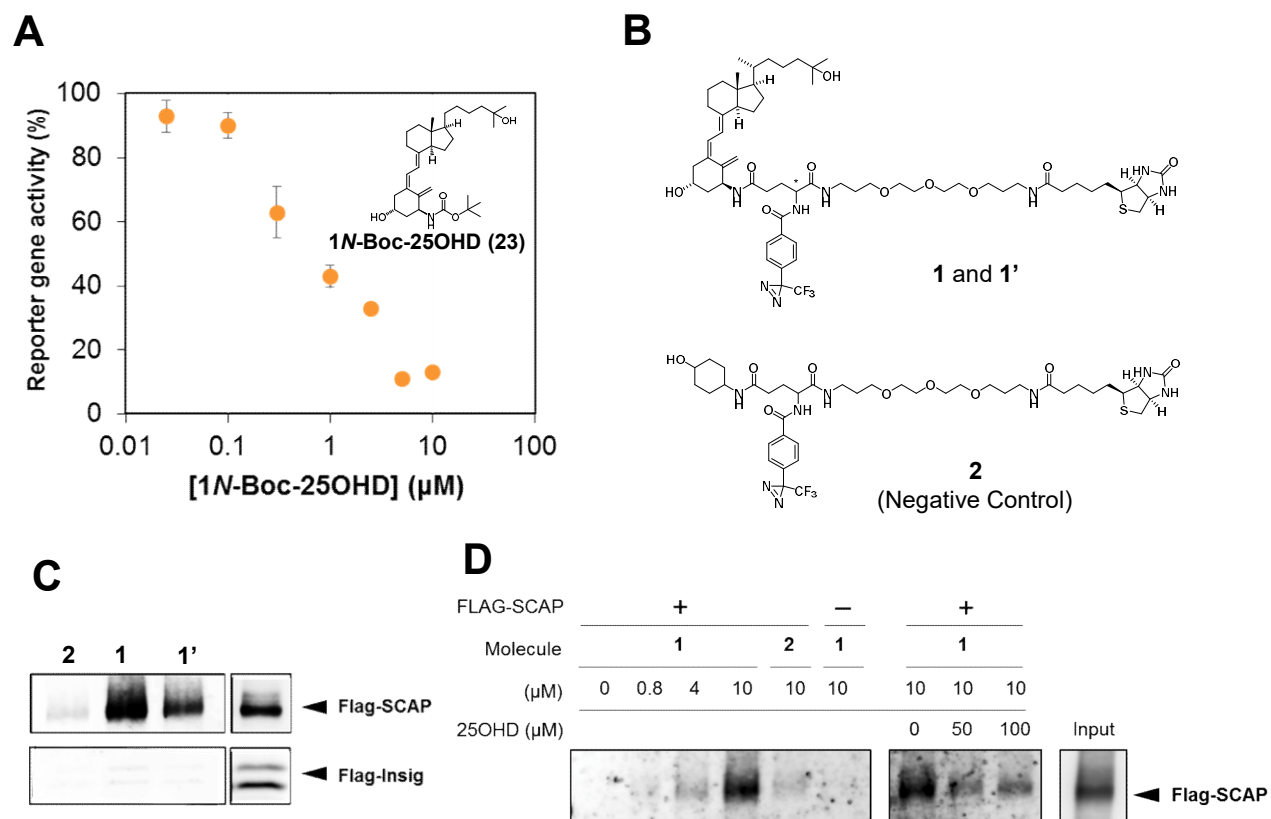
**E**

|        |             |   |             |   |
|--------|-------------|---|-------------|---|
|        | N-term FLAG |   | C-term FLAG |   |
| MG-132 | +           | + | +           | + |
| 25OHD  | -           | + | -           | + |

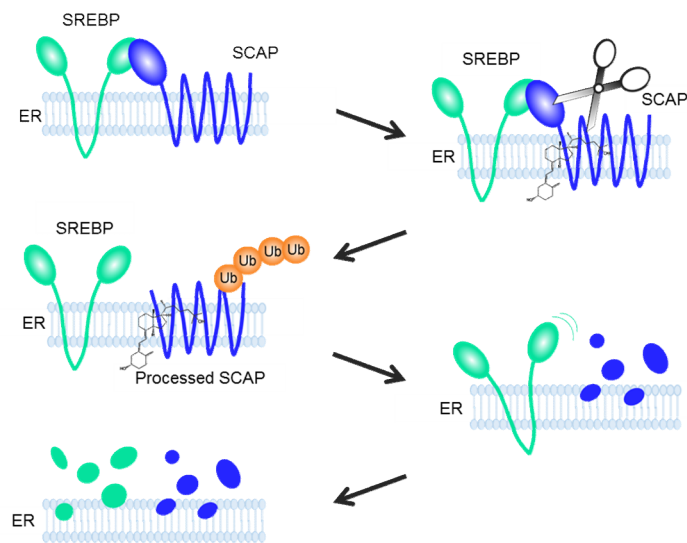
**Figure 3**



**Figure 4**



**Figure 5**



**Figure 6**

**Vitamin D metabolite, 25-Hydroxyvitamin D, regulates lipid metabolism  
by inducing degradation of SREBP/SCAP**

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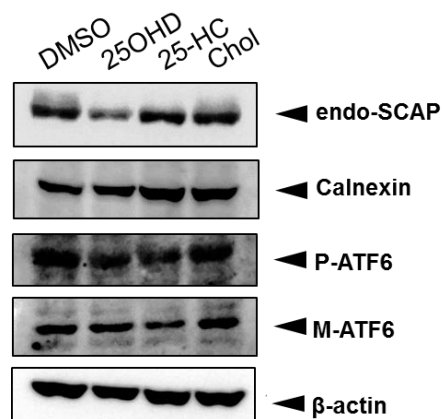
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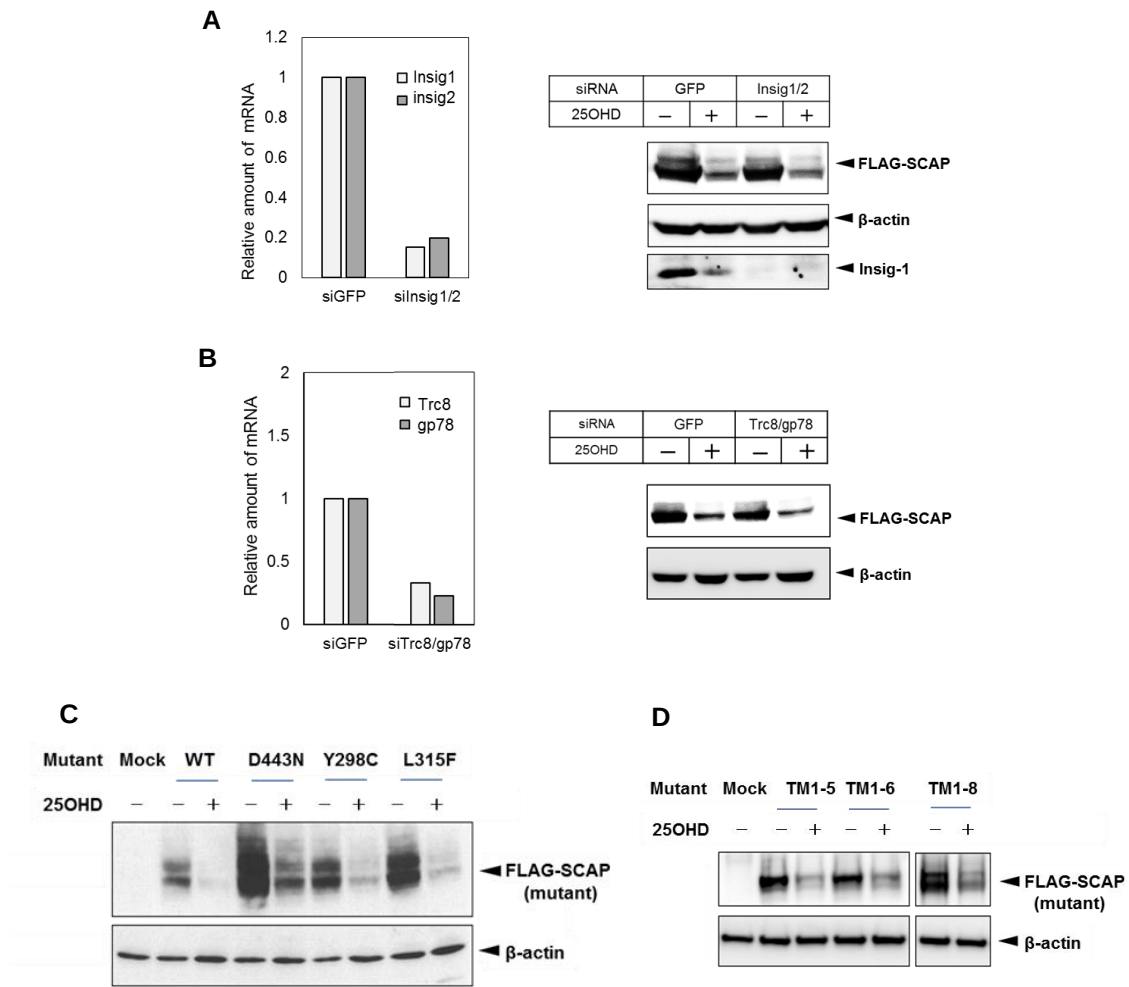
†These authors contributed equally to the work.

## Supplemental Figures

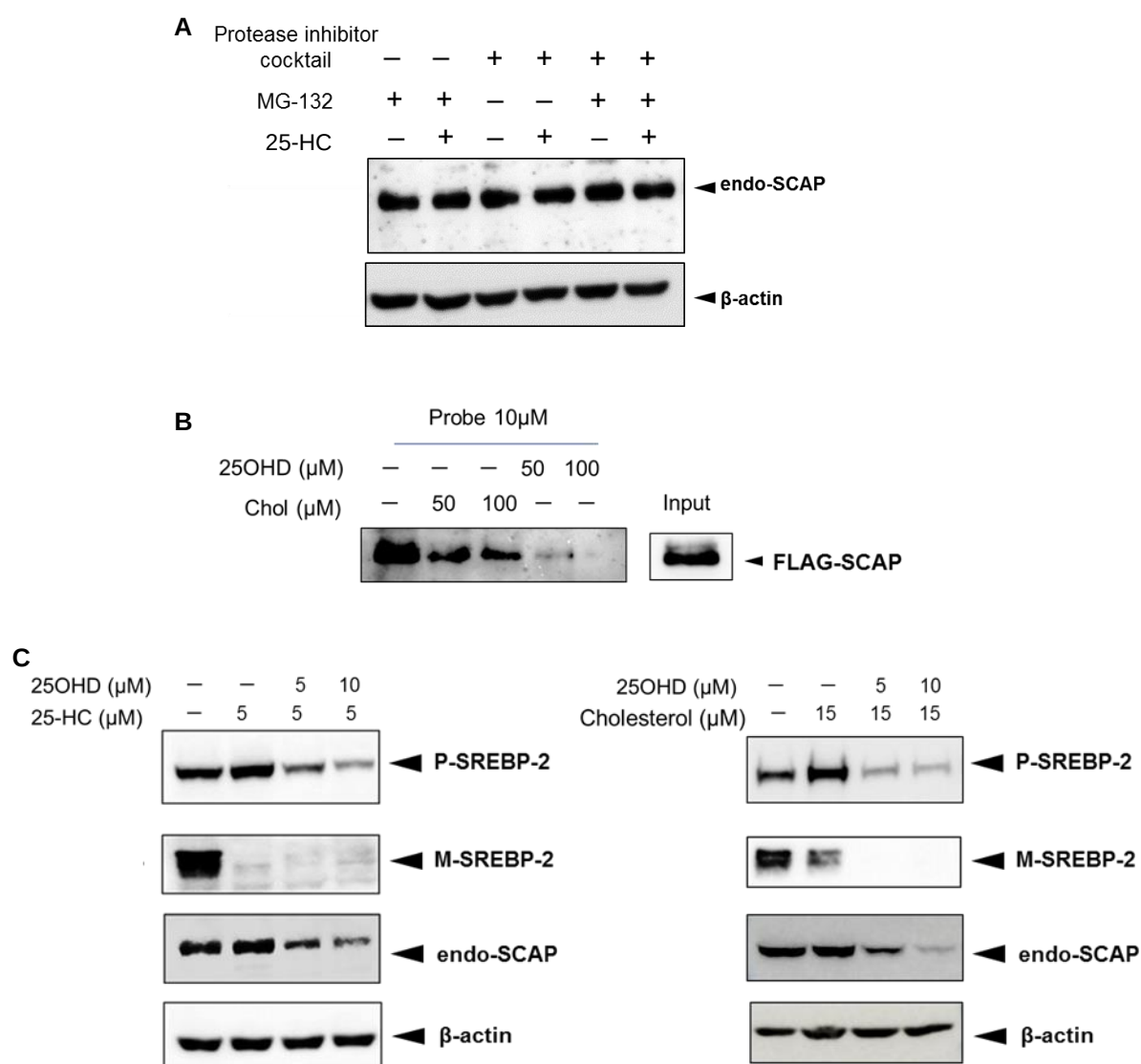


**Figure S1, related to Figure 1 and 2. The effects of 25OHD, 25-HC, and cholesterol on calnexin and ATF6.**

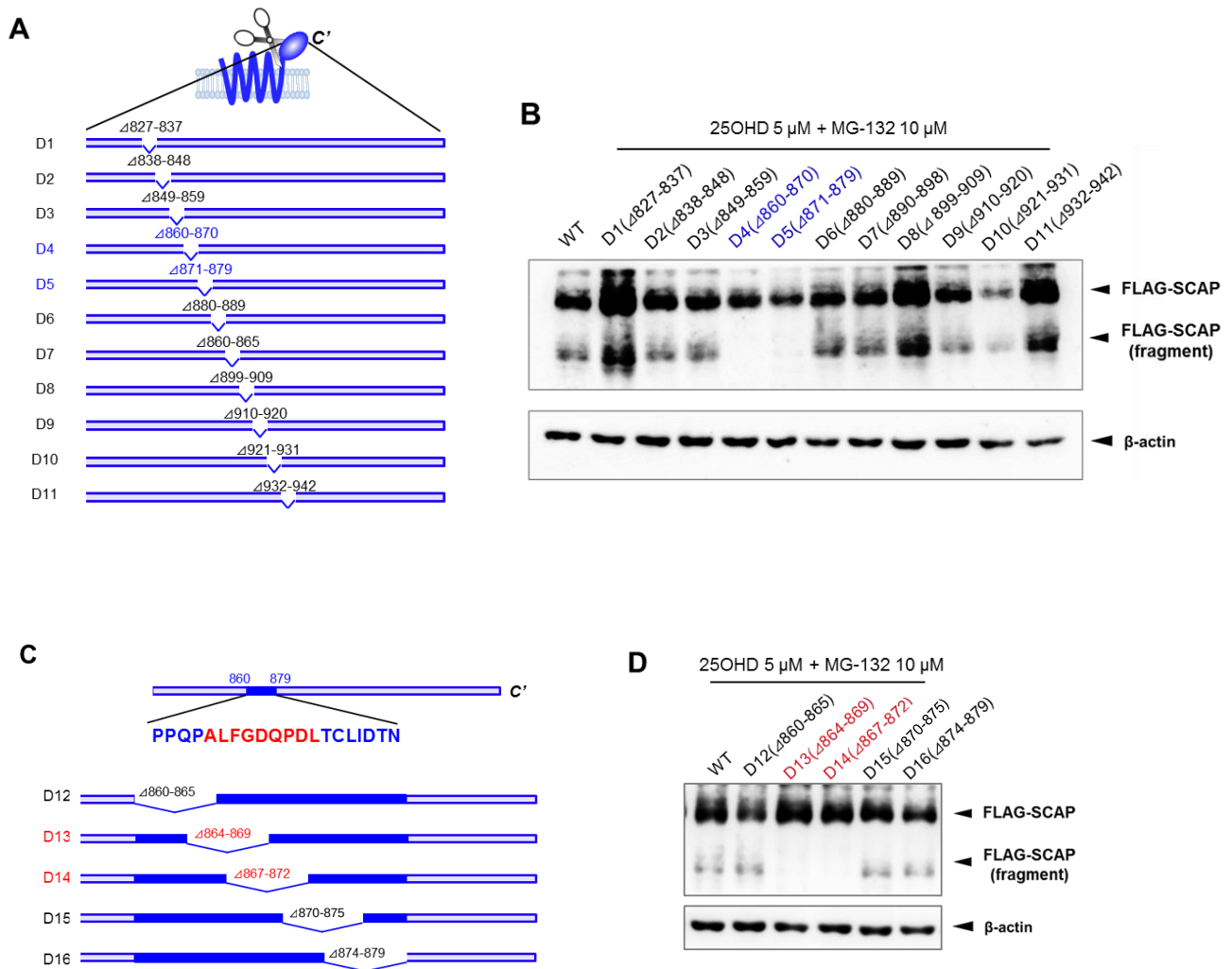
On day 0, CHO-K1 cells were added to 6-well plates at  $2.5 \times 10^5$  cells per well in medium A. On day 2, the cells were washed with PBS, and then incubated in medium C in the absence (–) or presence (+) of each molecule. After 18 h incubation, the cells were harvested and lysed, and the levels of SCAP, calnexin and precursor (P) and mature (M) forms of ATF6 were analyzed by western blots.



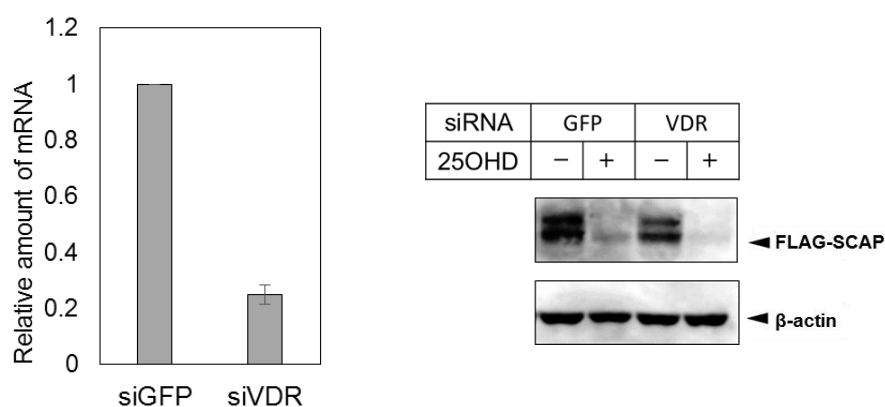
**Figure S2, related to Figure 2. Additional data on the ubiquitination of SCAP.** (A and B) The effects of siRNA knockdown on the 25OHD-induced degradation of SCAP. Knockdown efficiency assessed by qPCR (left panel) and western blot analysis (right panel). HEK293 cells were co-transfected with siRNAs targeting human INSIG-1 and -2 (A) or human Trc8 and gp78 (B) in medium B. After 12 h incubation, the cells were transfected with a plasmid encoding Flag-tagged SCAP in medium B. After 18 h incubation, the cells were washed with PBS, and then incubated in medium D in the absence (–) or presence (+) of 5  $\mu$ M 25OHD. After 6 h incubation, the cells were harvested and lysed, and the level of Flag-SCAP was analyzed by western blots. (C and D) The effects of 25OHD on point and deletion mutants of SCAP. Point mutants (D443N, Y298C, and L315F) and deletion mutant (TM1–5) of SCAP lack the INSIG-binding ability (Yabe et al., 2002; Yang et al., 2002; Yang et al., 2000). On day 0, CHO-K1 cells were added to 6-well plates at  $3.0 \times 10^5$  cells per well in medium A. On day 1, the cells were transfected with each plasmid encoding Flag-tagged SCAP mutant in medium A. On day 2, the cells were washed with PBS, and then incubated in medium C in the absence (–) or presence (+) of 5  $\mu$ M 25OHD. After 24 h incubation, the cells were harvested and lysed, and the levels of Flag-tagged SCAP mutants were analyzed by western-blot. Western-blots of actin are shown in the lower panel as a loading control.



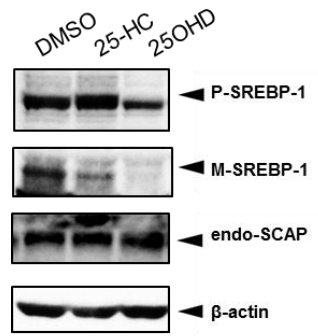
**Figure S3, related to Figure 3 and 5. Additional data on the effect of sterols.** (A) The effects of MG-132 and a protease inhibitor cocktail on the activity of 25-HC. On day 0, CHO-K1 cells were seeded onto 6-well plates at  $2.5 \times 10^5$  cells per well in medium A. On day 2, the cells were pre-incubated in medium C for 12 h, and then treated with 10 μM MG-132 and a protease inhibitor cocktail for 3 h in the absence or presence of 5 μM 25-HC. (B) Binding site of 25OHD and cholesterol to SCAP. CHO-K1 cells were co-transfected with plasmids encoding Flag-tagged SCAP. 16 h after transfection, cells were incubated in medium C. After incubation for 8 h, cells were harvested and lysed. 25OHD or cholesterol were added as competitors to the membrane fractions and incubated at 4 °C for 2 h prior to addition of the probe molecules. After 2 h, the lysates were exposed to UV at 365 nm for 1 h on ice. For the competition assay, 25OHD were added to the membrane fractions and incubated at 4°C for 2 h prior to addition of the probe molecules. After UV irradiation, the mixture were homogenized and incubated with avidin-conjugated agarose at 4 °C for 8 hr to pull down 25OHD-targeting protein. Western blot analysis was performed with anti-Flag antibodies. (C) The effect of sterols on the activity of 25OHD. On day 0, CHO-K1 cells were added to 6-well plates at  $2.5 \times 10^5$  cells per well in medium A. On day 2, the cells were washed with PBS, and then incubated in medium C containing each compound, as indicated above. After 18 h, the cells were harvested and lysed, and the levels of precursor (P) and mature (M) forms of SREBP-2 and SCAP were analysed by western blots.



**Figure S4, related to Figure3 and 4. Identification of SCAP deletion mutants resistant to 25OHD-induced processing.** (A) Design of a series of SCAP deletion mutants that lack a ~10-aa peptide segment in amino acids 827-942. Eleven expression vectors were constructed (**D1-11**). (B) Evaluation of the deletion mutants **D1-11** by western blot analysis. It is evident that **D4** and **D5** are resistant to the 25OHD-induced processing as highlighted in blue. (C) Design of a series of SCAP deletion mutants that lack a 6-aa peptide segment in amino acids 860-879. Five expression vectors were constructed (**D12-16**). The amino-acid sequence of 860-879 is shown. The peptide segment 864-872 is highlighted in red. (D) Evaluation of the deletion mutants **D12-16** by western blot analysis. **D13** and **D14**, whose deleted peptide sequences encompasses amino acids 864-872, were found to be resistant to the processing as highlighted in red.



**Figure S5, related to Figure 5. The effect of siRNA knockdown of VDR on the 25OHD-induced degradation of Flag-tagged SCAP.** HEK293 cells were transfected with siRNAs targeting human VDR in medium B. After 18 h incubation, the cells were washed with PBS, and then incubated in medium D in the absence (–) or presence (+) of 5  $\mu$ M 25OHD. After 12 h incubation, the cells were transfected with a plasmid encoding Flag-tagged SCAP in medium B. After 18 h incubation, the cells were harvested and lysed, and the levels of Flag-tagged SCAP were analyzed by western blots. Western blots of actin are shown as a loading control. The efficacy of knockdown of VDR by siRNA was assessed by qPCR analysis.



**Figure S6, related to 1 and 2. The effect of 25OHD on mouse hepatocyte cell.** On day 0, Hepa1-6 cells were seeded onto 6-well plates at  $2.5 \times 10^5$  cells per well in medium B. On day 2, the cells were washed with PBS, and then incubated in medium D in the absence (–) or presence (+) of 5  $\mu$ M 25OHD. After 18 h, the cells were harvested and lysed, and the levels of precursor (P) and mature (M) forms of SREBP-1 and SCAP were analyzed by western blots.

## **Supplemental Experimental Procedure**

### **Materials**

We obtained MG-132 and PR-619 were purchased from LifeSensors, Inc; Cycloheximide and ammonium chloride from Wako Pure Chemical Industries, Ltd; Lithium mevalonate, cholesterol, *N*-ethylmaleimide, Pepstatin A, Leupeptin A, AEBSF, Bestatin, PF429242, E-64d, and Aprotinin from Sigma-Aldrich Co; 25-hydroxycholesterol was purchased from Santa Cruz Biotechnology. 25-hydroxyvitamin D<sub>3</sub> and 24,25-dihydroxyvitamin D<sub>3</sub> were purchased from Enzo Life Sciences, Inc. 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> was purchased from Cayman Chemical Co. Nonidet® P-40 from Amresco, Inc. Urea and protease inhibitor Cocktail and SDS sample buffer from Nacalai Tesque, Inc. Compactin and sodium deoxycholate were purchased from Tokyo Chemical Industry Co., Ltd. Lipid-depleted serum was purchased from BioWest.

### **Media**

Medium A contains 1:1 mixture of Ham's F-12 medium and Dulbecco's modified Eagle medium (DMEM), supplemented with 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin sulfate, and 5% (v/v) fetal bovine serum. Medium B contains DMEM, supplemented with 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin sulfate, and 10% (v/v) fetal bovine serum. Medium C contains 1:1 mixture of Ham's F-12 medium and DMEM, supplemented with 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin sulfate, 5% (v/v) lipid-depleted serum, 50  $\mu$ M compactin, and 50  $\mu$ M lithium mevalonate. Medium D contains DMEM, supplemented with 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin sulfate, 10% (v/v) lipid-depleted serum, 50  $\mu$ M compactin, and 50  $\mu$ M lithium mevalonate. Medium E contains 1:1 mixture of Ham's F-12 medium and DMEM, supplemented with 100 units/mL penicillin, 100  $\mu$ g/mL streptomycin sulfate, 5% (v/v) lipid-depleted serum, 10  $\mu$ M compactin, and 10  $\mu$ M lithium mevalonate.

### **Buffer**

Buffer A contains 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, 8 M urea, and protease inhibitor cocktail. Buffer B is buffer A containing 5 mM EDTA, 5 mM EGTA, 10  $\mu$ M PR-619, and 10 mM *N*-ethylmaleimide. Buffer C is buffer B without urea and *N*-ethylmaleimide. Buffer D contains 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Nonidet P-40, 1% (w/v) sodium deoxycholate, and protease inhibitor.

### **Antibodies**

Primary antibodies used for immunoblotting were as follows: mouse monoclonal Anti-FLAG® (IgG-M2, Sigma); mouse monoclonal anti-c-Myc (IgG-MC045, Nacalai Tesque, Inc.); mouse monoclonal anti-hamster-SCAP (IgG-9D5, Santa Cruz Biotechnology); rabbit polyclonal anti-human-SREBP-1 (IgG-H-160, Santa Cruz Biotechnology); rabbit polyclonal anti-INSIG1 (Abcam); mouse monoclonal anti-calnexin (IgG-E-10, Santa Cruz Biotechnology); mouse monoclonal anti-actin (IgG-AC-40, Abcam); mouse monoclonal anti-ubiquitin (IgG-VU-1, LifeSensors); mouse monoclonal anti-hamster-SREBP-2 (IgG-7D4)(Sakai et al., 1996); mouse monoclonal anti-ATF6 $\alpha$  (IgG-37-1, kindly gifted by Prof. K. Mori of Kyoto University). Secondary antibodies used for immunoblotting were an ECL® peroxidase-labeled anti-mouse antibody (GE Healthcare Ltd.) and an anti-rabbit IgG, HRP-linked antibody (Cell Signaling).

### **Pulse-chase experiment**

HaloTag was fused to the N-terminus of SCAP using pHTN HaloTag® CMV-neo Vector (Promega). The resulting expression vector was transfected into CHO-K1 cells. After twenty-four hours, HaloTag-SCAP was labelled for 15 min

with 1.5  $\mu\text{M}$  TMR Ligand. The cells were washed with PBS and chased in medium C containing 5  $\mu\text{M}$  25OHD. Samples were harvested at different time points and analysed by SDS-PAGE and fluorescence imaging. Western blotting was also performed using Anti-HaloTag® Monoclonal Antibody (Promega).

### Exploration of SCAP deletion mutants resistant to 25OHD

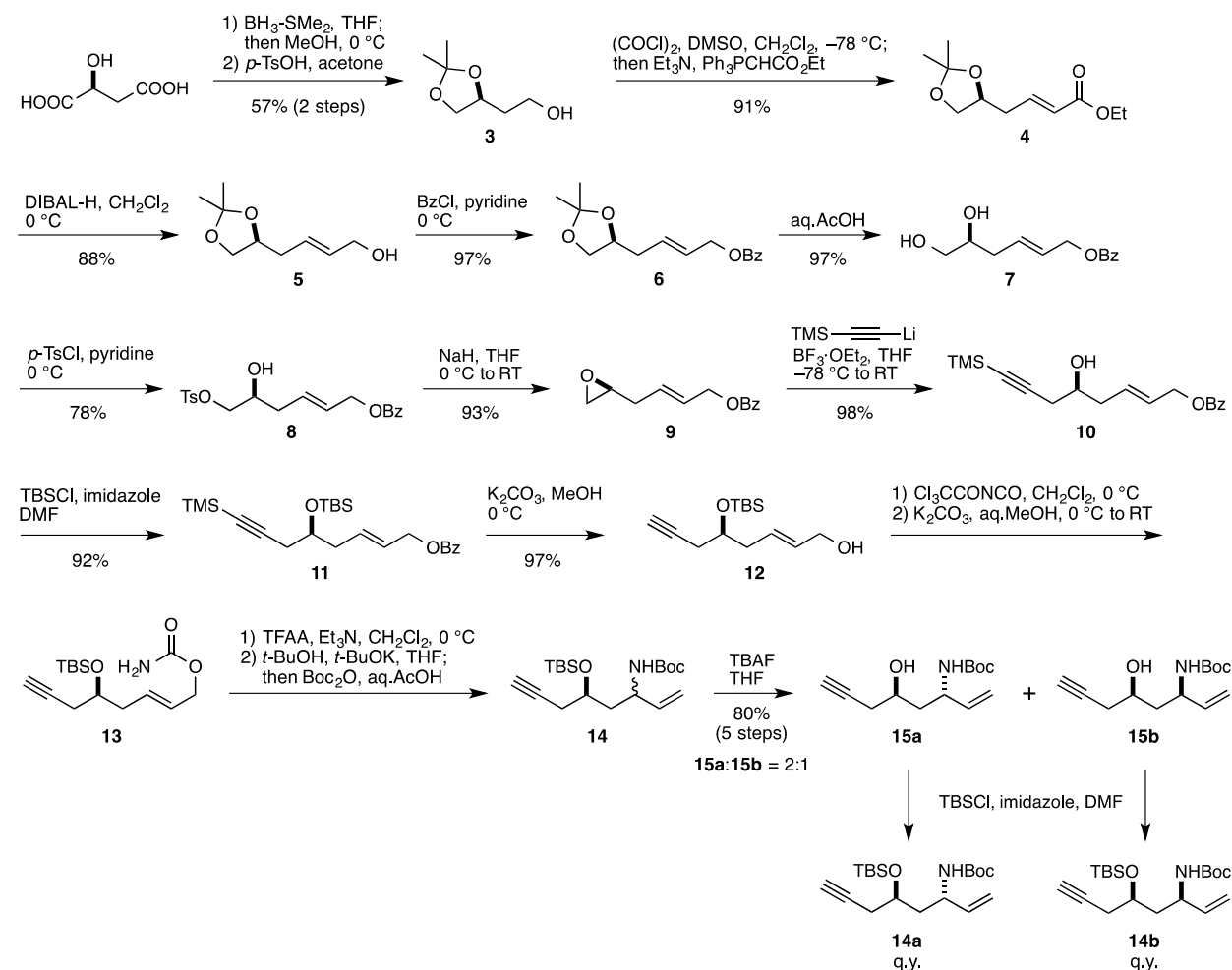
Each expression vector encoding a deletion mutant of SCAP was constructed using a PrimeSTAR® Mutagenesis Basal kit (TakaraBio Inc.). Each vector was transfected into CHO-K1. After 24 h, the cells were pre-incubated in medium C for 12 h and then treated with 5  $\mu\text{M}$  of 25OHD and 10  $\mu\text{M}$  of MG-132 for 3 h. The processing of each mutant SCAP was evaluated by western blot analysis.

### Evaluation of D14, a resistant deletion mutant of SCAP

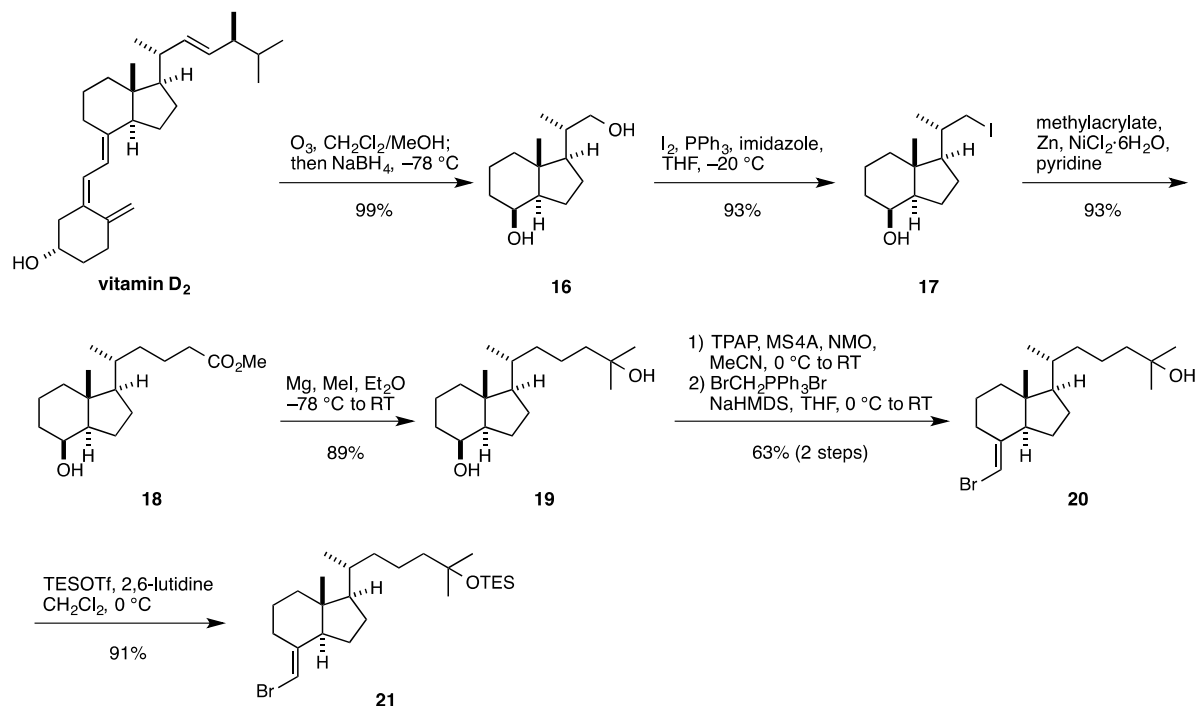
Three cell lines of CHO-K1 cells stably expressing wild type-SCAP (**WT**), a 25OHD-resistant mutant SCAP (**D14**), or a non-resistant mutant SCAP (**D12**) were generated through transient transfection of neomycin plasmid DNA vectors. For analyzing processing status of SCAP, the cells were pre-incubated in medium C for 12 h, and then treated with 5  $\mu\text{M}$  of 25OHD and 10  $\mu\text{M}$  of MG-132 for 3 h. For evaluating degradation of SCAP and SREBP, the cells were treated with 25OHD for 6 h and 18 h, respectively.

## Synthesis

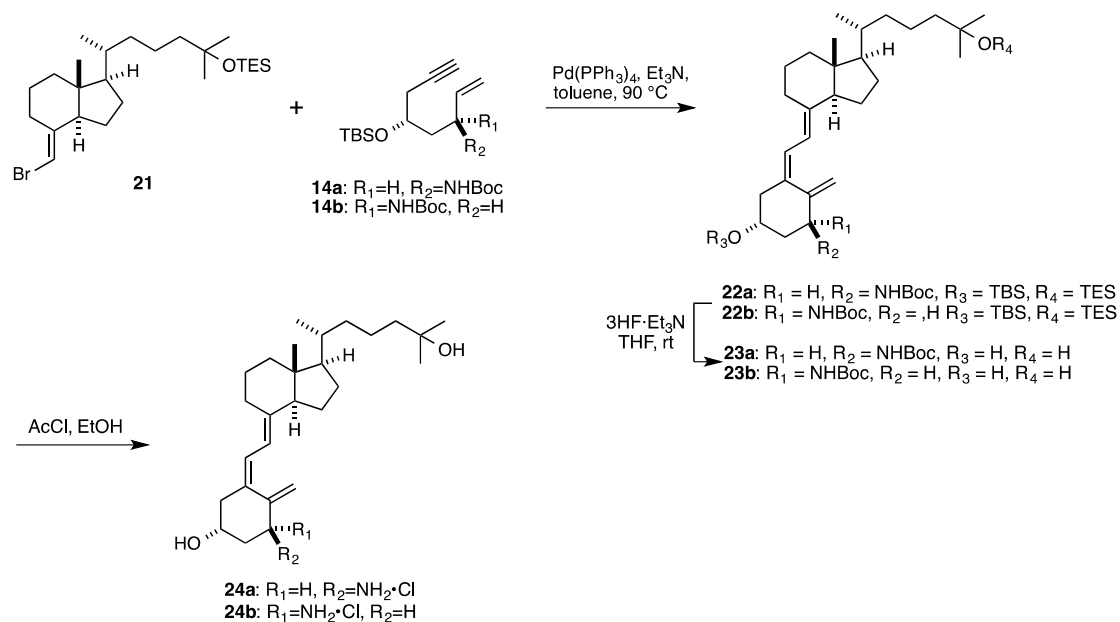
### Scheme 1. Preparation for a ring synthon 14a and 14b (single diastereomer).



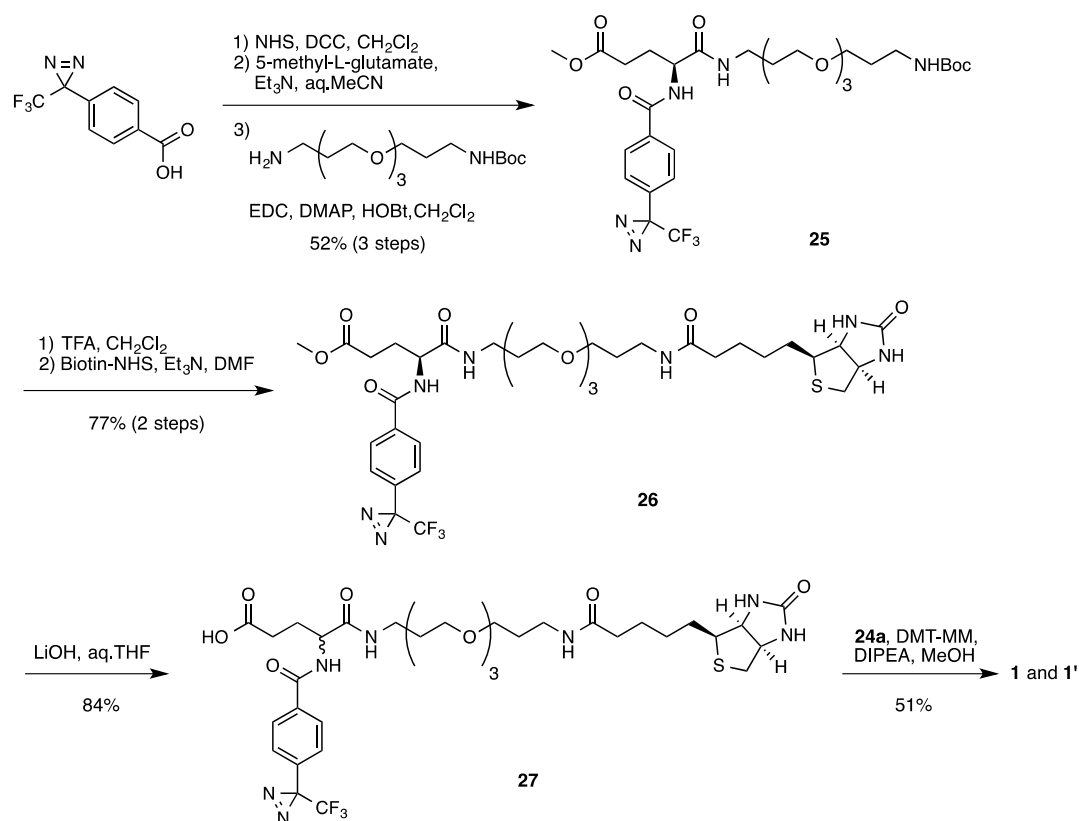
## Scheme 2. Preparation for intermediate compound 21.



## Scheme 3. Preparation for 1N-Boc-25OHD and 1-NH<sub>2</sub>-25OHD derivatives.



#### Scheme 4. Preparation for the photo-affinity probe.



#### Synthesis

Unless otherwise stated, reactions were performed under an argon atmosphere using freshly dried solvents. All reactions were monitored by thin-layer chromatography using Merck silica gel 60 F<sub>254</sub> pre-coated plates (0.25 mm) and were visualized by UV and *p*-anisaldehyde staining. Flash column chromatography was performed under pressurization using silica gel (particle size 40–100 μm) purchased from Cica. <sup>1</sup>H NMR spectra were recorded on a JNM-ECX 400 or a JNM-AL 300. The spectra are referenced internally according to residual solvent signals of CDCl<sub>3</sub> (<sup>1</sup>H NMR; δ = 7.26 ppm) or CD<sub>3</sub>OD (<sup>1</sup>H NMR; δ = 3.34 ppm). Data for <sup>1</sup>H NMR spectra are reported as follows: chemical shift (δ ppm) (multiplicity, coupling constant (Hz), integration). Multiplicity and qualifier abbreviations are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad. Mass spectra were recorded on a JEOL JMS-T100X spectrometer with ESI-MS mode using methanol as solvent.

#### 3

To a solution of BH<sub>3</sub>-SMe<sub>2</sub> (100 mL, 1.05 mol) in THF (200 mL) was added a solution of L-malic acid (50 g, 0.37 mol; purchased from Tokyo Chemical Industry Co., Ltd.) in THF (400 mL) with dropwise at 0 °C, then the reaction mixture was warmed to room temperature (RT). After stirring for 16 h, the reaction mixture was cooled to 0 °C before adding MeOH (400 mL). After additive 30 min at RT, the mixture was evaporated. Moreover, the residue was evaporated with MeOH (200 mL) six times and acetone (200 mL) two times. To the residue in acetone (600 mL) was added *p*-TsOH·H<sub>2</sub>O (4.0 g) and the mixture was stirred for 21 h. After addition of Et<sub>3</sub>N (2 mL), the mixture was stirred for 30 min and was evaporated. The residue was chromatographed on silica gel (hexane/ethyl acetate; 3:1 to 1:1) to give acetal **3** (31.0 g, 57%, 2 steps) as colorless oil.

#### 4

A solution of oxalyl chloride (2.1 mL, 24 mmol) in dichloromethane (110 mL) was cooled to  $-78\text{ }^{\circ}\text{C}$  and added dry DMSO (4.3 mL, 60 mmol) with dropwise. After stirring for 30 min at same temperature, a solution of acetal

**3** (1.8 g, 12 mmol) in dichloromethane (10 mL) was added with dropwise, and the reaction mixture was stirred for 1 h. To a suspension was added  $\text{Et}_3\text{N}$  (16.8 mL, 120 mmol) with dropwise, then the reaction mixture was warmed to RT. After additive 1 h, ethyl(triphenylphosphoranylidene)acetate (8.4 g, 24 mmol) was added and stirred for 1 day. The reaction was quenched by  $\text{H}_2\text{O}$ , and the aqueous layer was extracted with dichloromethane three times. The combined mixture was dried over  $\text{MgSO}_4$  and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 15:1 to 5:1) to give ethyl ester **4** (2.38 g, 92%) as yellow oil.

## 5

A solution of ethyl ester **4** (2.55 g, 11.9 mmol) in dichloromethane (120 mL) was added diisobutyl aluminum hydride in hexane (27.4 mL, 27.4 mmol, 1.0 M). After stirring for 2h, the reaction was quenched by careful addition of MeOH (19 mL). The mixture was added sat. Rochelle salt aq. (33 mL) and stirred for 30 min. The organic layer was washed with sat. Rochelle salt aq. three times, and the aqueous layer was extracted with  $\text{CHCl}_3$  three times. The combined mixture was dried over  $\text{MgSO}_4$  and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 6:1 to 2:1) to give alcohol **5** (1.80 g, 88%) as colorless oil.

## 6

To a solution of allyl alcohol **5** (636 mg, 3.69 mmol) in pyridine (3.7 mL) was added benzoyl chloride (0.64 mL, 5.54 mmol) with dropwise at  $0\text{ }^{\circ}\text{C}$ , then the reaction mixture was warmed to RT. After stirring for 2 h, the reaction mixture was added  $\text{H}_2\text{O}$ . The aqueous layer was extracted with ethyl acetate three times and organic layer was washed with brine. The combined mixture was dried over  $\text{MgSO}_4$  and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 30:1) to give benzoate ester (991 mg, 97%) as colorless oil.

## 7

To a benzoate ester **6** (991 mg, 3.59 mmol) was added acetic acid (15.4 mL) and  $\text{H}_2\text{O}$  (2.6 mL). After stirring at RT for 12 h, the reaction mixture was evaporated. The residue was chromatographed on silica gel (hexane/ethyl acetate; 1:1) to give diol **7** (825 mg, 97%) as colorless oil.

## 8

A solution of diol **7** (3.81 g, 16.1 mmol) in pyridine (32 mL) was added *p*-toluenesulfonyl chloride (3.69 g, 19.3 mmol) at  $0\text{ }^{\circ}\text{C}$ , then the reaction mixture was slowly warmed to RT. After stirring for 4h, the reaction was quenched by  $\text{H}_2\text{O}$ . The aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 5:1 to 1:1) to give tosylate **8** (4.93 g, 78%) as colorless oil.

## 9

A solution of tosylate **8** (4.93 g, 12.6 mmol) in THF (120 mL) was added NaH (1.01 g, 25.2 mmol, 60%) at  $0\text{ }^{\circ}\text{C}$ , then the reaction mixture was warmed to RT. After stirring for 8 h, the reaction was quenched by sat. aq.  $\text{NH}_4\text{Cl}$ . The aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 1:0 to 6:1) to give epoxide **9** (2.55 g, 93%) as colorless oil.

## 10

A solution of trimethylsilylacetylene (3.60 mL, 25.7 mmol) in THF (18 mL) was added *n*-BuLi in hexane (8.1 mL, 21.05 mmol, 2.6 M) with dropwise at  $-78^{\circ}\text{C}$  and stirred for 30 min. A solution of epoxide **9** (2.55 g, 11.7 mmol) in THF (5 mL) and  $\text{BF}_3\text{-OEt}_2$  (1.8 mL, 14 mmol) was added with dropwise at  $-78^{\circ}\text{C}$ , then the reaction mixture was warmed to RT over 2 h. The reaction was quenched with sat. aq.  $\text{NH}_4\text{Cl}$ , the aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 30:1 to 10:1) to give alcohol **10** (3.61 g, 98%) as colorless oil.

## 11

Alcohol **10** (3.61 g) was added imidazole (4.67 g, 68.5 mmol) and *tert*-butyldimethylsilyl chloride (6.88 g, 45.6 mmol) at RT, then DMF was added until the reagents were dissolved. After stirring for 4 h, the reaction mixture was diluted with diethyl ether and quenched with sat.  $\text{NaHCO}_3$  aq. The aqueous layer was extracted with diethyl ether three times. The combined organic extracts were washed with  $\text{H}_2\text{O}$  and brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 100:1 to 20:1) to give silyl ether **11** (4.52 g, 92%) as colorless oil.

## 12

A solution of **11** (8.28 g, 19.2 mmol) in MeOH (64 mL) was added potassium carbonate (7.97 g, 57.67 mmol) at  $0^{\circ}\text{C}$ , then the reaction mixture was cooled to RT. After stirring for 18 h, the reaction was quenched by  $\text{H}_2\text{O}$ . The aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 10:1 to 5:1) to give allyl alcohol **12** (4.74 g, 97%) as colorless oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.72-5.69 (m, 2H), 4.1 (d,  $J = 4.1$  Hz, 2H), 3.89-3.81 (m, 1H), 2.43-2.24 (m, 2H), 2.31 (dd,  $J = 6.18, 2.73$  Hz, 2H), 1.98 (t,  $J = 2.76$  Hz, 1H), 0.88 (s, 9H), 0.07 (s, 3H), 0.05 (s, 3H).

## 15

A solution of allyl alcohol **11** (1.46 g, 5.73 mmol) in dichloromethane (57 mL) was added trichloroacetyl isocyanate (1.40 mL, 11.5 mmol) at  $0^{\circ}\text{C}$ . The reaction was monitored by thin layer chromatography on silica gel plates (eluent: 4:1 ethyl acetate to hexane). After 15 min, the starting material spot completely converted to a new spot, then the reaction mixture was concentrated *in vacuo*. A solution of the residue in MeOH (36 mL) was added  $\text{H}_2\text{O}$  (8.2 mL) and potassium carbonate (3.2 g, 22.93 mmol) at  $0^{\circ}\text{C}$ , then the reaction mixture was warmed to RT. After stirring for 2 h, the reaction mixture was added  $\text{H}_2\text{O}$ , the aqueous layer was extracted with dichloromethane three times, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was filtered through a pad of silica gel to give a crude of carbamate **13** (5.73 mmol). To a solution of the crude and  $\text{Et}_3\text{N}$  (3.20 mL, 22.9 mmol) in dichloromethane (230 mL) was added trifluoroacetic anhydride (1.20 mL, 8.60 mmol) with dropwise at  $0^{\circ}\text{C}$ . After stirring for 1 h, the organic layer was washed with  $\text{H}_2\text{O}$  and brine. The combined organic extracts were dried over  $\text{Na}_2\text{SO}_4$ , and concentrated *in vacuo* to a crude of isocyanate. A suspension of *t*-BuOK (0.64 g, 5.7 mmol) and *t*-BuOH (1.10 mL, 11.5 mmol) in THF (30 mL) was cooled to  $0^{\circ}\text{C}$ . A solution of crude isocyanate (5.73 mmol) in THF (8 mL) was slowly added with dropwise. The reaction was monitored by thin layer chromatography on silica gel plates (eluent: 4:1 ethyl acetate to hexane). After stirring for 25 min, the starting material spot completely converted to new spots. To the reaction mixture was added acetic acid (0.33 mL, 5.7 mmol) and  $\text{H}_2\text{O}$  (19 mL), then was added  $\text{Boc}_2\text{O}$  until disappeared amine. After stirring for 13 h, the amine completely converted to product, then the reaction

mixture was diluted with ethyl acetate, the aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 100:1 to 20:1) to give Boc amine **14** as a diastereomeric mixture. To a solution of diastereomeric mixture **14** (5.73 mmol) in THF (11 mL) was added a solution of tetrabutylammonium fluoride in THF (5.7 mL, 1 M). After stirring for 2.5 h, the reaction mixture was evaporated. The residue was chromatographed on silica gel (hexane/ethyl acetate; 50:1 to 1:1) to give alcohol **15a** (0.72 g, 52%) and alcohol **15b** (0.39 g, 28%) as single diastereomer. **15a**:  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.90-5.78 (m, 1H), 5.24-5.12 (m, 2H), 4.76 (d,  $J$  = 8.9 Hz, 1H), 4.42 (br, 1H), 3.82 (br, 1H), 2.51-2.33 (m, 2H), 2.04 (t,  $J$  = 2.4 Hz, 1H), 1.79-1.60 (m, 2H), 1.45 (s, 9H). **15b**:  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.84-5.72 (m, 1H), 5.24-5.11 (m, 2H), 4.72 (br, 1H), 4.25 (br, 1H), 3.93-3.85 (m, 1H), 2.51-2.35 (m, 2H), 2.06 (t,  $J$  = 2.4 Hz, 1H), 1.78-1.65 (m, 2H), 1.44 (s, 9H).

#### A ring synthon **14a** and **14b** (single diastereomer)

To the alcohol **15a** (35 mg, 0.15 mmol) was added imidazole (60 mg, 0.88 mmol) and *tert*-butyldimethylsilyl chloride (88 mg, 0.58 mmol) at RT, then DMF was added until the reagents were dissolved. After stirring for 2 h, the reaction mixture was diluted with diethyl ether and quenched with sat. aq.  $\text{NaHCO}_3$ . The aqueous layer was extracted with diethyl ether three times. The combined organic extracts were washed with  $\text{H}_2\text{O}$  and brine, dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 30:1) to give **14a** (52 mg, q.y.) as colorless oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.83-5.72 (m, 1H), 5.18-5.07 (m, 2H), 4.26 (br, 1H), 4.01-3.93 (m, 1H), 2.43-2.27 (m, 2H), 2.01 (t,  $J$  = 2.4 Hz, 1H), 1.79-1.72 (br, 2H), 1.42 (s, 9H), 0.90 (s, 9H), 0.10 (s, 3H), 0.09 (s, 3H).

By following the same procedure described for **14a**, **14b** (35 mg, q.y.) was obtained from **15b** (23 mg, 0.094 mmol) as colorless oil.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.83-5.72 (m, 1H), 5.21-5.08 (m, 2H), 4.75 (br, 1H), 4.22-4.13 (m, 1H), 3.94-3.86 (m, 1H), 2.40-2.37 (m, 2H), 2.00 (t,  $J$  = 2.8 Hz, 1H), 1.86-1.59 (m, 2H), 1.43 (s, 9H), 0.91 (d,  $J$  = 3.5 Hz, 9H), 0.09 (s, 3H), 0.08 (d,  $J$  = 1.7 Hz, 3H).

#### 16

To a solution of vitamin D<sub>2</sub> (8.0 g, 20 mmol; purchased from Tokyo Chemical Industry Co., Ltd.) in dichloromethane (270 mL) was added methanol (112 mL) and the solution was cooled to  $-78^\circ\text{C}$ . Ozone was passed through the solution at  $-78^\circ\text{C}$  for 3 h. After flushing nitrogen to the solution to remove the residual ozone, the resulting mixture was treated with  $\text{NaBH}_4$  (4.56 g, 120 mmol) and stirred for 30 min at  $-78^\circ\text{C}$ , and then allowed to warm to RT. The reaction was quenched by the addition of aq. HCl (0.5 M), and extracted with dichloromethane. The organic phase was washed with water and dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 6:1 to 2:1) to give diol **16** (4.46 g, >99%).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  4.09 (d,  $J$  = 2.6 Hz, 1H), 3.64 (dd,  $J$  = 3.3, 10.6 Hz, 1H), 3.39 (dd,  $J$  = 6.6, 10.6 Hz, 1H), 1.99 (dd,  $J$  = 2.6, 13.2 Hz, 1H), 1.88-1.76 (m, 3H), 1.63-1.29 (m, 9H), 1.20-1.12 (m, 2H), 1.03 (d,  $J$  = 6.6 Hz, 3H), 0.95 (s, 3H).

#### 17

To a solution of diol **16** (1.00 g, 4.71 mmol) in THF (20 mL) were successively added imidazole (960 mg, 14.1 mmol), triphenylphosphine (1.20 g, 5.65 mmol), and iodide (1.32 g, 5.18 mmol) at  $-20^\circ\text{C}$  and the mixture was stirred at  $-20^\circ\text{C}$  for 15 min. The reaction mixture was warmed to RT and stirred for 2 h. The mixture was cooled to  $0^\circ\text{C}$ , and sat. aq.  $\text{NaHCO}_3$  was added. The aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with sat. aq.  $\text{Na}_2\text{S}_2\text{O}_3$  and  $\text{H}_2\text{O}$ , dried over  $\text{MgSO}_4$ , and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 10:1) to give iodide **17** (1.41 g, 93%).

## 18

A suspension of activated zinc (8.32 g, 127 mmol) in pyridine (90 mL) were successively added methyl acrylate (13.3 mL, 148 mmol) and NiCl<sub>2</sub>·6H<sub>2</sub>O (3.78 g, 15.9 mmol) at RT. The mixture was stirred at 60 °C for 1 h and then cooled to 0 °C. A solution of iodide **17** (3.42 g, 10.6 mmol) in pyridine (10 mL) was added with dropwise at 0 °C. The reaction mixture was stirred for 2 h at RT, and then diluted with ethyl acetate. The mixture was filtered through a pad of celite. The filtrate was washed with aq. HCl (1 M) two times, and the aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with H<sub>2</sub>O and brine, dried over MgSO<sub>4</sub>, and concentrated *in vacuo*. The pyridine hydrochloride was removed by filtering through a plug of cotton, and the filtrate was evaporated. The residue was chromatographed on silica gel (hexane/ethyl acetate; 8:1 to 4:1) to give methyl ester **18** (2.77 g, 93%).

## 19

Activated magnesium (2.91 g, 120 mmol) in dry diethyl ether (46 mL) was added methyl iodide (5.7 mL, 92 mmol) with dropwise at 0 °C. To a solution of methyl ester **18** (5.17 g, 18.3 mmol) in dry diethyl ether (90 mL) was added above Grignard reagent (46 mL, 2 M) with dropwise at –78 °C. The mixture was stirred for 15 min at –78 °C and then warmed to RT. After stirring for 2.5 h, the reaction was quenched by careful addition of sat. aq. NH<sub>4</sub>Cl. The aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over MgSO<sub>4</sub>, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 4:1 to 2:1) to give diol **19** (4.59 g, 89%).

## 20

A solution of diol **19** (300 mg, 1.06 mmol) in dry acetonitrile (10 mL) was added molecular sieves 4Å (530 mg) and *N*-methyl morpholine *N*-oxide (250 mg, 2.12 mmol) and stirred for 30 min. Tetraisopropylammonium perruthenate (15 mg, 0.043 mmol) was added, then the reaction mixture was warmed to RT. After stirring for 2.5 h, the mixture was filtered through a pad of silica gel, and the filtrate was evaporated to give a crude of ketone. A suspension of (bromomethyl)triphenylphosphonium bromide (2.32 g, 5.31 mmol) in toluene (50 mL) was sonicated for 30 min at RT and evaporated with toluene (50 mL) three times at 50 °C. The residue was dissolved in dry THF (7 mL) and cooled to 0 °C. To the solution was added a solution of sodium bis(trimethylsilyl)amide in THF (2.80 mL, 5.20 mmol, 1.9 M) with dropwise, and the resulting solution was stirred for 30 min at 0 °C. A solution of above crude ketone (1.06 mmol) in THF (1.2 mL) was added to the solution with dropwise. After additive 3 h, the reaction was quenched by the addition of a few drops of sat. aq. NH<sub>4</sub>Cl. The mixture was filtered, and the filtrate was evaporated. The residue was chromatographed on silica gel (hexane/ethyl acetate; 20:1 to 10:1) to give bromo olefin **20** (239 mg, 63%, 2 steps).

## 21

A solution of bromo olefin **20** (510 mg, 1.45 mmol) in dichloromethane (7.2 mL) was added 2,6-lutidine (0.34 mL, 2.9 mmol) and dropwised triethylsilyl trifluoromethanesulfonate (0.42 mL, 1.9 mmol). After stirring for 2.5 h, the reaction was quenched by sat. aq. NaHCO<sub>3</sub>. The aqueous layer was extracted with dichloromethane three times. The combined organic extracts were dried over MgSO<sub>4</sub>, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/dichloromethane; 10:1) to give **21** (0.62 g, 91%).

## 1-NHBoc-25OHD (23a,b)

To a mixture of **21** (109 mg, 0.231 mmol) and **14a** (36 mg, 0.10 mmol) in toluene-Et<sub>3</sub>N (1:1, 2.0 mL) was added Pd(PPh<sub>3</sub>)<sub>4</sub> (23 mg, 20 mol%) at RT, then the reaction mixture was stirred for 3.5 h at 90 °C. The reaction mixture was filtered through a pad of silica gel column and the filtrates were evaporated. The residue was chromatographed on silica gel (hexane/ethyl acetate; 100:1) to give

**22a** (19 mg, 0.025 mmol, not pure). To a solution of **22a** (19 mg, 0.025 mmol) in THF (1 mL) was added 3HF-Et<sub>3</sub>N (0.050 mL, 0.30 mmol) at RT. The reaction was monitored by thin layer chromatography on silica gel plates (eluent: 1:1 ethyl acetate to hexane). The reaction was quenched by sat. aq. NaHCO<sub>3</sub> and the aqueous layer was extracted with ethyl acetate three times. The combined organic extracts were washed with brine, dried over MgSO<sub>4</sub>, and concentrated *in vacuo*. The residue was chromatographed on silica gel (hexane/ethyl acetate; 5:2 to 2:1) to give 1 $\alpha$ -NHBoc-25OHD (**23a**, 7.2 mg, 14%, 2 steps) as a colorless oil. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>)  $\delta$  6.35 (d, *J* = 11.3 Hz, 1H), 5.99 (d, *J* = 11.3 Hz, 1H), 5.22 (s, 1H), 4.95 (s, 1H), 4.51-4.08 (m, 3H), 2.81 (d, *J* = 12.5 Hz, 1H), 2.56 (d, *J* = 12.5 Hz, 1H), 2.35-0.85 (m, 39H), 1.45 (s, 9H), 1.21 (s, 6H), 0.93 (d, *J* = 6.2 Hz, 3H), 0.53 (s, 3H); HRMS-ESI (*m/z*) calcd for C<sub>32</sub>H<sub>53</sub>NNaO<sub>4</sub>, 538.3872; found, 538.3882 [(M+Na)<sup>+</sup>].

By following the same procedure described for **23a**, 1 $\beta$ -NHBoc-25OHD (**23b**, 3.5 mg, 13%, 2 steps, colorless oil) was obtained from **16** (59 mg, 0.12 mmol) and **14b** (19 mg, 0.054 mmol).

#### 1-amino-25OHD (**24a,b**)

Acetyl chloride (0.080 mL, 1.1 mmol) was slowly added to EtOH (1.1 mL) with dropwise at 0 °C. After stirring for 30 min, the solution was added to **23a** (15.9 mg, 0.0308 mmol). After additional stirring for 6 h, the reaction mixture was evaporated. The residue was washed with diethyl ether, affording **24a** (13.0 mg, 93%) as a yellow solid. <sup>1</sup>H NMR (300 MHz, MeOH-*d*<sub>4</sub>)  $\delta$  6.45 (d, *J* = 11.0 Hz, 1H), 6.04 (d, *J* = 11.0 Hz, 1H), 5.28 (s, 1H), 5.17 (s, 1H), 4.19-4.11 (m, 1H), 4.07-4.02 (m, 1H), 2.89 (d, *J* = 10.5 Hz, 1H), 2.57 (d, *J* = 10.5 Hz, 1H), 2.41-1.13 (m, 27H), 0.97 (d, *J* = 6.2 Hz, 3H), 0.58 (s, 3H); HRMS-ESI (*m/z*) calcd for C<sub>27</sub>H<sub>46</sub>NO<sub>2</sub>, 416.3529; found, 416.3532 [(M+H)<sup>+</sup>].

By following the same procedure described for **24a**, **24b** (2.8 mg, 92%) was obtained from **23b** (3.5 mg, 0.0068 mmol) as a yellow solid. <sup>1</sup>H NMR (300 MHz, MeOH-*d*<sub>4</sub>)  $\delta$  6.50 (d, *J* = 11.0 Hz, 1H), 6.01 (d, *J* = 11.0 Hz, 1H), 5.38 (s, 1H), 5.19 (s, 1H), 4.11-4.01 (m, 1H), 3.92-3.86 (m, 1H), 2.89 (d, *J* = 10.5 Hz, 1H), 2.57 (d, *J* = 10.5 Hz, 1H), 2.42-1.14 (m, 27H), 0.97 (d, *J* = 6.2 Hz, 3H), 0.57 (s, 3H).

## 25

To a solution of 4-[3-(trifluoromethyl)-3*H*-diazirin-3-yl]benzoic acid (0.500 g, 2.17 mmol; purchased from Tokyo Chemical Industry Co., Ltd.) and *N*-hydroxysuccinimide (0.250 g, 2.17 mmol) in dichloromethane (15 mL) was added *N,N'*-dicyclohexylcarbodiimide (0.450 g, 2.17 mmol) at 0 °C and the mixture was stirred at RT overnight. The precipitation was filtered off and the filtrate was concentrated under reduced pressure to give a crude product. To a solution of 5-methyl-L-glutamate (0.380 g, 2.36 mmol) in acetonitrile/H<sub>2</sub>O (10:3, 13 mL) was added the crude product and triethylamine (0.660 g, 6.52 mmol) and the mixture was stirred at RT overnight. The mixture was evaporated and the residue was dissolved in ethyl acetate, washed with aq. HCl (1 M), water, brine, and dried (MgSO<sub>4</sub>). The solvent was evaporated to afford crude of carboxylic acid (0.890 g), which was used in next step without further purification. To a mixture of crude carboxylic acid (0.890 g) and *N*-Boc-4,7,10-trioxa-1,13-tridecanediamine (0.690 g, 2.15 mmol) in dichloromethane (10 mL) were added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.50 g, 2.6 mmol), 4-dimethylaminopyridine (0.32 g, 2.85 mmol) and 1-hydroxybenzotriazole monohydrate (0.35 g, 2.59 mmol) at 0 °C, and the resulting mixture was stirred at RT overnight. The mixture was washed with sat. aq. NaHCO<sub>3</sub>, water, and brine. The organic layer was dried over anhydrous

magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (ethyl acetate:hexane; 2:1) to yield **25** (0.760 g, 1.13 mmol, 52% for 3 steps).

## 26

To a solution of **25** (0.760 g, 1.13 mmol) in dichloromethane (10 mL) was added trifluoroacetic acid (2 mL) at 0 °C and the mixture was stirred at RT for 2 h. The solvent was evaporated and the residue was dissolved in chloroform, washed with sat. aq. Na<sub>2</sub>CO<sub>3</sub>, water and brine. The organic layer was dried over anhydrous magnesium sulfate and concentrated *in vacuo* to afford a crude product (0.60 g). To a solution of the crude product (0.60 g) in *N,N'*-dimethylformamide (5 mL) was added NHS-Biotin (0.36 g, 1.04 mmol) and triethylamine (0.32 g, 3.12 mmol), and the mixture was stirred at RT overnight. The solvent was evaporated and the residue was purified by column chromatography (methanol: chloroform; 1:10) to yield **26** (0.70 g, 0.87 mmol, 77% for 2 steps).

## 27

To a mixture of **26** (0.56 g, 0.70 mmol) in tetrahydrofuran (3 mL) and water (1 mL) was added lithium hydroxide monohydrate (59 mg, 1.4 mmol) at 0°C and the mixture was stirred at RT for 2h. The mixture was acidified with aq. HCl (1 M) and extracted with chloroform three times. The combined extracts were dried over anhydrous magnesium sulfate and concentrated *in vacuo*. The residue was purified by column chromatography (methanol:chloroform; 1:3) to yield **27** (0.46 g, 0.59 mmol, 84%, diastereomixture).

## 1

To a mixture of **27** (4.9 mg, 0.0062 mmol) and **24a** (1.4 mg, 0.0031 mmol) in methanol (1.0 mL) were added 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (1.7 mg, 0.0062 mmol) and *N,N*-diisopropylethylamine (0.54 µL, 0.0031 mmol) at 0 °C and the mixture was stirred at RT overnight. The solvent was evaporated and the residue was purified by HPLC to yield **1** (1.9 mg, 0.0016 mmol, 51%, major single diastereomer). <sup>1</sup>H NMR (CD<sub>3</sub>OD) δ 0.51 (s, 3H), 0.94-3.36 (m, 47H), 3.48-3.64 (m, 20H), 4.03-4.81 (m, 4H), 4.88 (s, 1H), 5.15 (s, 1H), 5.96 (d, 1H), 6.32 (d, 1H), 7.35 (d, 2H), 8.01 (d, 2H); HRMS-FAB (*m/z*) calcd for C<sub>61</sub>H<sub>90</sub>F<sub>3</sub>N<sub>8</sub>NaO<sub>10</sub>S, 1207.6429; found, 1207.6427 [(M+Na)<sup>+</sup>].

## Supplemental References

Sakai, J., Duncan, E.A., Rawson, R.B., Hua, X., Brown, M.S., and Goldstein, J.L. (1996). Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. *Cell* **85**, 1037-1046.

Sakai, J., Rawson, R.B., Espenshade, P.J., Cheng, D., Seegmiller, A.C., Goldstein, J.L., and Brown, M.S. (1998). Molecular identification of the sterol-regulated luminal protease that cleaves SREBPs and controls lipid composition of animal cells. *Mol Cell* **2**, 505-514.

Yabe, D., Xia, Z.P., Adams, C.M., and Rawson, R.B. (2002). Three mutations in sterol-sensing domain of SCAP block interaction with insig and render SREBP cleavage insensitive to sterols. *Proc Natl Acad Sci U S A* **99**, 16672-16677.

Yang, T., Espenshade, P.J., Wright, M.E., Yabe, D., Gong, Y., Aebersold, R., Goldstein, J.L., and Brown, M.S. (2002). Crucial step in cholesterol homeostasis: sterols promote binding of SCAP to INSIG-1, a membrane protein that facilitates retention of SREBPs in ER. *Cell* **110**, 489-500.

Yang, T., Goldstein, J.L., and Brown, M.S. (2000). Overexpression of membrane domain of SCAP prevents sterols from inhibiting SCAP.SREBP exit from endoplasmic reticulum. *J Biol Chem* **275**, 29881-29886.