Cite as: R. Yan *et al.*, *Science*
10.1126/science.abb2224 (2021).

A structure of human Scap bound to Insig-2 suggests how their interaction is regulated by sterols

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The SREBP pathway controls cellular homeostasis of sterols. The key players in this pathway, Scap and Insig-1/2, are membrane-embedded sterol sensors. 25-hydroxycholesterol (25HC)-dependent association of Scap and Insigs acts as the master switch for the SREBP pathway. Here, we present cryo-EM analysis of the human Scap and Insig-2 complex in the presence of 25HC, with the transmembrane (TM) domains determined at an average resolution of 3.7 Å. The sterol sensing domain (SSD) in Scap and all six TMs in Insig-2 were resolved. A 25HC molecule is sandwiched between the S4-S6 segments in Scap and TMs 3/4 in Insig-2 in the luminal leaflet of the membrane. Unwinding of the middle of the Scap-S4 segment is crucial for 25HC binding and Insig association.

The sterol regulatory element-binding protein (SREBP) pathway controls cellular homeostasis of sterols through a negative feedback mechanism (1, 2) (Fig. 1A). SREBPs (SREBP-1a, 1c, 2) are transcription factors in which a transmembrane hairpin connects the amino (N) terminal transcription factor domain and the carboxy (C) terminal regulatory domain (3–5). The C-terminal domain of SREBP is constitutively bound to the C-terminal WD40 domain of the SREBP-cleavage activating protein (Scap) (6, 7). Preceding the WD40 domain, Scap comprises eight transmembrane (TM) segments, with segments S2-S6 constituting the sterol-sensing domain (SSD) (8–10) (Fig. 1A).

The membrane-bound transcription factor SREBP-2 regulates cholesterol metabolism. When the cell is enriched with cholesterol, the SREBP-2/Scap complex is sequestered to the endoplasmic reticulum (ER) membrane through interaction between the Scap-SSD and Insig-1 or 2 (insulin-induced gene) (11–13). The association between Scap and Insig requires a cholesterol derivative like 25-hydroxycholesterol (25HC), or less favorably, cholesterol (14). Upon cholesterol depletion, the SREBP-2/Scap complex dissociates from Insig-1/2 and is transported by COPII-coated vesicles from the ER to the Golgi, where SREBP-2 undergoes two sequential cleavages.

The linker between the two TMs is first cut in the lumen by the site-1 protease (S1P), then TM1 is cleaved by the site-2 protease (S2P), an integral membrane metalloprotease, within the lipid bilayer (15–19). The transcription factor domain of SREBP-2 is released and translocated into the nucleus to activate the expression of genes encoding proteins for cholesterol uptake, exemplified by the low-density lipoprotein (LDL) receptor and enzymes for cholesterol synthesis, such as the rate-limiting enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) (1, 20–22).

HMGCR also contains a SSD that interacts with Insig-1/2 in a sterol-dependent manner (fig. S1A). Insig-1 bridges the interaction between an E3 ubiquitin ligase, GP78, and HMGCR for degradation of the latter (2, 23–25). Therefore, the interaction between Insig and the SSD in Scap and HMGCR is essential for the feedback inhibition to maintain cellular cholesterol homeostasis.

Although the pathway was discovered more than two decades ago, structural elucidation of the players has lagged behind the cellular and biochemical characterizations. Structural understanding of this pathway has mainly come from homologs in lower organisms, such as the crystal structures of an archaeal S2P MjS2P (26), a mycobacterial Insig

MvINS (27), and the C-terminal domains of the SREBP and Scap homologs in *S. pombe*, Sre1 and Scp1 (28, 29). The cryo-EM structures of several SSD-containing proteins (Niemann-Pick type C (NPC1), Patched 1 (Ptch1), NPC1L1, and Dispatched) have also been resolved (fig. S1A) (30–38). A putative sterol binding pocket is observed on the membrane-facing surface of the SSD in Ptch1 and NPC1L1, enclosed by S2-S4 (the S2-S4 site) (32–34, 38).

Despite these advances, the 25HC binding site and the interface between the SSD and Insig remain enigmatic. In addition, Insig was shown to bind to 25HC, but not cholesterol, whereas Scap can only bind to cholesterol through its luminal domain known as Loop 1 (39–41). Here we combine biochemistry and cryo-EM technology to obtain insight into the structural basis of the 25HC-dependent transmembrane interaction between human Scap and Insig-2.

Results

Structure of the 25HC-bound Scap/Insig-2 complex

The 25HC-dependent interaction between recombinant Scap and Insig-2 protein has been demonstrated using the insect cell-based pull-down assay, in which mouse Scap was used because of its higher expression level than the human ortholog (27). For the present study, the Scap and Insig variants used for binding assays were generated in the context of mouse Scap (residues 1-741) and full-length (FL) human Insig-2 (fig. S1B).

To obtain proteins for structural analysis, we tested a large number of constructs from different homologs with distinct boundaries, point mutations, fusion proteins, and affinity tags. Briefly, the combination of WD40 domain-deleted human Scap (residues 1-752 with N-terminal FLAG tag) and FL human Insig-2 (N-terminal His₁₀ tag, Bril fusion, and three point mutations, C14S, C90S, and C215S) yielded sufficient complex for structural studies when transiently co-expressed in HEK293F cells supplemented with 25HC at a final concentration of 1 µg/mL in the medium. For cryo-EM analysis, proteins were extracted from the membrane using 1% (w/v) lauryl maltose neopentyl glycol (LMNG) and 0.1 mg/mL 25HC. After tandem affinity purification and size exclusion chromatography (SEC), peak fractions were pooled and further concentrated for cryo-EM sample preparation (fig. S1C). If 25HC was omitted during extraction, the complex fell apart.

Cryo-EM data processing of the complex was challenging because of the small size of the TM region – 14 TMs without symmetry (8 from Scap and 6 from Insig-2) – and the flexibility of the soluble domain. Combining Relion, CryoSparc, and the “guided multi-reference 3D classification” method developed by C. Yan (42), a 3D reconstruction was obtained at an overall resolution of 4.2 Å (Fig. 1B). Refinement of a soft-masked TM region improved the resolution to 3.7 Å, while the

soluble domain was only poorly resolved, likely owing to its flexibility (Fig. 1C, figs. S2 and S3, and table S1).

The structural model was only built for the transmembrane domains based on the 3.7 Å-resolution map. In total, 13 TMs were resolved. Using the structures of MvINS and NPC1-SSD (PDB codes: 4XU6 and 6W5S, respectively) as references, the 6 TMs in Insig-2 and the first 6 TMs in Scap (Fig. 1D and fig. S3A) were unambiguously assigned. The one additional resolved TM is adjacent to TM1 in Scap. The resolution only supported modelling of poly-Ala, insufficient to distinguish whether this is TM7 or TM8. To facilitate structural illustration, we name the TMs in Insig-2 TM1-6, and those in Scap S1-S7.

Apart from the 13 TM segments, an elongated density reminiscent of a sterol was resolved on the interface between SSD and Insig-2. Because 25HC is indispensable for complex formation, we assigned a 25HC molecule into this density (Fig. 1C, right). Below, we describe individual structures of Scap and Insig-2 before analysis of the molecular basis for 25HC-dependent protein interaction.

A discontinuous S4 in Scap-SSD

In total, 180 side chains were assigned to the TMD of Scap. S1 is preceded by a membrane-attaching transverse helix, named TH1 (residues 5-25), and the last resolved residues are ⁴⁴⁷MELA₄₅₀ in the “MELADL” motif, marking the cytosolic end of S6 (19) (Fig. 2A and fig. S4A).

The resolved TM fold of Scap is similar to that of NPC1 and Ptch1, with the root-mean-square deviation (RMSD) values of 2.4 Å and 1.9 Å over 129 and 127 aligned Cα atoms, respectively (Fig. 2, B and C). However, Scap has a distinct feature – the S4 segment is broken in the middle, resulting in two half helices, designated S4a and S4b (Fig. 1C, Fig. 2A, and fig. S3A). In both NPC1 and Ptch1, the corresponding segment is an intact α helix (Fig. 2, B and C). Because of this kink, S4a leans toward the interior of the SSD, creating space for ligand accommodation (Fig. 1C and Fig. 2, A and B). This binding site, constituted by the luminal halves of S4-S6, is on the opposite side to the S2-S4 site in Ptch1 and NPC1L1. In Scap, the corresponding S2-S4 site is shielded by the S7 (or S8) segment, which is missing in the other SSD-containing proteins of known structures (Fig. 2C).

Among the well-characterized loss or gain of function (LOF or GOF) Scap mutations, four loci are resolved in our 3D EM reconstruction (Fig. 2D). Asp428, whose Ala substitution (D428A) confers binding to Insig in the absence of sterol (43), and Tyr298, whose replacement by Cys (Y298C) led to loss of Insig binding (13, 44), are positioned in the middle of S6 and S2, respectively; their side chains pointing to the unwound region of S4 from opposite sides. The other two LOF (L315F and D443N) loci (6, 13) are close to each other on the cytosolic termini of S3 and S6, respectively (Fig. 2D).

A hydrophobic pocket in Insig-2

The resolved Insig-2 contains residues 18-214, with the N-terminal Brill unresolved (Fig. 1B). TMs 1/2 and TMs 5/6 are related to each other by an approximately 180° rotation around an axis that is perpendicular to the membrane. TM3 and TM4, the counterparts of which mediate trimerization of MvINS, form an anti-parallel hairpin on one side of TM6. There are some shifts of the TM boundaries from predicted models, and TM3 and TM4 are one or two helical turns shorter than a regular transmembrane α helix (Fig. 3A and fig. S4B).

The overall structure of Insig-2 can be superimposed onto MvINS with RMSD of 3.3 Å over 141 C α atoms (Fig. 3B). The intervening sequences between TM1 and TM2 and between TM5 and TM6, designated L1-2 and L5-6, respectively, in Insig-2 are much longer than the MvINS counterparts, which form anti-parallel β -strands that cap the central cavity (Fig. 3B). We modeled the two linkers in the structure to indicate their length, but the sequences of residues 55-69 and residues 176-189 were barely visible in the EM map (fig. S3B).

In the crystal structure of MvINS, an endogenous diacylglycerol (DAG) molecule inserts one tail into the central pocket enclosed by TMs 1/2/3/5 and projects the other into the membrane through the cleft between TM2 and TM5 (27). Based on the DAG-bound MvINS structure, the central pocket in a homologous model of Insig-2 was predicted to be the accommodation site for sterols (27). Consistent with this model, replacement of a pocket-facing residue Phe115 by Ala led to resistance to 25HC-dependent inhibition of SREBP cleavage and abolished Scap binding in the presence of 25HC (27, 40). Although the structure of Insig-2 has a similar central pocket and a lateral cleft, no density that may correspond to a sterol is observed within the pocket even when the map is pass-filtered to low resolutions (fig. S3B). Instead, 25HC binds at the interface between Scap and Insig-2. Phe115, which interacts with a number of hydrophobic residues in the central pocket, is positioned on the back of the 25HC binding site on TM3 of Insig-2 (Fig. 3C). Replacement of the bulky Phe by Ala may disrupt the local interactions and lead to dislocation of TM3, hence altering the 25HC binding site.

To corroborate that the central pocket of Insig is not the binding site for 25HC, we conducted a series of structure-guided mutational characterizations. Insig was reported to bind to 25HC, but not cholesterol (40). Since the only difference between the two sterol molecules is the presence of a polar hydroxyl group at the end of the octyl tail of 25HC, it is reasonable to expect that one or more polar groups in Insig would specifically recognize the 25-OH group, should the central pocket be the accommodation site. However, the central pocket is highly hydrophobic, with only one polar residue, Ser81, nearby, but none inside (Fig. 3C). If Ser81 were the coordinating residue for the 25-OH of 25HC, then an Ala

substitution of this locus might impair 25HC response. To test this, we introduced a single point mutation S81A. The variant behaved just like WT protein in both 25HC-dependent Scap binding (Fig. 3D, lanes 1 to 4, and fig. S5A) and in the suppression of SREBP cleavage (Fig. 3E, lanes 1 to 6, and fig. S5B), thus excluding Ser81 as the 25-OH coordinating residue. An entirely hydrophobic pocket should not prefer 25HC to cholesterol.

If the pocket is not responsible for 25HC binding, blockage of the central pocket by bulky residues should have no effect on the 25HC-dependent complex formation. Based on the structure, two Insig-2 variants were generated, each containing a pair of point mutations that replaced small or linear side chains at similar depth within the pocket with Phe, L42F/C77F and L46F/V73F (Fig. 3C). There is no significant difference between these mutants and WT Insig-2 with regard to 25HC-dependent Scap interaction (Fig. 3D, lanes 1, 2, and 5 to 8, and fig. S5A) or SREBP cleavage suppression (Fig. 3E, lanes 1 to 3 and 7 to 12, and fig. S5B).

Taken together, our structural analysis and biochemical evidence suggests that the central pocket of Insig is not the accommodation site for the 25HC molecule that is required for the interaction between Scap and Insig.

25HC sandwiched by Scap-SSD and Insig-2

The interaction between Scap and Insig-2 appears highly asymmetric with limited contacts. Viewed from the lumen, S2, S4, S6, and S5 of Scap form a concave surface to interact with TMs 3/4 of Insig-2 (Fig. 4A, left). Insig-2 mainly engages TM3 to pack against S2 and S4a of Scap. The luminal tip of Insig-TM1 also contacts Scap-S2 (Fig. 4A, middle, and fig. S6, A and B). On the other side within the membrane, the interaction is mainly mediated through the bound 25HC molecule (Fig. 4A, right).

The resolved density allowed for discrimination of the sterol backbone, which is also featured with the two bulges corresponding to the C18 and C19 groups, from the linear tail of 25HC, leaving the 3 β -OH group on the luminal side (Fig. 1C and fig. S3C). The limited contacts between Scap and Insig result in a cytosol-facing cavity that is connected to the bound ligand (Fig. 4B). We placed the end of the iso-octanol tail adjacent to a cluster of polar and charged residues from TM3/4 of Insig-2 and S2/S4/S5 of Scap, exposing 25-OH to the aqueous cytosolic milieu. The hydrophilic environment in the mid-height of the membrane might explain the preference for a polar group to the hydrophobic tail of cholesterol (Fig. 4, B and C).

25HC is nearly parallel to the tilted S4a of Scap. The tetracyclic ring inserts into the hydrophobic pocket enclosed by S4a, S5, and S6 of Scap and TMs 3/4 of Insig-2 (Fig. 1C and Fig. 4, D and E). To validate the positioning of 25HC observed in the structure, we sought to introduce mutations to Scap

and Insig residues that are implicated in 25HC binding but not involved in direct contact of the proteins. We propose these may disrupt the 25HC-dependent interaction between WT proteins, while allowing interaction between Insig-2 and the GOF variant Scap(D428A) in the absence of 25HC (43).

Among the surrounding residues, three bulky ones, Tyr351 on S4a and Ile414 and Phe417 on S6 in Scap, and two small ones, Gly117 and Ala121 on TM4 of Insig-2, together define the contour for accommodating the tetracyclic ring and the protruding C18 and C19 groups of 25HC (Fig. 4E). However, substitution of these residues may also interfere with direct protein interaction. Based on our structure, His120 on the luminal end of TM3 of Insig-2 may contribute to the coordination of the 3 β -OH group of 25HC via water-mediated hydrogen bonds (Fig. 4E, left). Leu343 on the loop between S3 and S4a and Val355 on S4a of Scap appear to solely interact with 25HC. These three residues were selected for examination (Fig. 4E).

When treated with 0.1 μ g/mL 25HC, the WT Scap pulled down by Insig-2(H120A) was less than half than by WT Insig-2. When L343A and V355A were individually introduced to Scap, the amount of retained Scap mutants was each less than 25% of the WT protein (Fig. 4F, lanes 1 to 4, and figs. S7 and S8). Scap(D428A) maintains binding to Insig in the absence of 25HC, although the interaction is stronger in the presence of 25HC (Fig. 4F, lanes 9 and 13, and figs. S7 and S8). When the three mutations were each combined with Scap(D428A), complex formation in the absence of 25HC was only slightly affected (Fig. 4F, lanes 13 to 16, and figs. S7 and S8).

Consistently, these mutations alone also led to impaired 25HC-dependent suppression of SREBP cleavage. But when they were in combination with Scap(D428A), there was no detectable difference for suppression of SREBP activation in the absence of 25HC (fig. S9). Therefore, the pull-down and SREBP cleavage assays collectively consolidate the structure-elucidated binding site for 25HC.

Discontinuity of Scap-S4 is critical to Insig binding

Mid-point unwinding of S4, a distinct feature of Scap, may serve as a switch for Insig association. On the one hand, bending in the middle of Scap-S4 and the resulting tilt of S4a creates the cavity between Scap and Insig to accommodate 25HC (Fig. 2B and Fig. 4D). On the other hand, unwinding of Scap-S4 provides the space for the bulky side chains of Arg110 on TM3 of Insig-2 and Tyr298 on S2 of Scap (Fig. 5A and fig. S11A).

A previous report showed that Insig-2(R110A) did not affect the inhibition of 25HC-dependent SREBP cleavage (40). In our pull down assay, this mutation slightly weakened 25HC-mediated association with Scap (Fig. 5B, lanes 1, 2, 5, and 6, and figs. S8 and S10A). Examination of the EM map

suggested that Arg110, which was unambiguously resolved (Fig. 1C), may interact with Scap-Glu359 along with Lys102, a residue on the linker between TM3 and TM4 of Insig-2 (Fig. 5A). However, the side chains of Insig-Lys102 and Scap-Glu359 were not well resolved. To verify their potential interactions, we generated and examined additional mutants. Whereas Insig-2(K102A) behaved similarly to WT for 25HC-dependent Scap binding, E359A of Scap abolished 25HC-mediated interaction with Insig-2 (Fig. 5B, lanes 3, 4, 15, and 16; fig. S8; and fig. S10, A and B). The complex could no longer form in the presence of 25HC when both Lys102 and Arg110 were substituted with Ala (Fig. 5B, lanes 7 and 8, and fig. S10A), suggesting redundant interaction of these two basic residues with Scap-Glu359.

An intact Scap-S4, if similar with that in other SSD-containing proteins, would leave Insig-Arg110 in an unfavorable hydrophobic environment (fig. S11A), hence impeding interaction with Insig. Supporting a critical role of the S4 discontinuity in complex formation, when Gly357, the unwinding point, was mutated to the α helix-stabilizing residue Ala (45), the variant Scap(G357A) could not form a 25HC-mediated complex with Insig-2 (Fig. 5B, lanes 13 and 14, and figs. S8 and S10B).

Unwinding of a TM helix in the middle would be unfavored in the hydrophobic lipid bilayer if not stabilized. Both 25HC binding and the nearby residues appear to affect the stability of the discontinued helix. While tilting of S4a is required for generating the sterol binding site, insertion of the sterol into the cavity can in turn stabilize the kink (Fig. 4D). In addition, Tyr298 on S2 stabilizes the unwound segment through H-bonding to the exposed backbone amide of Leu358 (Fig. 5A). Scap(Y298C) is a well-characterized LOF mutant (44, 46). To investigate the importance of its hydroxy group in stabilizing the S4 kink, we generated a mutant Scap(Y298F), which, similar to Y298C, failed to interact with Insig-2 in the presence of 25HC (Fig. 5B, lanes 9 to 12, and figs. S8 and S10B).

On the other hand, S4 unwinding provides a potential explanation for the GOF of Scap(D428A). Asp428, with S4 unwound, is placed in an unfavorable environment constituted by hydrophobic residues (Fig. 5C). There is one nearby polar residue, Gln432, which is separated from Asp428 by one helical turn. In a 3.0-Å resolution structure of NPC1 (36), the side chains of the corresponding residues, Asp771 and Gln775, interact (Fig. 5D). In Scap, however, the side group of Gln432, which points away from Asp428 to avoid steric clash with Val361, projects into an unfavorable hydrophobic surrounding (Fig. 5D). If S4 were an intact helix, Gly357 would face these two residues, leaving enough space to allow for an Asp428-interacting conformation of Gln432 (Fig. 5D).

Because of the undesirable local environment for Asp428 and Gln432 with a broken S4, neutralization of them by Ala

may lower the energy barrier for S4 unwinding; whereas the penalty has to be compensated by 25HC binding for WT proteins. This analysis not only provides a molecular interpretation for the GOF of Scap(D428A), where the unwound conformation of S4 may be more favorable and compatible with Insig association even in the absence of 25HC, but also predicts that Q432A may lead to GOF. Indeed, the variant Scap(Q432A) could form a complex with Insig in the absence of 25HC, but to a lesser extent than Scap(D428A) (Fig. 5E and figs. S8 and S10C). Taken together, the published and our structure-derived LOF and GOF mutations support a function of S4 discontinuity in Insig binding.

Discussion

In this paper, we have limited our analyses to the TM regions of Scap and Insig-2 in the presence of 25HC. Studies in the past decade have revealed the regulatory role of Loop1 and Loop7 of Scap in response to cholesterol (10) (Fig. 5F). Loop1 and Loop7 interact with each other in the absence of cholesterol (47); upon cholesterol binding to Loop 1 (41), the SSD undergoes conformational changes that are compatible with Insig binding and cause separation of Loop1 and Loop7 (48). The conformation of Scap in the presence of 25HC and/or cholesterol prevents the MELADL motif from being recognized by COPII vesicles for trafficking to the Golgi (49). These sophisticated molecular events cannot be recapitulated from one structure, especially when the soluble domains were barely resolved (Fig. 1B). Nevertheless, structural comparison of Scap and the other SSD-containing proteins, such as NPC1 and Pth1, provides clues that may facilitate future investigation (fig. S11).

In addition to S4, the major conformational changes of SSD occur in S2 (fig. S11, A and B). The interaction between S2 and TM3 represents a major interface between Scap and Insig (Fig. 4A and fig. S6A). If the SSD of Scap adopts a similar conformation with that in NPC1 or Pth1, S2 can no longer reach Insig (Fig. 2, B and C, and fig. S11, A and B). In the conformation of NPC1 or Pth1, the equivalent residue of Tyr298 on S2 is distant from the straight S4 to avoid steric clash, thus unable to stabilize the unwinding of S4 (fig. S11A). Importantly, S2 is directly connected to Loop1. It remains to be tested whether cholesterol-dependent conformational changes of Loop1 may be coupled to the movement of S2 and S4.

Likewise, ⁴⁴⁷MELA₄₅₀ at the end of S6 are close to S2 and the putative S7 (or S8), two TM segments that are respectively contiguous with Loop 1 and Loop 7 (Fig. 2A). It is yet to be characterized whether conformational changes of Loop 1 and/or Loop 7 may be transmitted to the shifts of S2 and S7/S8 to induce structural shifts of the MELADL motif for regulated COPII recognition.

Although shown here is a heterocomplex between

monomeric Scap and Insig-2, Scap was reported to be a tetramer (39) and MvINS exists as a trimer (27). If S4 remains an integral helix in Scap alone, the Insig-facing surface has several conserved polar residues, the corresponding residues in NPC1 and Pth1 are protected by the SSD-like domain from the C-terminal half of the protein (figs. S1A and S11C) (30, 32). Homo-oligomerization of Scap-SSD may be required to shield the polar surface from the lipid bilayer. Whether Scap and Insig alone and as a complex can form a higher order oligomer remains to be determined.

The structure of the TM domains of human Scap and Insig-2 in the presence of 25HC suggests the importance of an interface between Scap's SSD and Insig, which would explain the requirement of 25HC for complex formation. Several GOF or LOF mutations can be reasonably interpreted in light of this model. Despite the advances in the mechanistic understanding of sterol sensing by Scap and Insig, there are a number of outstanding questions as discussed above. Our present structure provides clues to addressing these questions, but a comprehensive understanding awaits further investigation, including structure determination of the lone Scap and Insig proteins.

Materials and methods

Protein expression and purification

The cDNAs of full-length human Insig-2 (accession: NM_001321329), wild type (WT) or the 3CS mutant (C14S, C90S, and C215S), and WD40-deleted human Scap (accession: NM_012235, residues 1-752) were subcloned separately into the pCAG vector. Scap was tagged with an N-terminal FLAG, and the N terminus of Insig-2 was tandemly fused with 10×His and Bril. Point mutations were introduced using a standard two-step PCR.

HEK 293F cells (Invitrogen) were cultured in SMM 293T-I medium (Sino Biological Inc.) at 37°C under 5% CO₂ in a Multitron-Pro shaker (Infors, 130 rpm). When cell density reached 2.0×10⁶ cells per milliliter, the cells were transiently co-transfected with two plasmids and polyethylenimine (PEI) (Polysciences). The two plasmids, ~ 0.75 mg each, were premixed with 3 mg PEI in 50 mL fresh medium for 15 min before adding into 1 L cell culture for transfection. After 24 hours, 25-hydroxylcholesterol (25HC, Sigma) was added to the cell culture at a final concentration of 1 µg/mL. The transfected cells were cultured for another 48 hours before harvesting.

For purification of the Scap and Insig-2 complex, the cells were collected and resuspended in the buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 1% (w/v) LMNG (Sigma), 0.1 mg/mL 25HC, and a protease inhibitor cocktail containing aprotinin at 1.3 µg/mL, pepstatin at 0.7 µg/mL, and leupeptin at 5 µg/mL. After incubation at 4°C for 2 hours, the solution was centrifuged at 18,000×g at 4°C for 40 min. The

supernatant was loaded to the M2 resin, rinsed with the wash (W2) buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 0.005% LMNG, and 0.5 $\mu\text{g/mL}$ 25HC, and eluted by W2 buffer supplemented with 0.2 mg/mL FLAG peptide. The eluent was then loaded to the Ni-NTA resin and washed with the wash (W3) buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 0.005% LMNG, and 10 mM imidazole, followed by elution with W3 buffer plus 290 mM imidazole. The eluent was concentrated and applied to size exclusion chromatography (SEC, Superose 6 Increase 10/300 GL, GE Healthcare) in the buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, and 0.005% LMNG.

Cryo-EM sample preparation and data acquisition

Aliquots (3.5 μL) of the purified human Scap/Insig-2 complex, which was concentrated to approximately 10 mg/mL, were placed on glow-discharged holey carbon grids (Quantifoil Au R1.2/1.3, 300 mesh). After blotting for 3.0 s, the grids were plunge-frozen in liquid ethane cooled by liquid nitrogen in Vitrobot (Mark IV, Thermo Fisher Scientific). The cryo-grids were transferred to a Titan Krios operating at 300 kV equipped with Gatan K3 Summit detector and a GIF Quantum energy filter. Micrographs were recorded in the super-resolution mode with a normal magnification of 81,000 $\times$, resulting in a calibrated pixel size of 0.544 $\text{\AA}$. Each stack of 32 frames was exposed for 8 s, with an exposing time of 0.25 s per frame. The total dose rate was about 5.3 counts/sec/physical-pixel ($\sim 4.7 \text{ e}^-/\text{sec}/\text{\AA}^2$) for each stack. AutoEMation was used for the fully automated data collection (50). All 32 frames in each stack were aligned and summed using the whole-image motion correction program MotionCor2 (51) and binned to a pixel size of 1.09 $\text{\AA}$. The defocus value of each image was set to 1.5–2.0 μm and determined by Gctf (52).

Data processing

Out of 8,549 micrographs, 5,648,776 particles were automatically picked by Gautomatch (www2.mrc-lmb.cam.ac.uk/kzhang/Gautomatch/). Two rounds of 2D classification were performed using RELION 3.0 (53, 54) (fig. S2, A and B), and a small subset of the selected particles was used to generate the initial model by RELION 3.0. A good reference of low resolution and five bad references were generated using conventional 3D classification to perform the next-step “guided multi-reference classification” following a reported procedure (42). One round of guided multi-reference classification was performed, resulting in 606,502 particles (fig. S2B). After two additional rounds of multi-reference classification of the same 606,502 particles with a pixel size of 2.18 $\text{\AA}$, 216,819 particles were selected, and then re-centered and re-extracted with a pixel size of 1.09 $\text{\AA}$ (fig. S2B). These particles were further processed with CRYOSPARC (55). After two additional rounds of multi-reference classification,

153,168 resulting particles yielded a reconstruction at an average resolution of 4.2 $\text{\AA}$ after Non-Uniform refinement. The resolution of the TM region was improved to an average of 3.7 $\text{\AA}$ after focused refinement (fig. S2, B and C, and table S1). Reported resolutions were calculated on the basis of the FSC 0.143 criterion, and the FSC curves were corrected with high-resolution noise substitution methods (56). Local resolution variations were estimated using CRYOSPARC (55, 57). The angular distributions of the particles used for the final reconstruction of the Scap/Insig complex were reasonable (fig. S2D). The resulting map displayed clear features of secondary elements and side chains for the core region of the transmembrane (TM) domains of the Scap/Insig complex (Fig. 1C and fig. S3).

Model building and refinement

The atomic coordinates of the Scap/Insig complex were generated by combining homology modelling and de novo model building. The initial structure models for the Scap-SSD domain and Insig were generated by CHAINSAW (58) using homologous structures of NPC1 (PDB code: 6W5S, 28% identity with the SSD domain of Scap) and MvINS (PDB code: 4XU6, 26% identity with human Insig-2) as template. These structures were docked into the density map and manually adjusted and re-built in COOT (59). The model for the TM region was refined against the 3.7- $\text{\AA}$ map using PHENIX (60) in real space with secondary structure and geometry restraints. Overfitting of the model was monitored by refining the model in one of the two independent maps from the gold-standard refinement approach and testing the refined model against the other map (61) (fig. S2E). The structure of the Scap/Insig complex was validated through examination of the Molprobit scores (62) and statistics of the Ramachandran plots (table S1).

Pull-down of Scap by Insig-2

The pull-down assay was performed following our published protocol (27). Human Insig-2, WT or indicated variants, with an N-terminal His₁₀-tag and WD40-deleted mouse Scap (TMD, residues 1–741), WT or indicated variants, with an N-terminal FLAG tag were co-expressed using baculovirus-infected Sf-9 insect cells, which were cultured in the SF-900 II medium (Gibco) supplemented with 100 units/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin at 27°C. Where indicated, 25HC was added to the culture at final concentrations of 1 $\mu\text{g/mL}$ or 0.1 $\mu\text{g/mL}$ 24 hours after infection. Otherwise, 25HC was omitted throughout the experiments. Cells were collected and resuspended in the TBS buffer (25 mM Tris (pH 8.0) and 150 mM NaCl) plus protease inhibitor cocktail, 1% LMNG, and 0.1% (w/v) cholesteryl hemisuccinate Tris salt (CHS) 40 hours after infection. 25HC was added at indicated concentrations where it was required. After incubation at 4°C for 1 hour, the

mixture was applied to centrifugation at 18,000×g at 4°C for 30 min and the supernatant was applied to Ni-NTA. The resin was washed with 1 ml TBS buffer plus 30 mM imidazole, 0.01% LMNG, and 25HC at indicated concentrations for seven times. The proteins were eluted using wash buffer plus 300 mM imidazole, applied to 12% NuPAGE (Invitrogen), and analyzed by Western blot.

For Western blotting, the proteins were transferred to Immobilon-P transfer membranes (Millipore) after SDS-PAGE. The membranes were blocked by 5% (w/v) Bovine Serum Albumin (Amresco) at room temperature for 1 hour and incubated with the primary antibodies – anti-His tag mouse monoclonal antibody (CWBIO) for Insig-2 or anti-Flag mouse monoclonal antibody (CWBIO) for Scap – at 4°C overnight with a 1:2000 dilution. Then the membranes were washed with the TBST that contained 25 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% (w/v) Tween-20 for four times. The bound antibodies were visualized by chemiluminescence (SuperSignal West Dura Extended Duration Substrate; Thermo) using a 1:3000 dilution of goat anti-mouse IgG antibody, Horseradish Peroxidase (HRP) conjugates, as the secondary antibody (CWBIO). Membranes were exposed by ChemiDOC XRS+ System (Bio-Rad). For semiquantitative analysis shown in Fig. 4F, band intensities were measured by densitometry using the software ImageJ.

SREBP-2 cleavage assay

On day 0, SRD-13A cells, a Scap-deficient cell line derived from CHO-7 cells (63), were distributed in 6-well plates at a density of 2.5×10^5 cells per well in medium A that contained DMEM/F12 (1:1, Gibco) supplemented with 5% newborn calf serum (Gibco), 5 µg/ml cholesterol (Sigma), 1 mM sodium mevalonolactone (Sigma), and 20 µM sodium oleate (Sigma). After incubation at 37°C for 1-2 hours, cells in each well were transfected with plasmids as following: 0.8 µg pCMV-3×Myc-mScap (mouse) variants, 0.9 µg pCMV-His-hInsig-2 (human) variants, and 0.09 µg pCMV-3×Flag-hSREBP2 (human) by ViaFect™ transfection reagent (Promega). The medium was removed 16-20 hours later, and cells were washed twice with PBS that contained 135 mM NaCl, 4.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM NaH₂PO₄ (pH 7.3 ± 0.1) before changing for medium B (medium A supplemented with 5% lipoprotein-deficient serum, 50 µM mevastatin, and 50 µM sodium mevalonolactone) plus 1% hydroxypropyl-β-cyclodextrin (Sigma). After incubation at 37°C for 1 hour, cells were washed twice with PBS and switched to medium B in the absence or presence of 25-HC. Protease inhibitors, 1.5 µM FMK-Z-VAD (MedChemExpress) and 20 µg/mL N-acetyl-leucinal-leucinal-norleucinal (ALLN) (MedChemExpress), were added to prevent degradation of the N-terminal fragment of SREBP-2. After incubation for another 4-5 hours at 37°C, cells were washed twice with cold PBS and subsequently suspended in

100 µL lysis buffer (PBS supplemented with 0.5% SDS, protease inhibitors, and benzonase). The cell lysates were left on a shaker at 4°C for 15 min. The total protein concentration of each cell lysate was determined by UV280 using Nanodrop. The cell lysates were mixed with 5 × sample buffer (250 mM Tris-HCl (pH 6.8), 10% SDS, 50% glycerol, 0.2% bromophenol blue, and 500 mM dithiothreitol). Then an aliquot (10~20 µg total protein) of each sample was loaded to NuPAGE™ 4-12% Bis-Tris gradient Gel (Invitrogen). After electrophoresis, the proteins were transferred to Hybond-C nitrocellulose membranes (Bio-Rad). Strips were blocked with 4% (w/v) milk in TBST, and incubated with primary antibodies for 1 hour at room temperature. The following antibodies were used: anti His-tag mouse monoclonal antibody (CWBIO), anti-Flag-tag mouse monoclonal antibody (MBL International), and anti c-Myc tag mouse monoclonal antibody (9E10, Invitrogen). After incubation with the primary antibodies, the washed membranes were incubated with HRP conjugated goat anti-mouse secondary antibodies (CWBIO) at room temperature for 1 h. Membranes were exposed by ChemiDOC XRS+ System (Bio-Rad). The band intensities of precursor (P) and nuclear (N) form of SREBP2 were measured by densitometry using the software ImageJ. Resulting data were analyzed by GraphPad Prism 7.00. The mean ratio of the cleaved form to total SREBP2 (N/(N+P)) for WT proteins in the absence of 25HC in three experiments was set as 1, against which the ratio of other groups was normalized.

Circular dichroism (CD) characterization of purified protein

The recombinant N-terminal His₁₀-tagged human Insig-2 variants and the transmembrane domain of N-terminal FLAG-tagged mouse Scap variants were individually expressed in Sf-9 insect cells in the SF-900 II medium (Gibco) supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin at 27°C. 48 hours after infection, cells were collected and resuspended in the TBS buffer containing 25 mM Tris (pH 8.0) and 150 mM NaCl, the protease inhibitor cocktail, 1% LMNG, and 0.1% CHS. After incubation at 4°C for 2 hours, the mixture was centrifuged at 18,000×g at 4°C for 30 min. For Insig-2 variants, the supernatant was applied to Ni-NTA, which was then washed with the TBS buffer plus 30 mM imidazole, 0.01% LMNG, and 0.001% CHS. The proteins were eluted using wash buffer plus 300 mM imidazole. For Scap variants, the supernatant was applied to anti-FLAG M2 affinity resin, which was washed with the TBS buffer plus 0.01% LMNG and 0.001% CHS. The proteins were eluted using wash buffer plus 0.2 mg/mL FLAG peptide. The eluent was then concentrated and applied to SEC (Superose 6 Increase 10/300 GL, GE Healthcare) in the buffer containing 25 mM Tris (pH 8.0), 150 mM NaCl, 0.01% LMNG, and 0.001% CHS. The peak fractions were pooled and reinjected to SEC. The peak

fractions from the second-round SEC were concentrated and diluted for final buffer condition as 25 mM Tris (pH 8.0), 10 mM NaCl, 0.01% LMNG, and 0.001% CHS. The circular dichroism spectra of proteins at ~ 0.1 mg/mL were carried out using Chirascan plus (Applied Photophysics) at room temperature.

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ACKNOWLEDGMENTS

We thank Prof. Baoliang Song for sharing the SRD-13A cells and the plasmid pCMV-3×Flag-SREBP2. We thank the cryo-EM Facility of Westlake University and the Tsinghua University Branch of China National Center for Protein Sciences (Beijing) for providing cryo-EM and computation support. We thank Li Huang (Westlake University) and Xiaomin Li (Tsinghua University) for technical support during EM image acquisition. **Funding:** This work was funded by the National Key R&D Program of China (2020YFA0509301 to C.Y.) from Ministry of Science and Technology of China, Beijing Nova Program (Z201100006820039 to C.Y.), the China Postdoctoral Science Foundation (2020M681937 to R.Y.), the National Postdoctoral Program for Innovative Talents of China (BX20200304 to R.Y.), and the National Natural Science Foundation of China (project 31971123 to Q.Z.). N.Y. acknowledges the Ministry of Science and Technology of China and the National

Natural Science Foundation of China for multiple funds that have supported this research in 2008-2017. N.Y. has been supported by the Shirley M. Tilghman endowed professorship from Princeton University since 2017. **Author contributions:** N.Y. conceived and supervised the project. N.Y., R.Y. P.C., W.S., H.Q., and C.Y. designed the experiments. R.Y., H.Q., X.Z., Y.L., S.G., P.C., X.G., performed molecular cloning, protein purification, and cryo-EM data acquisition. P.C., W.S., and R.Y. performed all functional assays. X.D., H.C., A.J.B. and H.Y. contributed to the design and data analysis of the SREBP cleavage assay. X.L. and J.S. generated antibodies. J.L. and Q.Z. contributed to data acquisition and processing. C.Y. performed data processing and structural building and refinement. All authors contributed to data analysis. N.Y. wrote the manuscript. **Competing interests:** The authors declare no competing interests. **Data and materials availability:** The atomic coordinates and EM map have been deposited in the Protein Data Bank (www.rcsb.org) and EMDB (www.ebi.ac.uk/pdbe/emdb/) with the accession codes 6M49 and EMD-30074, respectively. Materials are available from the corresponding authors upon request.

SUPPLEMENTARY MATERIALS

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Figs. S1 to S11

Table S1

References (66, 67)

MDAR Reproducibility Checklist

8 February 2020; resubmitted 31 July 2020

Accepted 6 January 2021

Published online 14 January 2021

10.1126/science.abb2224

Fig. 1. Structure of the human Scap and Insig-2 complex in the presence of 25HC. (A) A simplified cartoon that illustrates several key players in the SREBP pathway. 25HC promotes the interaction between Scap and Insig-1/2, whereas sterol depletion leads to their dissociation and the COPII vesicle-mediated transport of the Scap and SREBP complex from the ER membrane to the Golgi. L1/L7: the luminal loops L1 and L7. NTD: the amino (N) terminal domain; CTD: the carboxy terminal domain. (B) The transmembrane (TM) region is better resolved than the soluble domains in the 3D EM reconstruction. The densities corresponding to Insig-2, the sterol sensing domain (SSD), other transmembrane (TM) segments, and the luminal domain of Scap are low pass filtered to 8 Å and colored cyan, yellow, blue, and light cyan, respectively. The same color scheme is applied to all figures. The LMNG micelle is shown as gray, semi-transparent surface to indicate the orientation of the TM domain. In all figures, the cytosolic side is placed on top in the side views. Because of the low resolution of the luminal domains, we focus on the TM region for analysis in this study. The EM map was generated in Chimera (64). (C) The interface between Scap and Insig-2 and the sandwiched 25HC are clearly resolved. Left: The local densities were calculated using Relion 3.0 and presented in Chimera. Right: The densities for the ligand and surrounding key residues, shown as blue mesh, are contoured at 5 σ . Note that S4 in Scap is broken in the middle, and the two half segments are designated S4a and S4b. (D) Overall structure of the TM domain of the complex. The TM segments in Insig-2 are denoted by numbers, while those in Scap are labeled S1-S7. All structure figures were prepared in PyMol (65).

Fig. 2. The S4 segment of Scap is broken in the middle. (A) Structure of the resolved TM domain of Scap. The last four visible residues in S6, ⁴⁴⁷MELA₄₅₀ from the MELADL motif, are colored orange. The unwound segment in the middle of S4 in Scap is highlighted with an orange circle. Please refer to fig. S4A for detailed annotation of the TM boundaries. (B) SSD comparison between Scap and NPC1 (light cyan). Major structural shifts, indicated by orange arrows, also occur in S2. Of particular note, S4, which is broken in Scap, is an intact helix in Ptch1 and NPC1. (C) SSD comparison between Scap and Patched1 (Ptch1, light green). It is noted that the potential sterol binding site on Ptch1-SSD, which is occupied by a cholesterol hemisuccinate molecule, is shielded by S7 (or S8) in Scap, while 25HC binds to the opposite surface of the SSD. The marked shift of S2 is indicated by red arrows. The PDB codes for NPC1 and Ptch1 are 6W5S and 6DMB, respectively. (D) Structural mapping of representative Scap mutations in the TM region. D428A confers Scap the Insig binding ability even in the absence of sterols. Single point mutations Y298C, L315F, and D443N all abolish 25HC-dependent Insig binding.

Fig. 3. Structure of human Insig-2. (A) Overall structure of Insig-2. Two opposing side views of Insig-2, rainbow-colored from blue to red, are shown. Please refer to fig. S4B for details of TM boundaries. (B) Structural comparison of human Insig-2 and MvINS, a mycobacterial homolog of Insigs (27). The two structures, shown in a luminal view, can be superimposed with the root-mean-square deviation of 3.3 Å over 141 Cα atoms. Note that the TM1-TM2 linker and TM5-TM6 linker in MvINS form antiparallel β strands, while the corresponding sequences, which are longer in mammalian Insigs and modeled in panels A and B to indicate the TM connections, were barely resolved in the EM map (fig. S3B). MvINS: Insig from *Mycobacterium vanbaalenii* (PDB code: 4XU4). (C) A hydrophobic pocket enclosed by TMs 1/2/3/6. A luminal view of Insig-2 is shown. The unresolved TM1-2 and TM5-6 linkers are indicated by dashed lines. (D) Removal of a polar group close to the pocket or blockage of the Insig-2 central pocket with aromatic residues does not interfere with 25HC-dependent Scap binding. The pull-down experiments were performed as reported previously (27) and described in detail in the Materials and methods. Loading and eluted samples are indicated by blue and orange lines on the right of the Western blots. The pull-down results in all figures are representatives of at least three repeated experiments. (E) SREBP cleavage suppression assay for Insig-2 variants containing single or double point mutations S81A, L46F/V73F, and L42F/C77F. The assays were performed using SRD-13A cells that were transfected with pCMV-3×Myc-mScap, indicated pCMV-His-hInsig-2 variants, and WT pCMV-3×Flag-hSREBP2. Top: A representative set of Western blots. “BP2”: human SREBP2. “P”: the SREBP precursor; “N”: the cleaved NTD of SREBP. Bottom: Semi-quantification of the band densities by Image J. The mean ratio of the cleaved form to total SREBP2 (N/(N+P)) for WT proteins in the absence of 25HC in three experiments was set as 1, against which the ratio of other groups was normalized. Please refer to fig. S5 for the original Western blot images. Experimental details are presented in the Materials and methods.

Fig. 4. 25HC mediates the interaction between Scap and Insig-2. (A) The interface between Scap and Insig-2. The direct interaction of the two proteins is mainly mediated by Scap-S2/4 and Insig-TM1/3. Please refer to fig. S6 for a detailed illustration of the interaction between Scap and Insig-2 residues. (B) Limited contact between Scap and Insig-2 within the membrane results in a cavity that exposes the 25OH-group of 25HC to the cytosol. Left: A fenestration on one side of the complex due to the limited contact between Scap-S5 and Insig-TM4. Right: The 25-OH end of the 25HC tail is exposed to the aqueous environment of the cytosol. Surface electrostatic potential of the complex was calculated in PyMol. (C) The end of the iso-octanol tail is in the vicinity of a cluster of polar and charged residues. The hydrophilic environment provides a potential interpretation for the preference of 25HC over cholesterol in facilitating the interaction between Scap and Insig-2. (D) Unwinding and bending of Scap-S4 results in a cavity on the Scap/Insig-2 interface for ligand accommodation. The SSD of NPC1 (PDB code: 6W5S, colored dark grey) is superimposed to Scap. A straight Scap-S4 in the complex would clash with 25HC. A cartoon that illustrates the bending of S4 is shown on the bottom left. (E) Coordination of 25HC by Scap and Insig-2. Left: The backbone of 25HC is surrounded by hydrophobic residues from both proteins. Middle: Coordination of 25HC by Insig-2. Potential water-mediated polar interaction between the 3 β -OH group of 25HC and His120 in Insig-2 is indicated by a red, dashed line, although water molecules cannot be resolved at this resolution. Right: Scap mainly engages hydrophobic residues to coordinate 25HC. (F) Functional validation of the structure-revealed 25HC binding site. The GOF variant Scap(D428A) can form complex with Insig-2 in the absence of sterols (43). Ala substitution of 25HC-coordinating residues in Scap (Leu343 and Val355) and Insig-2 (His120) significantly compromised 25HC-dependent complex formation (Lanes 1-4), but only had minor impact on the direct interaction between Scap and Insig-2 in the absence of 25HC in the context of Scap(D428A) (Lanes 9-13). Left: Representative Western blots for the pull down assays. Right: A semiquantitative analysis of the pull-down assays independently repeated for three times. In the two panels, the band densities of eluted Scap (bottom bot), WT in Lane #1 and D428A in Lane #9, were respectively set as 1 and served as the normalization reference for the other bands in the same gel. Please refer to the Materials and methods for experimental details, fig. S7 for full blots, and fig. S8 for biochemical characterization of the mutants.

Fig. 5. Unwinding of Scap-S4 is crucial for Insig binding. (A) Unwinding and bending of S4 is stabilized by the bound 25HC and nearby residues from both Scap and Insig. Lys102 and Arg110 of Insig-2 may simultaneously interact with Scap-Glu359. Unwinding of S4 is important to accommodate Arg110 in Insig-2 and Tyr298 in Scap, which in turn stabilize the mid-point discontinuity of S4. The polar interaction between Tyr298 and the exposed amide of Leu358 in Scap is indicated by a red, dashed line. (B) Functional validation of the key interacting residues shown in panel A. The Scap mutation G357A was designed to prevent helix unwinding. (C) A potential mechanistic interpretation for the GOF of Scap(D428A). Asp428 and Gln432 are both placed unfavorably in a hydrophobic environment within the SSD. Ala substitution of these two polar residues may lower the energy for S4 unwinding, allowing for complex formation with Insig even in the absence of 25HC. In a WT protein, the penalty has to be compensated by sterol binding. (D) Asp428 and Gln432 may prefer S4 in a straight conformation. Asp771 and Gln775, the NPC1 (light cyan) residues corresponding to Asp428 and Gln432, respectively, interact. Scap-Gln432 in a similar conformation as NPC1-Gln775 would clash with Val361 on S4b in the S4-unwound structure, whereas a straight S4 would place Gly357 to face these two residues, leaving enough space for them to interact. (E) Q432A of Scap is also a GOF mutation. Scap(Q432A), to a lesser extent than Scap(D428A), interacts with Insig-2 in the absence of 25HC. Please refer to fig. S8 for biochemical characterization of the mutants and fig. S10 for full blots. (F) A simplified model for 25HC-regulated interaction between Scap and Insig. Left: A cartoon of the structural finding reported in this paper. Middle: In the presence of 25HC and Insig, S4 of Scap unwinds in the middle, and the luminal Loop1 is anchored by cholesterol to the membrane (41). The MELADL motif (orange) is sequestered from being recognized by COPII. Right: In the absence of the sterols, S4 may exist as an intact helix as in other SSD-containing proteins. Conformational changes of multiple SSD segments expose the MELADL motif for COPII recognition.