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RESEARCH ARTICLE

COPI-mediated retrieval of SCAP is crucial for regulating lipogenesis under basal and sterol-deficient conditions

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ABSTRACT

Retrograde trafficking from the Golgi complex to endoplasmic reticulum (ER) through COPI-coated vesicles has been implicated in lipid homeostasis. Here, we find that a block in COPI-dependent retrograde trafficking promotes processing and nuclear translocation of sterol regulatory element binding proteins (SREBPs), and upregulates the expression of downstream genes that are involved in lipid biosynthesis. This elevation in SREBP processing and activation is not caused by mislocalization of S1P or S2P (also known as MBTPS1 and MBTPS2, respectively), two Golgi-resident endoproteases that are involved in SREBP processing, but instead by increased Golgi residence of SREBPs, leading to their increased susceptibility to processing by the endoproteases. Analyses using a processing-defective SREBP mutant suggest that a fraction of SREBP molecules undergo basal cycling between the ER and Golgi in complex with SREBP cleavage-activating protein (SCAP). Furthermore, we show that SCAP alone is retrieved from the Golgi and moves to the ER after processing of SREBP under sterol-deficient conditions. Thus, our observations indicate that COPI-mediated retrograde trafficking is crucial for preventing unnecessary SREBP activation, by retrieving the small amounts of SCAP–SREBP complex that escape from the sterol-regulated ER retention machinery, as well as for the reuse of SCAP.

KEY WORDS: COPI-coated vesicles, Golgi complex, Lipogenesis, SCAP, SREBP

INTRODUCTION

In eukaryotic cells, triacylglycerols (TAGs) and steryl esters are stored in cytoplasmic lipid droplets. According to the prevailing view of lipid droplet biogenesis, TAGs and steryl esters accumulate between the two leaflets of the endoplasmic reticulum (ER) membrane and gradually grow into globules. The nascent lipid droplets then bud into the cytoplasm to generate isolated lipid droplets (Ohsaki et al., 2014; Wilfling et al., 2014a). By contrast, neutral lipids in lipid droplets are mobilized through the action of lipases, including adipose triglyceride lipase (ATGL, also known as PNPLA2). Thus, lipid storage in lipid droplets is in dynamic equilibrium between the generation and mobilization of lipids.

Systematic studies using small interfering (si)RNA screening have revealed that knockdown of components of COPI-mediated Golgi-to-ER retrograde trafficking, such as COPI subunits, Arf GTPases and the Arf-guanine nucleotide exchange factor GBF1, gives rise to an increase in lipid droplet size (Beller et al., 2008; Guo et al., 2008). These data suggest that COPI-mediated trafficking directly regulates lipid storage and/or mobilization. Furthermore, COPI-dependent delivery of ATGL to lipid droplets has been implicated in lipolysis, suggesting that inhibition of COPI-mediated trafficking results in lipid storage (Ellong et al., 2011; Soni et al., 2009). We have recently confirmed that knockdown of COPI-mediated trafficking components results in increased lipid storage; however, we failed to show direct evidence for the involvement of COPI-mediated trafficking in the delivery of ATGL to lipid droplets (Takashima et al., 2011). Therefore, we were interested in lipogenesis as well as lipolysis in order to understand the role of COPI-mediated trafficking in lipid homeostasis.

Sterol regulatory element-binding proteins (SREBPs) are transcription factors that function as master regulators of genes that are responsible for the synthesis of fatty acids and cholesterol (Goldstein et al., 2006; Horton et al., 2002). SREBP1 and SREBP2 (also known as SREBF1 and SREBF2, respectively) are synthesized as transmembrane precursors; there are two SREBP1 variants, designated SREBP1a and SREBP1c, which are derived from a single gene through the use of alternative transcription start sites. Under sterol-rich conditions, they are retained in the ER in complex with an escort protein, SREBP cleavage-activating protein (SCAP), which in turn forms a complex with the ER-resident Insig proteins (of which there are two, Insig1 and Insig 2) (see Fig. 6A). When cellular sterol is depleted, SCAP undergoes a conformational change, leading to its release from Insig proteins. Liberated SCAP, which can interact with the COPII coat proteins, escorts SREBPs from the ER to the Golgi complex through COPII-coated vesicles. At the Golgi, SREBPs undergo sequential limited proteolysis by site-1 and site-2 proteases (S1P and S2P; also known as MBTPS1 and MBTPS2, respectively), thereby releasing the SREBP transcription factor domain (TFD) from the N-terminal cytosolic region. The TFD is then translocated into the nucleus and promotes the expression of various genes that are involved in lipid biosynthesis (see Fig. 6B). According to the prevailing model (Goldstein et al., 2006), the SCAP–SREBP complex is retained in the ER under sterol-rich conditions, preventing unnecessary SREBP activation (see Fig. 6A).

In this study, we found that inhibition of COPI-mediated retrograde trafficking promotes the processing of SREBPs to yield the mature TFDs, which are then translocated into the nucleus to upregulate the expression of downstream genes that are involved in lipid biosynthesis. More importantly, the data indicate that although a fraction of the SCAP–SREBP complex constitutively escapes the sterol-regulated ER-retention machinery, the proteins that have escaped are retrieved from the Golgi, back to the ER, through COPI-mediated trafficking. Furthermore, we found that SCAP alone

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undergoes COPI-mediated retrieval from the Golgi after regulated ER-to-Golgi trafficking of the SCAP–SREBP complex, and following SREBP processing.

RESULTS

A block in COPI-mediated trafficking increases nuclear SREBP accumulation and the expression of genes involved in lipid biosynthesis

We have previously shown that cells depleted of any component of COPI-mediated trafficking, including β-COP (also known as COPB1), exhibit enlarged lipid droplets associated with an increase in cellular TAG content (Takashima et al., 2011); COPI-coated vesicles are involved in retrograde protein trafficking from the cis-Golgi to the ER, whereas COPII-coated vesicles are involved in anterograde trafficking of proteins, including the transport of the SCAP–SREBP complex, from the ER to the Golgi (Harter and Wieland, 1996). This prompted us to examine whether SREBPs, which are master regulators of TAG and cholesterol biosynthesis, are implicated in the observed increase in cellular lipid storage, for the following reasons – (i) ER-to-Golgi trafficking regulates the activation of SREBPs; under sterol-depleted conditions, SCAP escorts SREBPs to the Golgi through COPII-coated vesicles, and at the Golgi, SREBPs undergo sequential processing by the Golgi-resident S1P and S2P endoproteases to liberate the cytosolic TFDs (Goldstein et al., 2006; Horton et al., 2002) (also see Fig. 6B). (ii) Inhibition of COPI-mediated trafficking often perturbs the dynamic equilibria of proteins between the ER and the Golgi (for example, see Saitoh et al., 2009), and can therefore perturb the subcellular distribution of SREBPs and/or the endoprotease(s).

To determine whether SREBPs are activated under COPI-deficient conditions, we treated HeLa cells with control small interfering (si)RNAs or siRNAs against β-COP (Fig. 1A,B), as described previously (Saitoh et al., 2009; Takashima et al., 2011). We then incubated these cells in medium containing lipoprotein-deficient serum (LPDS) and oleic acid that had been supplemented with or without 25-hydroxycholesterol (25-HC), which blocks the

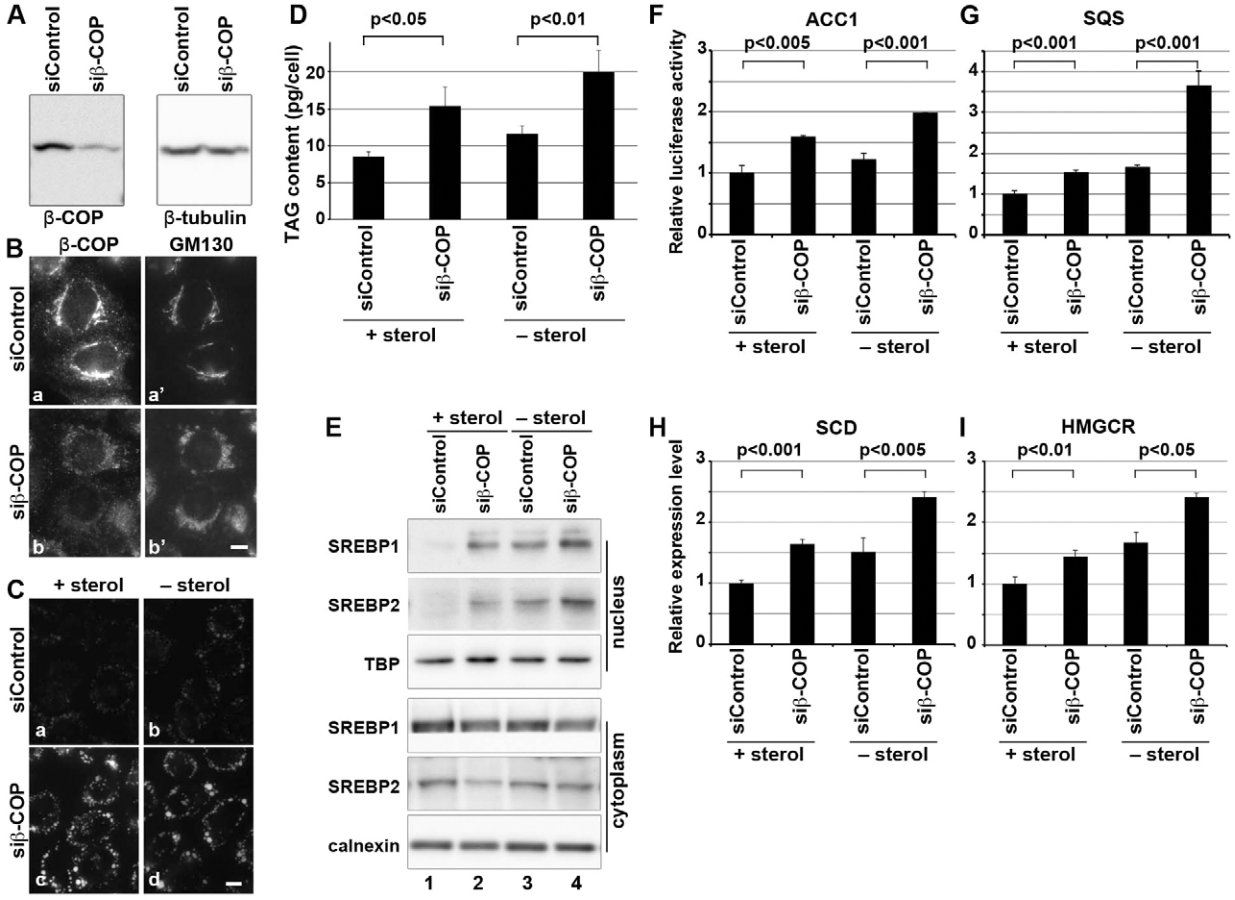

Fig. 1. β-COP knockdown increases nuclear SREBP accumulation and the expression of genes involved in lipogenesis. (A–D,H,I) HeLa cells that had been cultured in normal medium were transfected with control siRNAs (for LacZ, siControl) or siRNAs against β-COP (siβ-COP) and incubated for 20 h. (A) Lysates prepared from the siRNA-transfected cells were processed for immunoblot analysis with an antibody against β-COP (left) or β-tubulin as a control (right). (B) The siRNA-transfected cells were co-immunostained for β-COP (a,b) and GM130 as a Golgi marker (a′,b′). (C,D,H,I) After switching the medium to MEM supplemented with 10% LPDS and 400 µM oleic acid, with and without 1 µg/ml 25-HC (+ and – sterol, respectively), the siRNA-transfected cells were incubated for 16 h and processed for staining with BODIPY 493/503 (C), TAG quantification (D), or RT-qPCR analyses for *SCD* (H) or *HMGCR* (I). (E) After the medium switching, the siRNA-transfected cells were incubated for 6 h, and after addition of ALLN (a final concentration, 100 µM) to inhibit protein degradation by the proteasome, were further incubated for 6 h. The cells were then fractionated into nuclear and cytoplasmic fractions, which were processed for immunoblot analysis for SREBP1, SREBP2, TBP (a nuclear protein) or calnexin (an ER protein). (F,G) Cells were transfected with a reporter construct for the *ACC1* (F) or *SQS* (G) promoter, transfected with siControl or siβ-COP and cultured in MEM supplemented with 10% LPDS and 400 µM oleic acid, with or without 1 µg/ml 25-HC. The cells were then subjected to luciferase assays. (D,F–I) The data are means±s.d. of three independent experiments. *P*-values were calculated using one-way ANOVA followed by Tukey's test. Scale bars: 10 µm (B,C).

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exit of SREBPs from the ER and thereby suppresses their processing and activation at the Golgi. The cells were then stained with BODIPY 493/503 in order to visualize lipid droplets, assayed for cellular TAG content or subjected to fractionation to separate the nuclear and cytoplasmic (containing the ER) fractions, which were then processed for immunoblot analysis of SREBP1 or SREBP2. As shown in Fig. 1C, lipid deposition in lipid droplets was elevated in the β-COP-knockdown cells (Fig. 1Cc,Cd) relative to control cells (Fig. 1Ca,Cb) irrespective of the presence (Fig. 1Ca,Cc) or absence (Fig. 1Cb,Cd) of sterol. Almost in parallel with the increased lipid deposition, the cellular TAG content was significantly increased by β-COP knockdown, again irrespective of the presence or absence of sterol (Fig. 1D).

Under sterol-depleted conditions, SREBPs undergo COPII-mediated anterograde trafficking from the ER to the Golgi, where they are processed by S1P and S2P to liberate their cytosolic TFDs, which are then translocated into the nucleus (Goldstein et al., 2006; Horton et al., 2002). As shown in Fig. 1E, the nuclear mature forms of SREBP1 and SREBP2 were barely detectable in control cells that had been incubated in the presence of sterol (lane 1), whereas substantial amounts of the mature forms were found in the nuclear fraction of control cells that had been incubated in the absence of sterol (lane 3). When β-COP-knockdown cells were incubated in the absence of sterol, the levels of the nuclear mature forms of SREBP1 and SREBP2 were slightly higher than those in control cells that had been incubated in the absence of sterol (compare lane 4 with lane 3). The most striking changes were observed in β-COP-knockdown cells that had been incubated with sterol (compare lane 2 with lane 1) – substantial amounts of the mature forms of SREBP1 and SREBP2 were found in the nuclear fractions, even in the presence of sterol. Thus, processing of SREBPs to yield the mature TFDs was enhanced by knockdown of β-COP, irrespective of the cellular sterol level.

The results described above implicate SREBP activation in the observed increases in lipid deposition and TAG content in β-COP-knockdown cells. To further test this hypothesis, cells were transfected with a luciferase reporter plasmid containing the promoter region of the gene encoding acetyl-CoA carboxylase 1 (*ACC1*, also known as *ACACA*) or squalene synthase (*SQS*, also known as *FDFT1*), which are primarily controlled by SREBP1 and SREBP2, respectively (Horton et al., 2002). The transfected cells were treated with siRNAs against β-COP and subjected to luciferase assays to assess the transcriptional activity of endogenous SREBPs. Under sterol-depleted conditions, the activity of both the *ACC1* and *SQS* promoters was approximately doubled by knockdown of β-COP (Fig. 1F,G). Furthermore, knockdown of β-COP increased the activity of both promoters even in the presence of sterol, although the basal activities were lower than those in the absence of sterol.

We also examined the effects of β-COP knockdown on the levels of the mRNAs encoding stearoyl-CoA desaturase (*SCD*, also known as *SCD5*) and HMG-CoA reductase (*HMGCR*), which are regulated mainly by SREBP1 and SREBP2, respectively (Horton et al., 2002), by using quantitative reverse-transcription PCR (RT-qPCR) analyses. As shown in Fig. 1H,I, the results were generally consistent with those obtained using the reporter assay – i.e. β-COP knockdown resulted in an increase in the levels of both mRNAs under both sterol-rich and sterol-depleted conditions – although the relative expression levels were lower under the sterol-rich conditions. Thus, both the reporter assay and RT-qPCR analyses revealed that the activity of SREBP1 and SREBP2 is increased by knocking down β-COP, even under sterol-rich conditions, under which the exit of SCAP–SREBP from the ER is normally suppressed.

Localization of S1P or S2P is not altered in COPI-compromised cells

To explore the mechanism underlying the increased activation of SREBPs under COPI-compromised conditions, we first addressed the possibility that Golgi-to-ER retrograde trafficking of the processing endoproteases S1P and S2P is elevated. This hypothesis was based on the fact that the treatment of cells with brefeldin A (BFA), which causes the Golgi complex to collapse into the ER by inhibiting Arf guanine nucleotide exchange factors (Jackson, 2000; Sciaky et al., 1997), results in increased processing of SREBP2 owing to mislocalization of S1P to the ER (DeBose-Boyd et al., 1999), as well as in increased lipid deposition and TAG content (Beller et al., 2008; Takashima et al., 2011). As shown in supplementary material Fig. S1, exogenously expressed hemagglutinin (HA)-tagged S1P and S2P were localized predominantly in the perinuclear Golgi region in control cells (supplementary material Fig. S1Aa–a‴, Ba–a‴), as revealed by staining for the Golgi protein giantin (also known as GOLGB1). Consistent with a previous report (Bartz et al., 2008; DeBose-Boyd et al., 1999), treatment with BFA altered the staining pattern of S1P or S2P so that it matched that of the ER (supplementary material Fig. S1Ac–c‴,Bc–c‴). In cells treated with BFA or golgicide A, which is a more specific Arf guanine nucleotide exchange factor inhibitor, processing of SREBP1c was observed even in the presence of sterol (supplementary material Fig. S1C), as reported for SREBP2 in BFA-treated cells (DeBose-Boyd et al., 1999). In contrast to the effect of BFA, β-COP knockdown (supplementary material Fig. S1Ab–b‴,Bb–b‴) did not alter the Golgi localization of S1P, S2P or giantin. These observations are consistent with our previous study showing that β-COP knockdown does not affect the localization of other Golgi proteins (GM130 and golgin-97; Saitoh et al., 2009) and indicate that, in COPI-compromised cells, a mechanism other than redistribution of S1P and/or S2P contributes to SREBP activation; however, they do not completely exclude the possibility that a small fraction of S1P and/or S2P molecules are redistributed to the ER.

COPI-dependent retrieval of SCAP and SREBPs from the Golgi to the ER

In light of the observations described above, we investigated whether SREBP trafficking is altered in COPI-compromised cells. To this end, we exploited a SREBP1c mutant (R503A) that is defective in S1P-catalyzed cleavage (Hua et al., 1996b) and compared the subcellular localizations of wild-type (WT) and mutant SREBP1c proteins in control and β-COP-knockdown cells. The presence of exogenously expressed SREBP in large excess of the endogenous level of the escort protein SCAP prevents the expressed SREBP from exiting the ER, even under sterol-depleted conditions (Hua et al., 1996a; also see supplementary material Fig. S3A); to circumvent this problem, we co-infected HeLa cells with recombinant retroviruses encoding red fluorescent protein (mRFP)-tagged SREBP1c and SCAP–HA in order to achieve moderate expression levels of both proteins (Fig. 2).

In the control cells, under both sterol-rich (Fig. 2Aa–Aa‴,Ba) and sterol-depleted (Fig. 2Ab–Ab‴,Bb) conditions, mRFP–SREBP1c (WT) was primarily found in the nucleus (Fig. 2Aa,Ba, and Ab,Bb, respectively), whereas SCAP–HA was mainly localized in the Golgi (Fig. 2Aa′,Ab′). This observation indicates that exogenously expressed SREBP1c and SCAP are constitutively trafficked to the Golgi, and that the former is subsequently cleaved by the S1P and S2P endoproteases and translocated into the nucleus. This result is in agreement with previous studies showing that overexpression of

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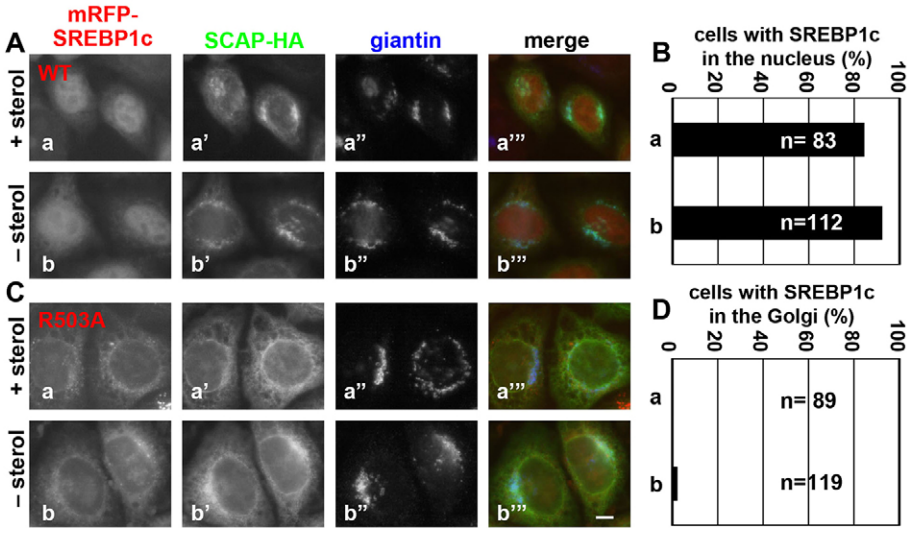

Fig. 2. Constitutive exit of exogenously expressed SREBP and SCAP from the ER. HeLa cells were co-infected with recombinant retroviruses for either mRFP–SREBP1c(WT) (A,B) or –SREBP1c(R503A) (C,D) and SCAP–HA, and then cultured in MEM supplemented with 10% LPDS and 400 µM oleic acid in the presence (+ sterol, a–a‴) or absence (– sterol, b–b‴) of 1 µg/ml 25-HC. The cells were then stained for HA (a′,b′) and giantin (a″,b″). Cells with typical nuclear fluorescence of mRFP–SREBP1c(WT) (A) or Golgi fluorescence of mRFP–SREBP1c(R503A) (C) were counted, and the percentages were expressed as bar graphs (B,D, respectively). Scale bar: 10 µm.

SCAP leads to a marked increase in the level of the nuclear mature SREBP2, even under sterol-rich conditions (Hua et al., 1996a; Yang et al., 2002), because overexpressed SCAP saturates the endogenous level of Insig proteins, and excess free SCAP escorts SREBPs to the Golgi, irrespective of the cellular sterol level.

When mRFP–SREBP1c(R503A) and SCAP–HA were co-expressed, both the SREBP1c mutant and SCAP were predominantly found in the ER under both sterol-rich (Fig. 2Ca–Ca‴,Da) and sterol-depleted (Fig. 2Cb–Cb‴,Db) conditions. These observations were somewhat unexpected given the results that have been described above (Fig. 2A,B) and in previous studies (Hua et al., 1996a; Yang et al., 2002), which indicate that exogenously expressed SREBPs and SCAP constitutively exit the ER. Two models might explain the observations obtained by co-expressing SREBP1c(R503A) and SCAP – (i) the complex of SCAP and SREBP1c(R503A) could not exit the ER; (ii) although the SCAP–SREBP1c(R503A) complex was constitutively transported from the ER to the Golgi, the complex was efficiently retrieved back to the ER. The results of the following experiments support the latter possibility.

To achieve sterol-regulated transport of the SCAP–SREBP complex, we then triple-infected cells with recombinant retroviruses encoding mRFP–SREBP1c, SCAP–HA and Insig2–FLAG (Fig. 3). In contrast to cells that lacked exogenous Insig2 expression, in which SREBP1c(WT) and SCAP constitutively exited the ER (Fig. 2A,B), cells co-expressing Insig2 exhibited regulated changes in the localization of SREBP1c and SCAP – under sterol-rich conditions (Fig. 3Aa–Aa‴,Ba), SREBP1c(WT) and SCAP were mainly found in the ER (Fig. 3Aa,Aa′), and the former was barely detectable in the nucleus (Fig. 3Aa), whereas under sterol-depleted conditions (Fig. 3Ac–Ac‴,Bc), SREBP1c (WT) and SCAP were found primarily in the nucleus (Fig. 3Ac) and the Golgi (Fig. 3Ac′), respectively. When the triple-infected cells were subjected to knockdown of β-COP, under either sterol-depleted (Fig. 3Ad–Ad‴,Bd) or sterol-rich (Fig. 3Ab–Ab‴,Bb) conditions, SREBP1c(WT) and SCAP were localized predominantly in the nucleus (Fig. 3Ab,Ad) and the Golgi (Fig. 3Ab′,Ad′), respectively. Comparison of the localization of SREBP1c(WT) and SCAP in control cells (Fig. 3A,Ca–Ca‴), and in β-COP-knockdown cells (Fig. 3A,Cb–Cb‴) under sterol-rich conditions revealed that a block in COPI-mediated transport caused an accumulation of SCAP–SREBP and increased SREBP1c processing in the Golgi. It is noteworthy that, in β-COP-knockdown cells, the Golgi structure revealed by staining of giantin did not undergo any substantial changes compared with that of control cells, nor did giantin redistribute to the ER (Fig. 3Ab″,Ad″), consistent with our previous report showing that β-COP knockdown does not affect the localization of other Golgi proteins (Saitoh et al., 2009). A possible explanation for these observations is that, even under sterol-rich conditions, a fraction of the SCAP–SREBP complex traffics to the Golgi complex through COPII-coated vesicles (Espenshade et al., 2002; Goldstein et al., 2006), but that the escaped complex is returned to the ER through COPI-coated vesicles.

To demonstrate basal cycling of the SCAP–SREBP complex between the ER and the Golgi, we exploited the processing-defective SREBP1c(R503A) mutant (Fig. 3C,D). In control cells that had been subjected to triple infection with retroviruses for mRFP–SREBP1c(R503A), SCAP–HA and Insig2–FLAG, both SCAP (Fig. 3Ca′,Cc′) and SREBP1c(R503A) (Fig. 3Ca,Cc) were mainly localized in the ER under both sterol-rich (Fig. 3Ca–Ca‴, Da) and sterol-depleted (Fig. 3Cc–Cc‴,Dc) conditions. In striking contrast to the control cells, both SREBP1c(R503A) (Fig. 3Cb,Cd, Db,Dd) and SCAP (Fig. 3Cb′,Cd′) were found predominantly in the Golgi, both under sterol-rich (Fig. 3Cb–Cb‴) and sterol-depleted (Fig. 3Cd–Cd‴) conditions in the β-COP-knockdown cells. To exclude the possibility that, in sterol-depleted control cells expressing mRFP–SREBP1c(R503A), the absence of SCAP and SREBP1c(R503A) in the Golgi was due to degradation of these proteins (see Discussion), and that the presence of these proteins in the ER reflected their *de novo* synthesis, cells that expressed either SREBP1c(WT) or SREBP1c(R503A), as well as SCAP and Insig2 were incubated in the presence of cycloheximide (CHX) to block *de novo* protein synthesis, and we examined the protein levels. As shown in supplementary material Fig. S2, neither the SREBP1c nor SCAP level was substantially different between cells expressing SREBP1c(WT) or SREBP1c(R503A) (supplementary material Fig. S2, lanes 1 and 3), although the SCAP level was slightly lower under sterol-depleted conditions than under sterol-rich conditions (compare supplementary material Fig. S2, lane 2 vs lane 1 and lane 4 vs lane 3). Taken together, the data shown in Fig. 3 and supplementary material Fig. S2 indicate that, even under sterol-rich conditions, a fraction of the SCAP–SREBP complex leaks out of the ER into the Golgi, but the complex is returned to the ER through COPI-coated vesicles (see Fig. 6C).

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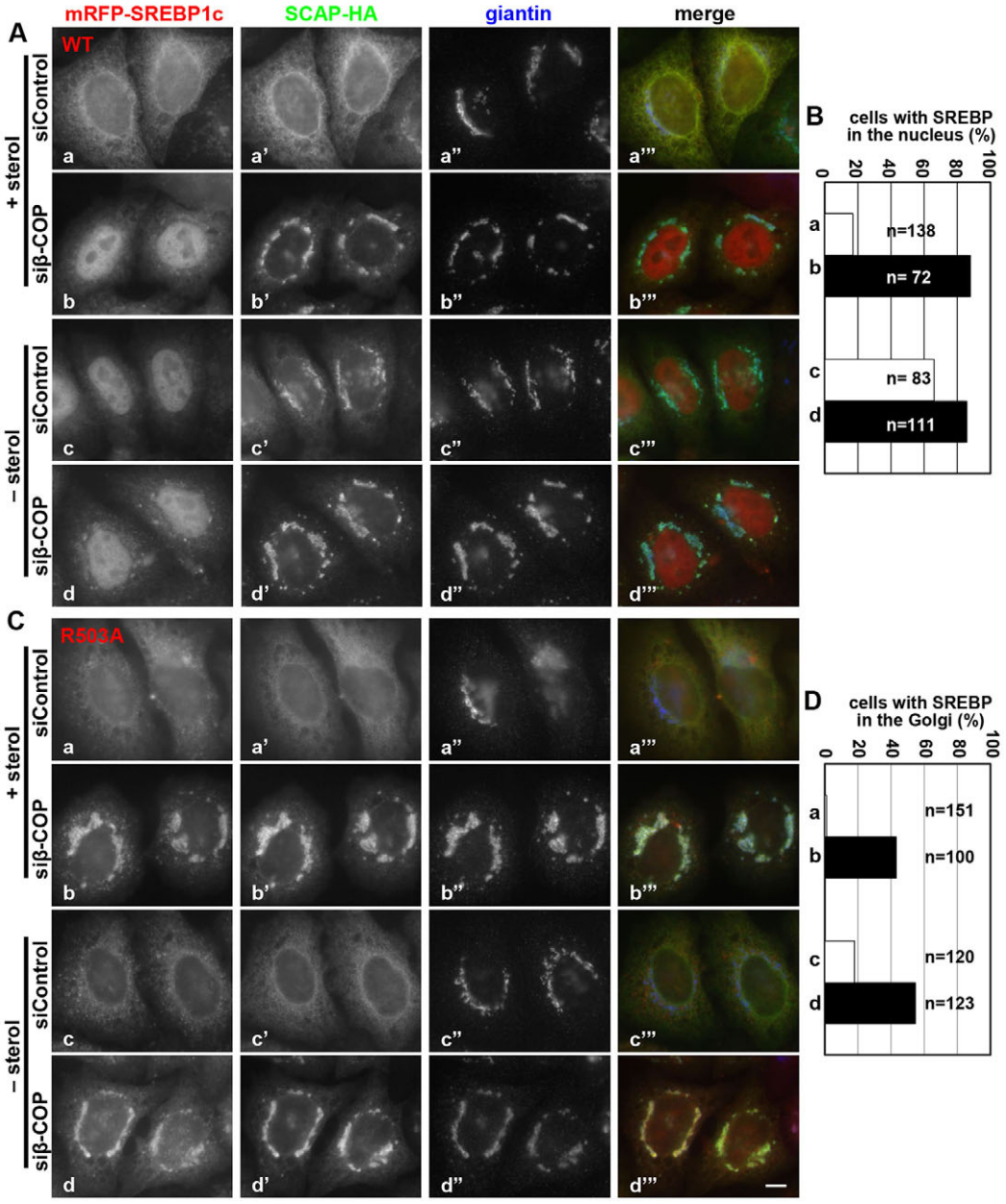

Fig. 3. COPI-dependent recycling of SREBP and SCAP to the ER. Cells were triple-infected with retroviruses for either mRFP–SREBP1c (WT) (A,B) or –SREBP1c(R503A) (C,D), SCAP–HA and Insig2–FLAG; transfected with control siRNAs (siRNAs against LacZ, siControl; a–a‴,c–c‴) or siRNAs against β-COP (siβ-COP; b–b‴,d–d‴); cultured in MEM supplemented with 10% LPDS and 400 µM oleic acid, with (+ sterol, a–a‴,b–b‴) or without (– sterol, c–c‴,d–d‴) 1 µg/ml 25-HC; and then stained for HA (a′–d′) and giantin (a″–d″). The cells with typical nuclear fluorescence of mRFP–SREBP1c(WT) (A) and those with Golgi-localized fluorescence of mRFP–SREBP1c(R503A) (C) were counted, and the percentages were expressed as bar graphs (B and D, respectively). Scale bar: 10 µm.

Suppression of SREBP activation reverses the increase in TAG content that is induced by COPI knockdown

If a basal fraction of SCAP–SREBP escapes the ER retention machinery, we predicted that depletion of SCAP would block the export of SREBPs and thereby limit its accessibility to the Golgi-resident endoproteases. This prediction is supported by the observation that when cells overexpressing the wild type or R503A mutant of mRFP-SREBP1c alone were treated with siRNAs against β-COP, both SREBP1c constructs were mainly found in the ER (supplementary material Fig. S3); even in β-COP-knockdown cells, SREBP1c(WT) was never found in the nucleus (supplementary material Fig. S3B) and SREBP1c(R503A) was never found in the Golgi (Fig. S3D).

We therefore knocked down SCAP in addition to β-COP; the knockdown efficiencies were examined by immunoblotting (supplementary material Fig. S4). As shown in Fig. 4, simultaneous knockdown of SCAP substantially suppressed the increase in the levels of the nuclear mature forms of endogenous SREBPs (Fig. 4A, compare lane 2 vs lane 4 and lane 6 vs lane 8) and suppressed the increase in the expression levels of the reporter genes (Fig. 4B,C) induced by β-COP knockdown, confirming that the increased activation of SREBPs requires prior export from the ER of SREBPs, escorted by SCAP.

Moreover, if this aberrant activation of SREBPs contributes to the increase in TAG content that is induced by β-COP knockdown, inhibition of SREBP activation by simultaneous

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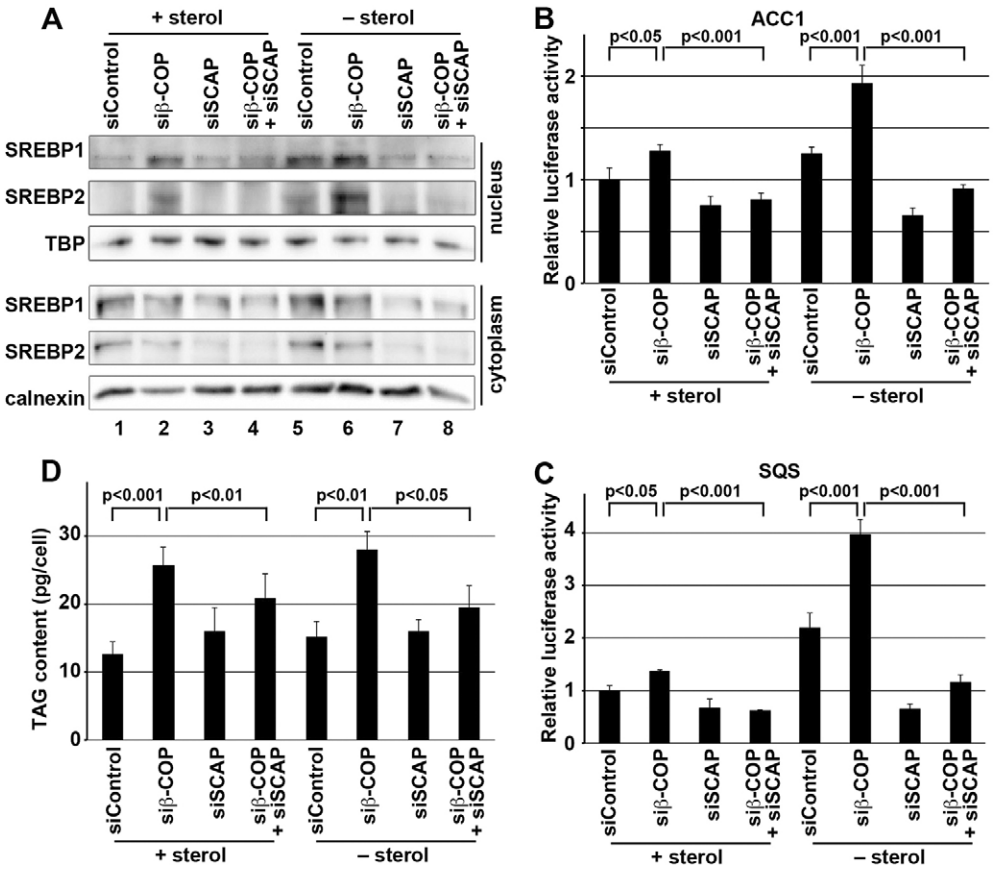

Fig. 4. SCAP knockdown attenuates SREBP activation, and β-COP knockdown increases TAG content. (A,D) Cells were transfected with control siRNAs (siControl), siRNAs targeting β-COP (siβ-COP) or SCAP (siSCAP), or a combination of siRNAs against β-COP and SCAP; they were then cultured in MEM supplemented with 10% LPDS and 400 µM oleic acid, with and without 1 µg/ml 25-HC (+ and – sterol, respectively). The cells were subjected to analysis of SREBP processing (A) or TAG quantification (D). (B,C) Cells were transfected with the *ACC1* (B) or *SQS* (C) reporter construct; transfected with control siRNAs, siRNAs targeting β-COP or SCAP, or a combination of siRNAs targeting β-COP and SCAP; cultured in MEM supplemented with 10% LPDS and 400 µM oleic acid, with or without 1 µg/ml 25-HC; and then subjected to luciferase activity assays. (B–D) The data are means±s.d. of three independent experiments. *P*-values were calculated using one-way ANOVA followed by Tukey's test.

knockdown of SCAP and β-COP should suppress the increase. In fact, in parallel with the levels of mature SREBPs, the increase in the cellular TAG content induced by β-COP knockdown were significantly reversed by simultaneous knockdown of SCAP (Fig. 4D). Thus, the increase in TAG content that is induced by the block in COPI-mediated retrograde transport depends on the aberrant activation of SREBPs.

Retrograde trafficking of SCAP after processing and activation of SREBP

Little information is available for the fate of SCAP after processing and activation of SREBP at the Golgi, although a previous cell-fractionation study reports that, even under low-sterol conditions, most SCAP appears to be present in the ER fraction (Nohturfft et al., 1999). To unequivocally determine whether SCAP that has been liberated from SREBP at the Golgi returns to the ER, we adopted the following protocol – HeLa cells that had been infected with recombinant retrovirus vectors encoding mRFP–SREBP1c, SCAP–HA and Insig2–FLAG were cultured in minimum essential medium (MEM) that had been supplemented with fetal bovine serum (FBS) and were then incubated for 90 min in MEM containing LPDS, 2-hydroxypropyl β-cyclodextrin (HPCD) and CHX in order to thoroughly deplete cellular sterols and block *de novo* protein synthesis. After washing twice with MEM containing LPDS and CHX, the cells were incubated in MEM containing FBS, 25-HC and CHX for 30 min to replenish cellular sterol. Before sterol deprivation, both SCAP and SREBP1c were predominantly localized in the ER (Fig. 5Aa,Aa′). After sterol deprivation for 90 min, a major fraction of SCAP was found in the Golgi, whereas SREBP1c was predominantly found in the nucleus, indicative of its processing at the Golgi and subsequent translocation into the nucleus (Fig. 5Ab). Thus, the SCAP–SREBP complex appeared to be trafficked from the ER to the Golgi in response to a decrease in the cellular sterol level. After sterol replenishment for 30 min, the Golgi localization of SCAP was completely lost and only its ER localization was evident (Fig. 5Ac′). Immunoblot analysis of cell lysates indicated that the protein level of SCAP was not substantially changed during the period of sterol depletion and re-addition (Fig. 5B), excluding the possibility that the SCAP protein in the Golgi undergoes degradation after sterol re-addition. The most plausible explanation for these observations is that SCAP molecules, once trafficked to the Golgi under sterol-depleted conditions, recycle back to the ER upon sterol replenishment. Taking into account the fact that SCAP accumulates in the Golgi – even under sterol-rich conditions – in β-COP-knockdown cells (Fig. 3Cb′), SCAP is most likely recycled back to the ER by COPI-mediated trafficking.

We also followed sterol-dependent changes in the localization of SCAP by using time-lapse recording. Cells expressing mRFP–SREBP1c, enhanced green fluorescent protein (EGFP)-tagged SCAP and Insig2–FLAG were incubated for 90 min in MEM containing LPDS, HPCD and CHX in order to deplete the cellular sterols and block *de novo* protein synthesis. After washing twice with MEM containing LPDS and CHX, the cells were subjected to time-lapse recording in MEM containing FBS, 25-HC and CHX (+sterol) or that containing LPDS, HPCD and CHX (−sterol). At time=0, SCAP–EGFP exhibited Golgi-localization, whereas mRFP–SREBP1c exhibited nuclear localization (Fig. 5C,D, left panels). Under continually sterol-deprived conditions, the fluorescence signals of SCAP–EGFP at the Golgi did not change substantially, although both the signals at the ER and Golgi gradually decreased, probably owing to basal levels of degradation

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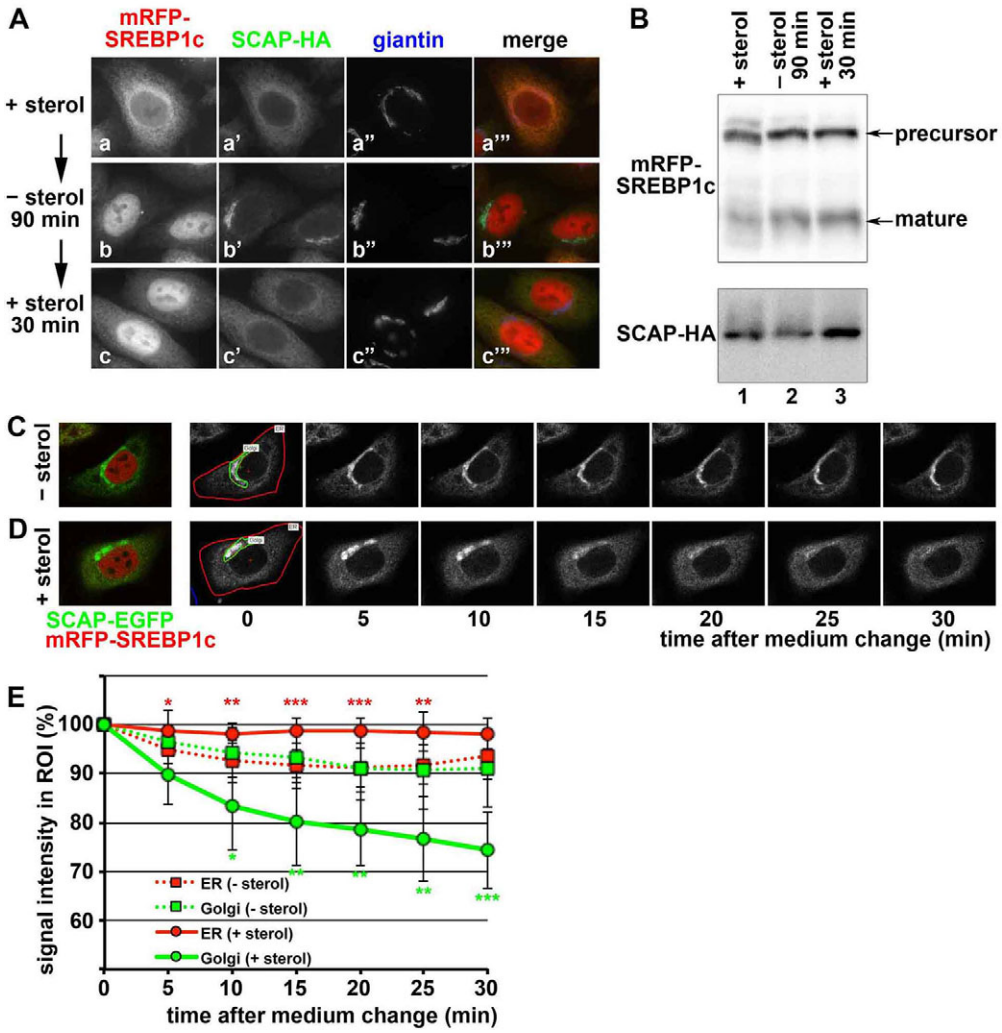

Fig. 5. SCAP recycling to the ER after processing of SREBP at the Golgi. (A,B) Cells were triple-infected with retroviruses encoding mRFP–SREBP1c, SCAP–HA and Insig2–FLAG, and cultured in MEM supplemented with 10% FBS (Aa-a‴; B, lane 1). The cells were then incubated in MEM containing 10% LPDS, 1% HPCD and 50 µg/ml CHX for 90 min to deplete cellular sterols and inhibit *de novo* protein synthesis (Ab-b‴; B, lane 2). After washing twice with MEM containing LPDS and CHX, the cells were incubated with MEM containing FBS, 1 µg/ml 25-HC and CHX for 30 min (Ac-c‴; B, lane 3). The cells were stained for HA (Aa′–Ac′) and giantin (Aa″–Ac″), or processed for immunoblot analysis with an antibody against RFP (upper panel) or HA (lower panel). After sterol depletion, a considerable fraction of SCAP–HA became localized in the Golgi region (Ab′), and after sterol re-addition, the Golgi signals for SCAP–HA were no longer detectable, and only ER signals were prominent (Ac′). (C,D) Cells that had been triple-infected with retroviruses encoding mRFP–SREBP1c, SCAP–EGFP and Insig2–FLAG were depleted of cellular sterols and washed as in A,B. The cells were then incubated in MEM containing LPDS and CHX (C, – sterol) or in that containing FBS, 25-HC and CHX (D, + sterol) and subjected to time-lapse recording. Images were acquired every 5 min. (E) The total intensities of the EGFP signal in the Golgi region (surrounded by green lines) and in the ER (the rest of the cell area) were estimated using NIS-elements (Nikon). The results are expressed as percentages of signals remaining in the Golgi region (green lines) and in the ER (red lines) in the cells incubated with sterol-deficient (– sterol, dotted lines) or -replenished medium (+ sterol, solid lines) at the indicated time points. The data are means±s.d. from five independent cells. **P*<0.05; ***P*<0.02; ****P*<0.005; Student's *t*-test.

of SCAP–EGFP and/or photobleaching of the EGFP moiety in the absence of its *de novo* synthesis (Fig. 5C,E, dotted lines; supplementary material Movie 1). In striking contrast, when the medium was switched to one that was sterol-rich, SCAP–EGFP in the Golgi region was virtually undetectable within 10 min (Fig. 5D,E, green solid line; supplementary material Movie 2). We failed to observe a significant increase in the SCAP–EGFP signal at the ER that correlated with a marked decrease in its signal in the Golgi region; this was attributable, at least in part, to the fact that the surface area of the ER was estimated to be at least fivefold larger than that of the Golgi (Griffiths et al., 1989; Sciaky et al., 1997). However, the signals at the ER after sterol replenishment (Fig. 5E, red solid line) were significantly higher than those in the continual absence of sterol (red dotted line). Taking into account that the SCAP protein level did not change during the period of sterol depletion and re-addition (Fig. 5B), these observations indicate that SCAP–EGFP molecules, once delivered to the Golgi, were recycled back to the ER, concomitant with the recovery of the cellular sterol level.

DISCUSSION

Previous studies by our group and others have shown that a block in Golgi-to-ER retrograde trafficking through COPI-coated vesicles causes an increase in lipid deposition (Beller et al., 2008; Guo et al., 2008; Soni et al., 2009; Takashima et al., 2011). Here, we extended our previous study and showed that aberrant activation of SREBPs, master regulators of biosynthesis of fatty acids and cholesterol, can

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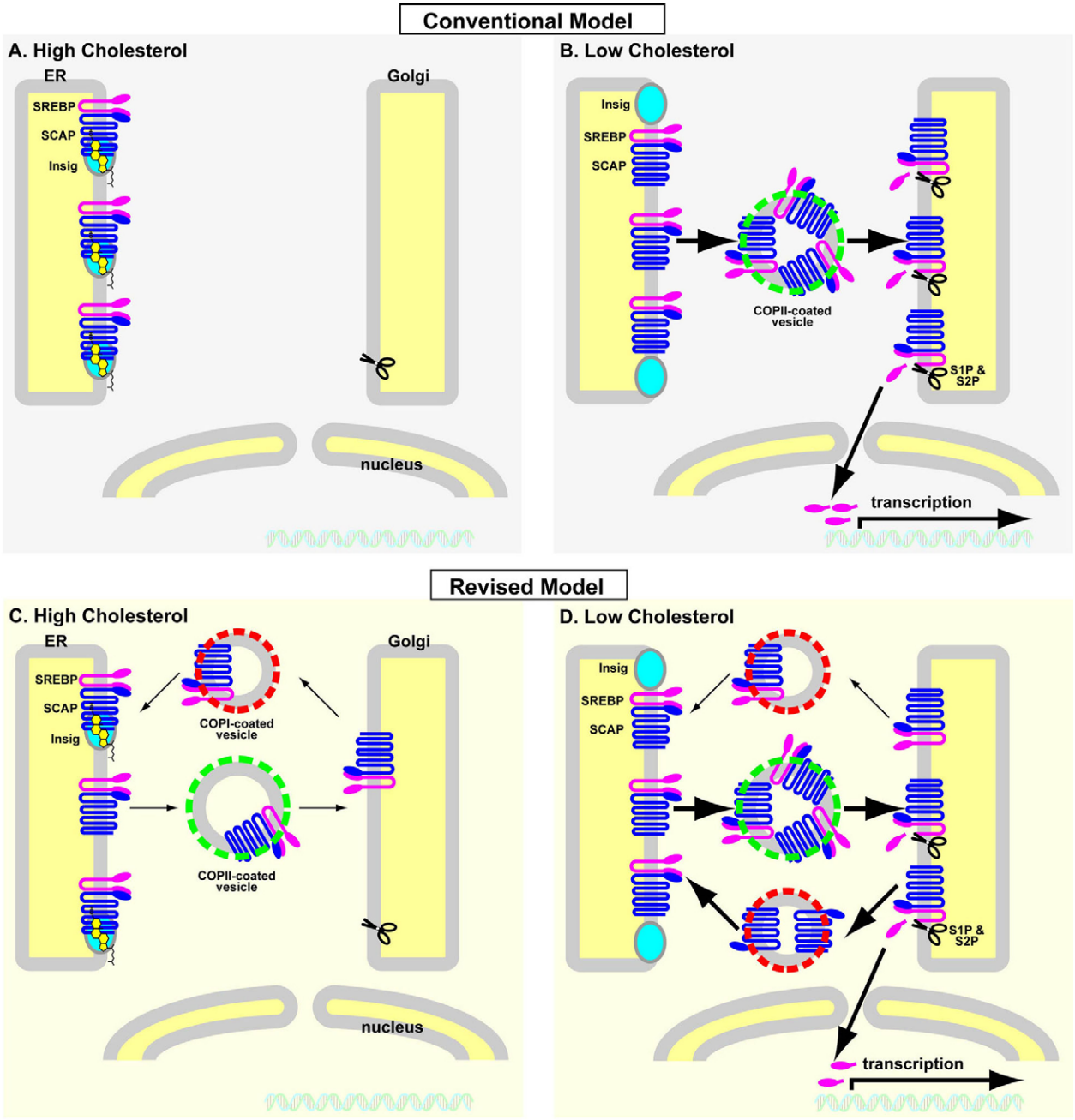

Fig. 6. Models for SCAP–SREBP trafficking and SREBP activation. In the conventional model (A,B), (A) the SCAP–SREBP complex is retained in the ER by interacting with Insig proteins under high-sterol conditions, or (B) the complex is exported from the ER through COPII-coated vesicles, and SREBP is processed by S1P and S2P at the Golgi and translocated into the nucleus under low sterol-conditions. In the revised model based on the present study (C,D), (C) a basal fraction of the SCAP–SREBP complex leaks out of the ER even under high-sterol conditions but is retrieved from the Golgi to the ER through COPI-coated vesicles before SREBP can be processed by S1P and S2P. (D) Under low-sterol conditions, the proportion of the SCAP–SREBP complex exiting the ER is increased, and a substantial fraction of the SREBP molecules that arrive at the Golgi are processed by S1P and S2P, and are translocated into the nucleus, whereas SCAP molecules are recycled to the ER through COPI-coated vesicles for reuse. (A,D) Cholesterol is represented by a schematic steroid backbone.

be ascribed, at least in part, to the increased lipid deposition that occurs under COPI-compromised conditions.

The current conventional model for regulated activation of SREBPs, followed by the stimulation of biosynthesis of fatty acids and cholesterol, was proposed by Brown, Goldstein and colleagues based on a series of elegant studies (Goldstein et al., 2006; Horton et al., 2002) (see Fig. 6A,B). In this model, when cholesterol builds up in ER membranes, the sterol causes SCAP to adopt a conformation that forces its binding to Insig proteins and prevents its binding to the COPII coat proteins. Consequently, the SCAP–SREBP complex fails to exit the ER (Fig. 6A). When cells are depleted of sterol, SCAP dissociates readily from Insig proteins and can bind to the COPII coat proteins, and the SCAP–SREBP complex is then transported towards the Golgi complex (Fig. 6B).

One important point to be stressed about this conventional model is that the SCAP–SREBP complex is not assumed to escape the sterol-regulated ER retention machinery. However, it is known that a number of ER proteins have ER ‘retention signals’ that actually

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serve as 'retrieval signals' from the Golgi in the regulated ER–Golgi recycling system (Harter and Wieland, 1996). For example, most soluble 'ER-resident proteins' have a C-terminal KDEL sequence that serves as a retrieval signal by binding to the KDEL receptor at the Golgi. Some ER transmembrane proteins have a C-terminal KKxx/KxKxx sequence that serves as a binding site for the COPI coat proteins, and another population of ER transmembrane proteins are recycled back to the ER by binding to the Rer1 protein at the Golgi. An early study of Brown, Goldstein and colleagues indirectly supports the view that a small fraction of the SCAP–SREBP complex can exit the ER even under sterol-rich conditions; they report that, even under sterol-rich conditions, a proportion of the *N*-linked oligosaccharide chains attached to SCAP are partially resistant to digestion by endoglycosidase H, although a high proportion of them are sensitive to the glycosidase (Nohturfft et al., 1998).

We first noticed that a fraction of SCAP–SREBP undergoes basal cycling between the ER and the Golgi in cells that exogenously co-expressed SREBP1c(R503A) and SCAP (Fig. 2C,D). In the absence of exogenous expression of Insig proteins, SCAP–SREBP constitutively exits the ER and traffics towards the Golgi, irrespective of the cellular sterol level (Goldstein et al., 2006; Hua et al., 1996a; Yang et al., 2002), as observed in cells that co-expressed SREBP1c(WT) and SCAP (Fig. 2A,B). By contrast, both SREBP1c(R503A) and SCAP were predominantly found in the ER under both sterol-rich and sterol-depleted conditions (Fig. 2C,D). Furthermore, essentially the same results were obtained when SREBP1c(R503A), SCAP and Insig2 were co-expressed (Fig. 3Ca–Ca‴,Cc–Cc‴,Da,Dc).

In striking contrast to the control cells, when cells that co-expressed SREBP1c(R503A), SCAP and Insig2 were subjected to knockdown of β-COP, both the SREBP1c mutant and SCAP were predominantly found in the Golgi complex under both sterol-rich (Fig. 3Cb–Cb‴,Db) and sterol-depleted (Fig. 3Cd–Cd‴,Dd) conditions. In addition, when cells that co-expressed SREBP1c (WT), SCAP and Insig2 were treated with control siRNAs or siRNAs against β-COP, and then cultured in the presence of sterol, both SREBP1c(WT) and SCAP were primarily found in the ER in the control cells (Fig. 3Aa–Aa‴,Ba), whereas SREBP1c(WT) and SCAP were found mainly in the nucleus and in the Golgi, respectively, in β-COP-knockdown cells (Fig. 3Ab–Ab‴,Bb). Taken together, these observations strongly suggest that, even under sterol-rich conditions, a fraction of the SCAP–SREBP complex escapes the sterol-regulated ER-retention machinery, which is then retrieved from the Golgi to the ER through COPI-coated vesicles, without the majority of SREBP molecules undergoing processing by the Golgi-resident proteases S1P and S2P (Fig. 6C); however, it is currently unknown why the SCAP–SREBP complex can return to the ER before SREBP processing by the endoproteases. One possible mechanism that prevents proteolytic processing of SREBPs despite their continual shuttling is that a large fraction of the SCAP–SREBP complex is retrieved before the complex reaches a compartment in which the S1P endoprotease is resident, as COPI-coated vesicles mediate retrieval of proteins not only from the cis-Golgi but also from the ER–Golgi intermediate compartment (Harter and Wieland, 1996; Lowe and Kreis, 1998; Watson and Stephens, 2005).

The model shown in Fig. 6C is compatible with the fact that, even under sterol-rich conditions, *N*-linked oligosaccharide chains of SCAP molecules are partially resistant to digestion with endoglycosidase H (Nohturfft et al., 1998). By contrast, under low-sterol conditions, SCAP that has been liberated from SREBP at the Golgi probably returns to the ER for reuse (Nohturfft et al., 1999), and the basal cycling of the SCAP–SREBP complex is likely to continue (Fig. 6D).

What determines the COPI-mediated retrograde trafficking of the SCAP–SREBP complex? Neither SREBP nor SCAP has a canonical KKxx/KxKxx sequence, and our preliminary analysis failed to show that knockdown of Rer1 affects the localization of SREBP and SCAP. This question should be addressed in future studies.

While this current study was in progress, Shao and Espenshade have suggested that uncleaved SREBP inhibits SCAP recycling from the Golgi to the ER, and that instead, SCAP escorts SREBP to lysosomes for degradation, although the authors do not present any data on the subcellular localization of SREBP or SCAP (Shao and Espenshade, 2014). By contrast, we show here that SREBP1c (R503A) – a mutant defective in S1P-catalyzed cleavage – and SCAP are primarily found in the ER under control conditions, whereas both accumulate in the Golgi when COPI-mediated retrograde trafficking is inhibited (Fig. 3). Furthermore, the SCAP level in the presence of co-expressed SREBP1c(R503A) was comparable to that in the presence of SREBP1c(WT) even when *de novo* protein synthesis was blocked (supplementary material Fig. S2; compare lane 1 vs lane 3 and lane 2 vs lane 4). Thus, our results unequivocally show that a large fraction of uncleaved SREBP molecules are recycled back to the ER, along with SCAP, through COPI-coated vesicles, although they do not exclude the possibility that a small fraction of SREBP–SCAP undergoes lysosomal degradation. The apparent discrepancy between our data and those of Shao and Espenshade might result from a difference in the experimental systems used. We reached our conclusions by using SREBP1c(R503A), which is not cleaved by S1P, whereas Shao and Espenshade have drawn their conclusions by using an S1P inhibitor (PF-429242) (Shao and Espenshade, 2014). Because S1P acts on multiple substrates in addition to SREBPs, there could be other factor(s) that need to be processed by S1P activity to allow SCAP to recycle properly. Although it is beyond the scope of the current study, this possibility should be addressed in a future study.

In this study, we showed that activation of SREBPs is mainly responsible for increased levels of lipid deposition under COPI-depleted conditions. However, in view of the data that lipid deposition, as revealed by using BODIPY 493/503 staining, was much stronger in β-COP-knockdown cells under sterol-rich conditions compared with that of control cells under sterol-deficient conditions (Fig. 1C) even though the nuclear SREBP levels were comparable in these two situations (Fig. 1E), it is possible that other mechanism(s) also underlie the increased lipid deposition in COPI-depleted cells. For example, it has been reported previously that COPI is required for the delivery of a lipolytic enzyme, ATGL, to lipid droplets (Soni et al., 2009). By contrast, other studies have shown that COPI can act directly on lipid droplets to promote the budding of nano-droplets and, consequently, prime the targeting of proteins or membranes to lipid droplet surfaces (Thiam et al., 2013; Wilfling et al., 2014b). Nevertheless, our data presented here unequivocally show that aberrant activation of SREBP contributes, at least in part, to lipid deposition under COPI-compromised conditions.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies were obtained from the indicated vendors – polyclonal rabbit anti-β-COP, Thermo Scientific; polyclonal rabbit anti-SCAP, Abgent; polyclonal rabbit anti-giantin, COVANCE; monoclonal mouse antibodies against SREBP1, β-tubulin and β-actin, Millipore;

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monoclonal mouse anti-SREBP2, MBL; monoclonal mouse antibodies against calnexin and GM130, BD Biosciences; monoclonal mouse anti-TATA-binding protein (TBP), Santa Cruz Biotechnologies; polyclonal rabbit anti-RFP, MBL; monoclonal rat anti-HA, Roche Applied Science; Alexa-Fluor-conjugated secondary antibodies, Molecular Probes; and horseradish-peroxidase-conjugated secondary antibodies, Jackson ImmunoResearch Laboratories. BODIPY 493/503 was purchased from Molecular Probes. BFA, 25-HC, CHX and oleic acid were from Sigma-Aldrich. ALLN was from Nacalai Tesque. HPCD was from Wako Pure Chemical Industries.

Recombinant plasmids and viruses

Expression vectors for C-terminally HA-tagged S1P and N-terminally HA-tagged S2P were constructed by subcloning a cDNA fragment containing the entire coding sequence of human S1P or S2P into pcDNA3-HAC (Hosaka et al., 1996) or pcDNA4-HAN (Shinotsuka et al., 2002), respectively. The expression vector for C-terminally EGFP-tagged SCAP was constructed by subcloning a cDNA fragment containing the entire coding sequence of human SCAP into pEGFP-N1. The expression vector for N-terminally mRFP-tagged SREBP1c was constructed by subcloning a cDNA fragment containing the entire coding sequence of human SREBP1c into the pcDNA3-Kozac-mRFP-C vector (Makyio et al., 2012). The R503A mutation of SREBP1c was introduced into the SREBP1c cDNA using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). The expression vector for C-terminally FLAG-tagged Insig2 was constructed by subcloning a cDNA fragment covering the entire coding sequence of human Insig2 into pCAG-sk-FLAG-N3. Construction of pCMV5-SCAP-HA and Firefly luciferase reporter vectors, pGL4-ACC1 and pGVB2-SQS, have been described previously (Irisawa et al., 2009). pGL4.74(hRluc/TK), which encodes *Renilla* luciferase, was purchased from Promega.

For production of mRFP–SREBP1c-encoding retrovirus, the mRFP–SREBP DNA fragment was amplified by using PCR of pcDNA3-mRFP-SREBP1c (described above), which was then inserted into the pMXs-puro vector (a kind gift from Toshio Kitamura, The University of Tokyo, Tokyo, Japan) (Kitamura et al., 2003) using an In-Fusion HD Cloning kit (TaKaRa). For production of the retroviruses for SCAP–HA, SCAP–EGFP or Insig2–FLAG, the SCAP–HA, SCAP–EGFP or Insig2–FLAG DNA fragment was amplified from pCMV5-SCAP-HA, pEGFP-N1-SCAP or pCAG-Insig2-FLAG, and inserted into pMXs-bla or pMXs-neo. The pMXs vector for mRFP–SREBP1c, SCAP–HA, SCAP–EGFP or Insig2–FLAG was co-transfected with pEF-gag-pol and pCMV-VSVG-Rsv-Rev (a kind gift from Hiroyuki Miyoshi, RIKEN BRC, Tsukuba, Japan) into HEK293T cells to produce recombinant virus.

Plasmid transfection, virus infection and RNA interference

β-COP was knocked down using a pool of siRNAs, as described previously (Saitoh et al., 2009; Takashima et al., 2011). For knockdown of SCAP, a pool of siRNAs directed against nucleotides 1215–1725 (when the A nucleotide of the initiation methionine codon is assigned as residue 1) of human *SCAP* mRNA was prepared using the BLOCK-iT RNAi TOPO transcription kit (Invitrogen) and a PowerCut Dicer kit (Thermo Scientific). Cells were transfected for 24 h with the siRNA pool using Lipofectamine 2000 (Invitrogen). The cells were then subcultured, incubated for a further 24 h and re-transfected with the siRNA pool for 24 h. The cells were then subcultured, incubated for 48 h and subjected to subsequent analyses. Plasmid transfection was performed using the X-tremeGENE 9 DNA Transfection Reagents (Roche Applied Science). Cells were transfected with expression plasmids 8 h before, or infected with recombinant retroviruses 24 h before, transfection with siRNAs against β-COP.

Cell culture

HeLa cells were maintained in MEM (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS (Invitrogen). Before experiments, cells were cultured in MEM containing 10% LPDS (Sigma-Aldrich) and 400 µM oleic acid in the presence or absence of 1 µg/ml 25-HC for 12 or 16 h. BFA (a final concentration of 10 µM) and a proteasome inhibitor, ALLN (a final concentration of 100 µM) were directly added to the medium 1 and 6 h before beginning the assay, respectively.

Immunoblotting

For immunoblot analyses of SREBPs, cells were fractionated into nuclear and cytoplasmic fractions using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Scientific). In other experiments, cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 1% Triton X-100, 150 mM NaCl) containing a protease inhibitor cocktail (EDTA-free) (Nacalai Tesque). Proteins in the fractions or the lysates were separated by using SDS-PAGE and were then electroblotted onto an Immobilon-P transfer membrane (Millipore). The membrane was blocked in 5% BSA and incubated sequentially with primary and horseradish-peroxidase–conjugated secondary antibodies. Detection was performed using the Chemi-Lumi One L kit (Nacalai Tesque).

Immunofluorescence analysis and visualization of lipid droplets

Cells grown on coverslips were fixed with 3% paraformaldehyde for 15 min, treated with 50 mM NH_4Cl for 20 min and permeabilized with 0.1% Triton X-100 for 5 min. The processed cells were blocked with 10% FBS for 30 min, incubated sequentially with primary and secondary antibodies for 1 h each, and then subjected to immunofluorescence analysis using an Axiovert 200 MAT microscope (Carl Zeiss) or an A1-RMP confocal laser-scanning microscope (Nikon). To visualize lipid droplets, BODIPY 493/503 (10 µg/ml) was included in the secondary antibody solution.

TAG quantification

To determine cellular TAG contents, TAGs were extracted from cells as described previously by Tsuboi et al. with some modifications (Tsuboi et al., 2004). Cells that had been pre-incubated with oleic acid as described above were trypsinized and collected. The collected cells were counted, and the cells were then suspended in 500 µl of isopropanol and sonicated to extract intracellular TAGs. Following centrifugation, a portion (300 µl) of the supernatant was subjected to quantification of TAGs using a Triglyceride E-test kit (Wako Pure Chemical Industries).

Luciferase assay

Cells were transfected with a reporter plasmid and pGL4.74[hRluc/TK], followed by transfection with siRNAs, and then subjected to assays for firefly and *Renilla* luciferase activities using the Dual-Luciferase Reporter Assay System (Promega), as described previously (Irisawa et al., 2009).

Semi-quantitative real-time PCR

Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen) and subjected to reverse transcription using a SuperScript VILO cDNA Synthesis Kit (Invitrogen). The resultant cDNA was used as a template for PCR using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.T., A.S., S.H. and T.F. designed and performed experiments; C.Y. and S.N. assisted with experiments; and R.S., H.-W.S. and K.N. designed experiments and prepared the manuscript.

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Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.164137/-/DC1

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SUPPLEMENTARY MATERIALS

COPI-mediated retrieval of SCAP is critical for regulating lipogenesis under basal and sterol-deficient conditions

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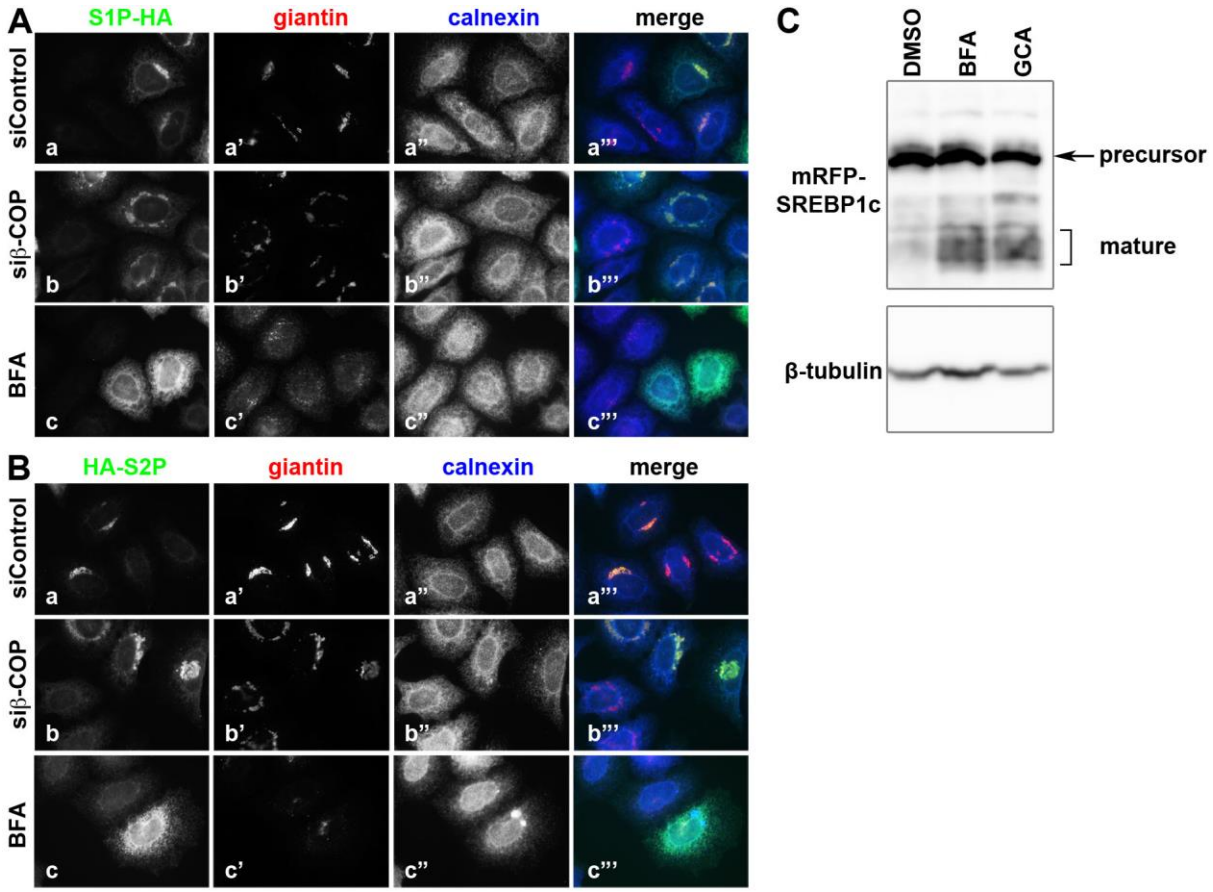


Fig. S1. ***Localization of S1P or S2P is not altered by a block in COPI-mediated trafficking***

(A and B) HeLa cells were transfected with an expression vector for C-terminally HA-tagged S1P (A) or N-terminally HA-tagged S2P (B), treated with control siRNAs or siRNAs for β-COP, and cultured in MEM supplemented with LPDS, OA, and 25-HC. In addition, cells not subjected to siRNA treatment were incubated in the presence of BFA. The cells were then subjected to triple staining with antibodies against HA, giantin (a Golgi marker), and calnexin (an ER marker). Bar = 10 µm. (C) Effects of brefeldin A and golgicide A on processing of SREBP1c. HeLa cells were triply infected with retroviruses for mRFP-SREBP1c, SCAP-HA and Insig2-FLAG, cultured in MEM supplemented with 10% LPDS, 1 µg/mL 25-HC, and 400 µM OA for 6 h, and mock-treated with DMSO or treated with 10 µM brefeldin A (BFA) or 30 µM golgicide A (GCA) for 6 h. Lysates prepared from the cells were processed for immunoblot analysis with antibody against RFP (upper) or β-tubulin (lower). The band for mature mRFP-SREBP1c was rather heterogeneous as reported for that of mature SREBP2 detected in cells treated with BFA (DeBose-Boyd et al., 1999).

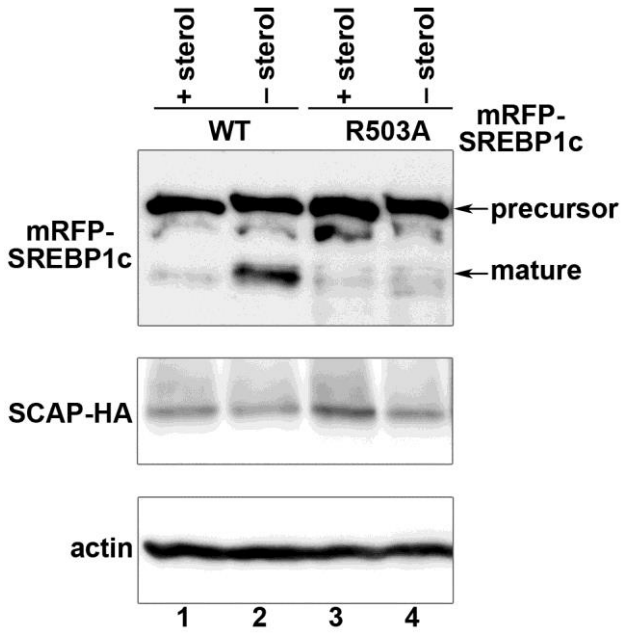


Fig. S2. ***Levels of the SREBP1c or SCAP protein are not different between cells expressing SREBP1c(WT) and SREBP1c(R503A)***

HeLa cells were triply infected with retroviruses for either mRFP-SREBP1c(WT) (lanes 1 and 2) or -SREBP1c(R503A) (lanes 3 and 4), SCAP-HA and Insig2-FLAG; cultured in MEM supplemented with 10% LPDS and 400 μM OA, with (lanes 1 and 3) or without (lanes 2 and 4) 1 μg/mL 25-HC for 13 h. After addition of CHX (a final concentration, 50 μg/mL), the cells were further incubated for 3 h and the cell lysates were processed for immunoblot analysis with antibody against RFP (top), HA (middle) or β-actin (bottom).

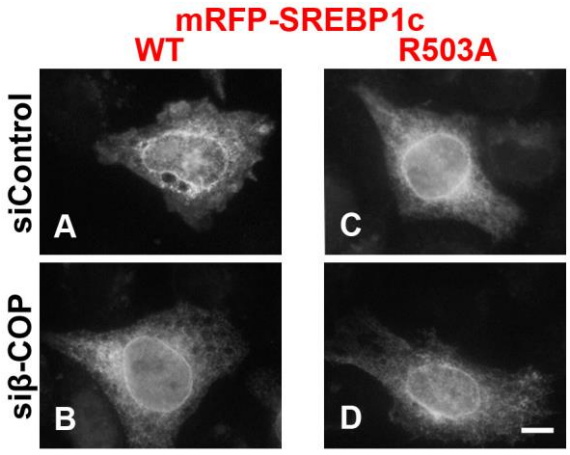


Fig. S3. ***Exogenously expressed SREBP cannot exit the ER***

HeLa cells were transfected with an expression vector for mRFP-SREBP1c(WT) (A and B) or -SREBP1c(R503A) (C and D) and control or β-COP siRNAs, cultured in MEM supplemented with 10% LPDS and 400 μM OA in the absence of 25-HC, and fixed for observation of mRFP fluorescence. Bar = 10 μm.

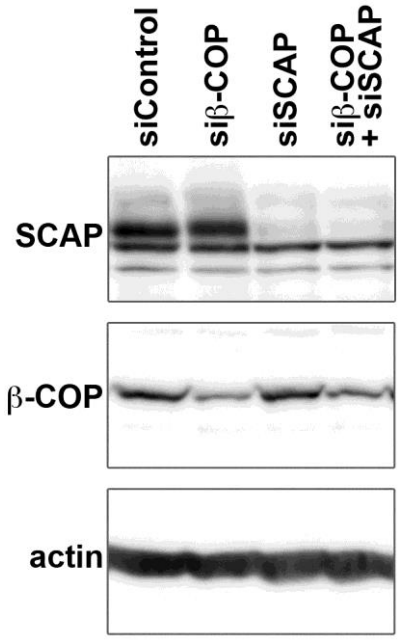
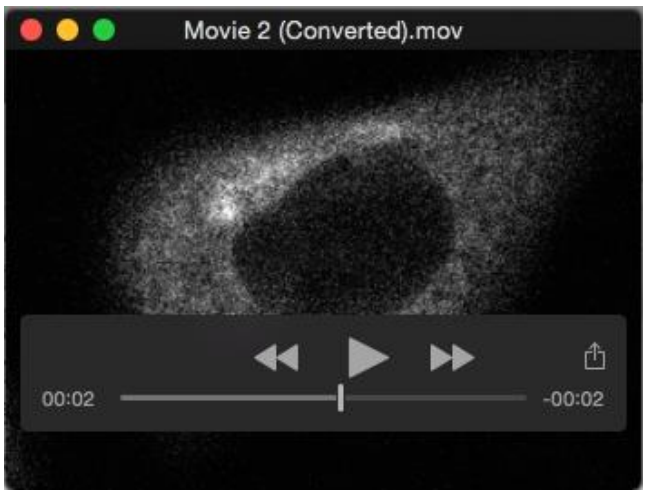


Fig. S4. ***Examination of knockdown efficiencies of β-COP and SCAP***

HeLa cells were treated with control siRNAs, siRNAs targeting β-COP or SCAP, or a combination of β-COP and SCAP siRNAs. Lysates prepared from the cells were subjected to immunoblot analysis for SCAP (top panel), β-COP (middle panel) or β-actin (bottom panel). In the SCAP blot, the lower two bands are likely to be non-specific, because the SCAP siRNA treatment did not affect the intensity of these bands.

Movie 1. Retention of SCAP-EGFP at the Golgi in the continual presence of sterol

Cells were triply infected with retroviruses for mRFP-SREBP1c, SCAP-EGFP and Insig2-FLAG, and cultured in MEM supplemented with 10% FBS. The cells were then incubated in MEM containing 10% LPDS, 1% HPCD and 50 µg/mL CHX for 90 min to deplete cellular sterols and inhibit *de novo* protein synthesis. After washing twice with MEM containing LPDS and CHX, the cells were incubated in the washing medium and subjected to time-lapse recording for 30 min using an A1-RMP confocal microscope. Images were acquired every 5 min.



Movie 2. Retrieval of SCAP-EGFP from the Golgi to the ER upon sterol replenishment

After washing as described in the legend for Video S1, the medium was changed to MEM containing FBS, 25-HC and CHX and the cells were subjected to time-lapse recording for 30 min. Images were acquired every 5 min.