Heat Shock Protein 90 Modulates Lipid Homeostasis by Regulating the Stability and Function of Sterol Regulatory Element-binding Protein (SREBP) and SREBP Cleavage-activating Protein

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Sterol regulatory element-binding proteins (SREBPs) are the key transcription factors that modulate lipid biosynthesis. SREBPs are synthesized as endoplasmic reticulum-bound precursors that require proteolytic activation in the Golgi apparatus. The stability and maturation of precursor SREBPs depend on their binding to SREBP cleavage-activating protein (SCAP), which escorts the SCAP-SREBP complex to the Golgi apparatus. In this study, we identified heat shock protein (HSP) 90 as a novel SREBP regulator that binds to and stabilizes SCAP-SREBP. In HepG2 cells, HSP90 inhibition led to proteasome-dependent degradation of SCAP-SREBP, which resulted in the down-regulation of SREBP target genes and the reduction in intracellular triglyceride and cholesterol levels. We also demonstrated in vivo that HSP90 inhibition decreased SCAP-SREBP protein, down-regulated SREBP target genes, and reduced lipids levels in mouse livers. We propose that HSP90 plays an indispensable role in SREBP regulation by stabilizing the SCAP-SREBP complex, facilitating the activation of SREBP to maintain lipid homeostasis.

Lipids are essential biomolecules that serve as a form of stored energy and comprise the fundamental components of cellular membranes, signaling molecules, and hormones (1). Dysregulation of lipid homeostasis can lead to metabolic diseases such as atherosclerosis and obesity (2). Emerging evidence also shows the link between hyperactivated lipid biosynthesis and enhanced tumor growth (3–5).

SREBPs are a family of transcription factors that modulate the transcription of the key lipogenic enzymes (6). Mammalian cells produce three SREBPs isoforms: SREBP1a, SREBP1c, and SREBP2. Whereas SREBP1c modulates transcription of genes involved in fatty acid biosynthesis, SREBP2 preferentially activates genes involved in cholesterol uptake and biosynthesis (7). SREBPs are synthesized as membrane-bound precursors residing in the ER. The activation of SREBP is regulated by SCAP. Upon synthesis, SREBP forms a stable complex with SCAP that interacts with coated protein II (COPII) proteins under cholesterol depletion and facilitates the transport of SREBP-SCAP complex to the Golgi apparatus, where SREBP is sequentially cleaved by site 1 and site 2 proteases. The proteolytic processing of SREBP releases its N-terminal transcription factor domain (8). When intracellular cholesterol levels rise, binding of cholesterol to SCAP changes its conformation to one hindering COPII binding and facilitates interaction with insulin-induced gene 1 protein (Insig), anchoring the SREBP-SCAP complex to ER and thereby inhibiting SREBP processing (9, 10).

The interaction between SREBP and SCAP is critical for the stability and activation of SREBP. SREBP uses its C-terminal regulatory domain to interact with the cytosolic C-terminal domain (CTD) of SCAP (11). Intriguingly, the crystal structure of yeast SCAP CTD has recently been resolved, revealing a unique WD40 domain structure comprising eight WD40 repeats (12). With a β-propeller structure that creates a large interface for protein-protein interactions, WD40 domains are characterized by their ability to act as a platform for multimeric protein complex formation (13). However, aside from SREBP, no other SCAP CTD-binding protein has yet been reported.

HSP90 is a highly conserved molecular chaperone that is expressed in a variety of tissues and can account for over 1% of...
total cellular protein (14). As one of the most promiscuous chaperones in cells, HSP90 interacts with more than 200 protein substrates, known as “clients,” and modulates their stability, folding, and maturation (15). HSP90 clients include kinases, nuclear receptors, and transcription factors that participate in the regulatory circuits of a wide range of cellular processes including cell cycling, differentiation, and death (16). HSP90 is indispensable for normal cell function, and it has been reported that deletion or knockdown of HSP90 and its co-chaperones resulted in cellular or embryonic lethality (17). Because of its crucial functions in cell survival and growth, HSP90 is also highly expressed in cancerous cells, maintaining the functional stability of clients that enhance tumorigenesis (18–21).

In this study, we identified HSP90 as a novel SREBP regulator that associates with SCAP-SREBP. We studied the binding domain, intracellular localization, and regulation of protein-protein interaction of the HSP90-SCAP-SREBP complex. We also demonstrated that HSP90 is required for the stability of SCAP-SREBP and modulates the activity of SREBPs to maintain lipid homeostasis in vitro and in vivo.

Results

HSP90 Interacts with the C Termini of SCAP and SREBP—To search for potential regulators of SCAP-SREBP that bind to the WD40 domain of SCAP, we transfected HEK293 cells with expression plasmids encoding FLAG-tagged C-terminal domain of SCAP (FLAG-C-SCAP) or empty vector as control and performed immunoprecipitation (IP) using anti-FLAG affinity resin. Silver staining of the precipitated samples revealed a number of specific proteins that were bound to FLAG-C-SCAP (Fig. 1A and B). The precipitated protein samples were trypsinized, and the resulting peptides were analyzed with an electrospray ionization-quadrupole time of flight mass spectrometry system as described under “Experimental Procedures.”

HSP90 Regulates SCAP and SREBP—In this study, we identified HSP90 as a novel SREBP regulator that associates with SCAP-SREBP. We studied the binding domain, intracellular localization, and regulation of protein-protein interaction of the HSP90-SCAP-SREBP complex. We also demonstrated that HSP90 is required for the stability of SCAP-SREBP and modulates the activity of SREBPs to maintain lipid homeostasis in vitro and in vivo.

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Mass spectrum analysis revealed 28,262 sequences matching 293 proteins from the SCAP sample and 25,373
sequences matching 128 proteins from the control sample. Proteins identified only in the SCAP sample were assigned as potential SCAP-binding partners. Table 1 shows the proteins with high sequence coverage among candidate proteins. These included the protein chaperones HSP90-β, HSP90-α, and HSP70. A list of proteins that were specifically identified in the FLAG-C-SCAP sample is shown in supplemental Table S1. We were particularly interested in HSP90, given emerging evidence for the involvement of HSP90 in the regulation of lipid metabolism (22–24).

Immunoblot analysis of the anti-FLAG IP samples showed that endogenous HSP90-α/β was indeed bound to FLAG-C-SCAP and that SREBP precursors were also presented in the anti-FLAG immunoprecipitates (Fig. 1B). We next employed an overexpressed protein system to further study the protein-protein interactions in HSP90-SCAP and HSP90-SREBP. We used overexpressed HSP90-β for the study because it is the constitutively expressed isof orm of HSP90 (15). Myc-tagged HSP90 was co-overexpressed with FLAG-tagged full-length (fl) SCAP, SREBP1, or SREBP2 in HEK293 cells for anti-Myc IP. As a result, FLAG-fl-SCAP, as well as FLAG-fl-SREBP1 and FLAG-fl-SREBP2, was co-precipitated with Myc-HSP90 (Fig. 1C). These results demonstrate the ability of HSP90 to interact with both SCAP and SREBP, suggesting the formation of a HSP90-SCAP-SREBP complex. To verify the formation of endogenous HSP90-SCAP-SREBP complex, anti-HSP90 IP was performed using HepG2 cells. The IP experiment shows that both SCAP and SREBP precursors were co-precipitated with HSP90 (Fig. 1D). The co-chaperone HSP70 was also co-precipitated with HSP90 (Fig. 1D).

We showed that HSP90 binds to the membrane-bound full-length and the cytosolic C-terminal domain of SCAP (Fig. 1, B–D). To exclude the possibility that HSP90 might also bind to the N-terminal transmembrane domain of SCAP, we examined the binding between overexpressed FLAG-tagged N-terminal SCAP (N-SCAP) to Myc-HSP90. We demonstrated only that the C-terminal and full-length SCAP but not the N-terminal domain of SCAP were capable of interacting with Myc-HSP90 (Fig. 1E).

Because the C-terminal domain of SCAP bound to HSP90 and interacted with the C-terminal domain of SREBPs, it seemed plausible that HSP90 associates with the C-terminal domain of SREBPs. To rule out the possibility of the interaction between HSP90 and the N-terminal transcription factor domain of SREBPs, we examined the binding between FLAG-tagged N-terminal domain of SREBPs (N-SREBP) to Myc-HSP90. Anti-Myc IP experiments revealed that only the C-terminal domains of SREBP1 and SREBP2 but not the N-terminal domains were co-precipitated with Myc-HSP90 (Fig. 1, F and G), indicating that HSP90 interacted with SREBPs via the C-terminal regulatory domains. Nonetheless, because HSP90 is a cytoplasmic chaperone, and N-SREBP is localized in the nucleus, the different localization could also explain the lack of HSP90-N-SREBP interaction.

### TABLE 1

| Protein    | Accession no. | No. of identified peptides | No. of matching sequences | Sequence coverage % |
|------------|---------------|---------------------------|--------------------------|---------------------|
| SCAP       | NM_012235     | 49                        | 14                       | 32                  |
| HSP90-β    | NM_00127969   | 32                        | 17                       | 31                  |
| HSP90-α    | NM_00107963   | 21                        | 14                       | 21                  |
| HSP70      | NM_002155     | 16                        | 8                        | 15                  |

**HSP90 Regulates SCAP and SREBP**

**Mass spectrometric identification of SCAP and its binding heat shock proteins**

**Protein**  | **Accession no.** | **No. of identified peptides** | **No. of matching sequences** | **Sequence coverage %**
--- | --- | --- | --- | ---
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HSP70 | NM_002155 | 16 | 8 | 15

**HSP90 REGULATES LIPIDS BIOSYNTHESIS BY CONTROLLING SCAP AND SREBP PROTEIN LEVELS**

To investigate the potential role of HSP90 in SCAP-SREBP regulation, HepG2 cells were treated with HSP90 inhibitor 17-αllylamino-17-demethoxygeldanamycin (17-AAG), and the protein levels of SCAP and SREBPs were determined. Dose-dependent decreases of SCAP protein and the precursor, N-terminal, and C-terminal SREBP1 and SREBP2 proteins were observed in the cells treated with 0–2 μM of 17-AAG, whereas the protein level of HSP70 was dose-dependently increased by HSP90 inhibition (Fig. 2A). The increase in HSP70 protein served as a positive control of HSP90 inhibition (24, 25). Corresponding to the decrease of matured N-terminal SREBP, the mRNA expression of SREBP target genes was down-regulated, and the intracellular total triglyceride and cholesterol levels were decreased by 17-AAG (Fig. 2, B and C). Treating another hepatoma cell line, Huh-7, and a CHO cell line with 17-AAG also resulted in dose-dependent reductions in SCAP and SREBP proteins (supplemental Fig. S1), indicating that the effect was not limited to HepG2 or hepatoma cells.

To confirm the regulatory effect of HSP90 on SCAP and SREBP, HSP90 was knocked down by siRNA transfection. HSP90 knockdown dramatically reduced the protein levels of SCAP and both the precursor and matured N-terminal of SREBP1 and SREBP2 (Fig. 2D) and caused a significant decrease in intracellular triglyceride and cholesterol levels (Fig. 2E).

It has been observed in alcohol-induced livers that the protein and mRNA levels of HSP90 were increased in parallel to the elevated triglyceride level (24). However, the causal relation between the increase of HSP90 and triglycerides was not demonstrated. To investigate whether increased expression of HSP90 protein directly affects SREBP and lipid homeostasis, we infected HepG2 cells with HSP90-expressing lentivirus.

As compared with the cells infected with control virus, the protein levels of HSP90, SCAP, and both the precursor and matured N-terminal of SREBP1 and SREBP2 and the total triglyceride and cholesterol levels were higher in the cells infected with HSP90-expressing lentivirus (Fig. 2, F and G). These results indicated that HSP90 played a critical role in regulating SCAP and SREBP proteins and lipid homeostasis.

**17-AAG INHIBITS HSP90-SCAP-SREBP INTERACTION AND INDUCES PROTEASOME-DEPENDENT SCAP-SREBP DEGRADATION**

To further study the effect of HSP90 on SCAP-SREBP proteins, we performed a time course experiment revealing the different
kinetics of SCAP and SREBP proteins in response to HSP90 inhibition. A significant reduction in SCAP protein level was observed after 6 h of 17-AAG treatment, whereas SREBP protein level was decreased only after 10 h of 17-AAG treatment (Fig. 3A).

To test whether HSP90 affects protein stability of SCAP and SREBP, we performed a cycloheximide chase assay to track the degradation rate of SCAP and SREBP. This assay revealed that treating HepG2 cells with 17-AAG accelerated the degradation of SCAP and SREBP precursors by 4- and 2-fold, respectively. The protein half-life of SCAP decreased from 32 to 8 h, precursor SREBP1 decreased from 14 to 6 h, and precursor SREBP2 decreased from 19 to 9 h, respectively, upon 17-AAG treatment (Fig. 3B).

It was reported that SCAP can be degraded through both the lysosome-dependent (26) and the proteasome-dependent (3) pathways. We thus investigated whether 17-AAG-induced SCAP and SREBP degradation was through either of these pathways.

HepG2 cells were treated with 17-AAG in the presence or absence of proteasome inhibitor MG132. Although 17-AAG dramatically decreased SCAP and SREBP precursors, co-treatment of MG132 abolished the effect, suggesting that HSP90 protects SCAP and SREBP precursors from proteasome-dependent degradation (Fig. 3C). HepG2 cells were also cultured with 17-AAG in the presence of lysosomal inhibitors chloroquine or ammonium chloride. However, these inhibitors had little effect on 17-AAG-induced SCAP and SREBP degradation (Fig. 3D), ruling out the involvement of the lysosomal pathway.

17-AAG binds to the N-terminal ATP-binding pocket of HSP90, which can cause the inhibition of the binding between HSP90 and its clients (15). To investigate whether 17-AAG hindered the interaction between HSP90 and SCAP-SREBP, HepG2 cells were treated with 17-AAG in the presence of MG132 to protect SCAP and SREBP from degradation, and the binding between HSP90 and SCAP-SREBP was studied by anti-HSP90 IP. As demonstrated in Fig. 3C, in the presence of MG132, SCAP and SREBP precursors were protected from 17-AAG-mediated degradation (Fig. 3D), ruling out the involvement of the lysosomal pathway.

17-AAG is required for HSP90-mediated SREBP regulation but is dispensable for HSP90-SREBP interaction—Because the protein level of SCAP directly affects the stability of SREBP precursors and SREBP processing (27), we investigated the possibility that HSP90 regulates SREBP proteins through SCAP. To this end,
end, we took advantage of an SCAP-deficient cell line SRD-13A derived from Chinese hamster cell line CHO-7 (27). Compared with CHO-7 cells, SRD-13A cells expressed no detectable SCAP, relatively low amounts of SREBP1 precursor, and comparable HSP90 and actin (supplemental Fig. S2).

When treated with 17-AAG, SREBP1 precursor was dose-dependently degraded in CHO-7 cells but not in SRD-13A cells (Fig. 4, A and B, lanes 1–6), suggesting that 17-AAG-induced SREBP degradation was SCAP-dependent. To demonstrate the direct involvement of SCAP, we transfected FLAG-fl-SCAP expression plasmids or an empty vector into SRD-13A cells and then treated the cells with 17-AAG. In cells transfected with an empty vector, the protein level of SREBP1 precursor was nearly undetectable and remained at a steady level even in the presence of 17-AAG (Fig. 4B, lanes 1–6). On the other hand, in the cells transfected with FLAG-fl-SCAP, the protein level of SREBP1 precursor was restored to a comparable level with its CHO-7 counterpart and was dose-dependently reduced by 17-AAG (Fig. 4B, lanes 7–9). In addition, FLAG-fl-SCAP was dose-dependently decreased by 17-AAG. These results indicate that HSP90 stabilizes SREBP1 precursor protein through a SCAP-dependent mechanism.

Because HSP90 regulated SREBP through SCAP, we next investigated whether it also interacted with SREBP via SCAP. To this end, anti-HSP90 IP was performed using CHO-7 and SRD-13A cells. Unexpectedly, we were able to recover SREBP1 precursor in the anti-HSP90 precipitates from both CHO-7 and SRD-13A cell lysates (Fig. 4C). This finding suggests that HSP90 interacts with SREBP independently of SCAP; however, this interaction alone is insufficient to fulfill its regulatory function on SREBP.

**HSP90 Interacts with and Regulates SCAP-SREBP Independently of Intracellular Sterol Levels**—The conformation of SCAP is modulated by its binding to cholesterol and to Insig, a sterol sensing protein that anchors SCAP to the ER membrane. The binding of cholesterol and 25-hydroxycholesterol (25-HC)-bound Insig to the transmembrane domain of SCAP alters the conformation of ER luminal loop 1 and loop 7 of SCAP,

![Image](https://via.placeholder.com/150)
tightening the binding between SCAP and Insig, blocking the access of COPII vesicle proteins to the MELADL motif of cytosolic loop 6 of SCAP, and leading to the inhibition of ER to Golgi transport of the SCAP-SREBP complex (28, 29).

To further study the regulatory function of HSP90 on SCAP and SREBP, we investigated whether changes in the intracellular sterol level modulate the binding between HSP90 and SCAP-SREBP or the effect of 17-AAG. HepG2 cells were cultured under sterol-depleted conditions in the absence or presence of 25-HC or cholesterol, and the interaction between HSP90 and SCAP-SREBP was examined by anti-HSP90 IP. A substantial increase in SREBP1 precursor and a modest increase in SREBP2 precursor proteins were observed when 25-HC was applied to the cells, whereas cholesterol moderately increased both SREBP precursor proteins (Fig. 5A, Input). The increases in SREBP precursors were a result of the inhibition of SREBP processing, judging from the corresponding decreases of processed SREBPs. Nonetheless, the amount of SCAP and SREBP precursor proteins that were co-precipitated with HSP90 remained constant upon either 25-HC or cholesterol treatment (Fig. 5A, IP: HSP90).

To study the regulation of HSP90 on SCAP-SREBP during active or halted SREBP processing, we examined the effect of 17-AAG under sterol-depleted conditions in the absence or presence of 25-HC. SREBP processing was highly activated in HepG2 cells cultured under sterol-depleted conditions and was drastically inhibited by application of 25-HC as observed by the phenomenal rise of precursor to processed SREBP ratio (Fig. 5B). Nevertheless, 17-AAG consistently reduced SCAP and SREBP precursor independently to the state of intracellular sterol level and SREBP processing.

We next examined the intracellular localization of the HSP90-SCAP-SREBP complex. Because HSP90 is known to be a cytoplasmic protein, we first examined the existence of HSP90 on the membrane of ER and Golgi apparatus. HepG2 cells were cultured in complete or sterol-depleted medium for 24 h and then harvested. The cells were lysed and then separated by sucrose density gradient ultracentrifugation into 10 fractions. The total cell lysates (input, fraction 0) and the density fractions were analyzed by immunoblot. The ER fractions (fractions 8–10) and the Golgi fractions (fractions 3 and 4) were determined by the predominant existence of calnexin and Golgi 58K protein, respectively (Fig. 5C). SCAP was predominantly localized in the ER fractions under complete medium condition, whereas it was predominantly localized in the Golgi fractions under sterol-depleted conditions. Under both conditions, HSP90 was enriched in the light fractions (fractions 2–5), whereas it was still detectable in the heavy fractions (Fig. 5C). These findings suggest the co-existence of HSP90 and SCAP on the membrane of both the ER and the Golgi apparatus.

To examine the binding between HSP90 and SCAP, the input, ER, and Golgi fractions shown in Fig. 5C were analyzed by anti-HSP90 IP. As a result, HSP90 was readily recovered by IP in the input (fraction 0) and the Golgi (fractions 3 and 4) fractions (Fig. 5D, lanes 3 and 9). Although the protein level of HSP90 is relatively low in the ER (fractions 8–10) fractions, it was enriched by IP (Fig. 5D, lanes 4 and 6). Under both complete medium and sterol-depleted conditions, we were able to detect SCAP in the anti-HSP90 precipitates of the input (fraction 0), ER (fractions 8–10), and Golgi (fractions 3–4) fractions (Fig. 5D, lanes 3, 6, and 9). This result indicates that SCAP is bound to HSP90 on the ER membrane and in the Golgi apparatus. Furthermore, using antibodies against the C terminus of SREBP, we were able to detect the cleaved C-terminal SREBP in...
the anti-HSP90 IP precipitates (Fig. 5E). This finding reveals that HSP90 stays associated with SCAP and SREBP after the cleavage of N-terminal SREBP. These findings also support the idea that HSP90 functions to regulate the stability of SCAP and SREBP proteins rather than their response to sterol alteration.

The proposed role of HSP90 to maintain lipid homeostasis is illustrated in Fig. 5F. HSP90 binds to SCAP-SREBP precursor on the ER membrane, stabilizing the protein complex throughout the ER to Golgi transport and continues to bind and stabilize SCAP and the cleaved C-terminal SREBP after SREBP processing in the Golgi apparatus. The dramatic decreases of SCAP-SREBP proteins and intracellular lipid levels underline the importance of HSP90 in preserving SREBP to act as the master transcription regulator of lipid biosynthesis. Nonetheless, the mechanism by which the cleaved C-terminal SREBP dissociates from SCAP and HSP90 and how it affects SCAP recycling to the ER require further research.

**HSP90 Inhibition Decreases SCAP and SREBP Protein Levels and Down-regulates SREBP Target Gene Expression in Mouse Liver**—Having confirmed the effect of HSP90 inhibition in human hepatoma cells, we next investigated whether HSP90 had similar functions in mouse primary hepatocytes and livers.

Primary mouse hepatocytes were prepared from C57/BL6NCrl mice and treated with 0–3 μM of 17-AAG for 24 h. Moreover, 17-DMAG down-regulated the mRNA levels of SREBP target genes in mouse primary hepatocytes (Fig. 6B).

To examine the in vivo effect of HSP90 inhibition, C57/BL6NCrl mice were intraperitoneally injected with 5 mg/kg
body weight of 17-DMAG or equivalent volume of PBS as a control for times at 12-h intervals. Livers were excised from euthanized mice 4 h after the last injection. Corresponding to our \textit{in vitro} findings, 17-DMAG substantially reduced the protein level of SCAP, SREBP1, and SREBP2. A marked increase of HSP70 protein indicated that 17-DMAG was delivered and inhibited HSP90 activity in the mouse livers (Fig. 6, \textit{C} and \textit{D}).

We also quantified the mRNA levels of SREBP target genes in the liver tissues. Expression of SREBP1 target genes \textit{Fasn} (fatty acid synthase), \textit{Acc1} (acetyl-CoA carboxylase 1), and \textit{Scd1} (stearyl-CoA desaturase 1) was down-regulated by 40–60%, and expression of SREBP2 target genes \textit{Hmgcr} (3-hydroxy-3-methylglutaryl-CoA reductase) and \textit{Sqs} (squalene synthase) was reduced by 30%. \textit{Srebp1c} and \textit{Srebp2} expressions were also down-regulated by 30%, whereas \textit{Srebp1a}, which is not an SREBP target, was not affected by 17-AAG (Fig. 6\textit{E}). Finally the triglycerides and total cholesterol levels in mouse livers were measured. In accordance with the decreased SREBP proteins and the down-regulated SREBP target mRNA expression, both the triglycerides and total cholesterol levels in the mouse livers were reduced by 17-DMAG treatment (Fig. 6\textit{F}). Taking these results together, we conclude that HSP90 activity is essential in stabilizing SCAP and SREBP proteins and facilitating SREBP function as a transcription regulator of lipid biosynthesis \textit{in vivo}.

### Discussion

SREBP is the master transcription regulator of the genes required for lipid biosynthesis and metabolism. SCAP, the SREBP chaperone, is a membrane-bound sterol sensor that regulates SREBP function. SCAP is reported to bind to cholesterol via its ER luminal loop 1, which interacts with loop 7 and causes a change in the conformation of COPII binding domain on the cytosolic loop 6, resulting in the inhibition of the ER to Golgi transport of the SCAP-SREBP complex (28, 29). Although the transmembrane domains of SCAP have been studied in molecular detail, less attention has been paid to the cytosolic C terminus of SCAP. In the present study, we showed that HSP90 binds to the C termini of SCAP and SREBP to form a stable
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complex, thereby exerting a regulatory effect on the stability of SCAP-SREBP complex and the activation of SREBP. The direct causal relation between elevated HSP90 and increased SREBP protein level and activity provides important evidence for the development of potential therapeutic target to treat metabolic diseases or cancers in which both SREBP and HSP90 were highly expressed.

Taipale et al. (30) colleagues previously uncovered the possibility that the WD40 domain might comprise a novel HSP90 client protein fold. We demonstrate here the interaction between HSP90 and WD40 repeats containing SCAP C terminus. We emphasize here that because the structure of human SCAP has not been solved and the predicted WD40 domain is only a part of the SCAP C terminus, HSP90 might interact with other portion of SCAP C terminus. Although HSP90 is a cytoplasmic protein chaperone, it is capable of binding to ER- and Golgi membrane-bound SCAP and SREBP (Fig. 5D). The ability of cytoplasmic protein chaperone HSP90 to modulate an ER membrane-bound protein has been demonstrated previously. The HSP90 chaperone system was reported to control the ER-associated degradation and maturation of the thiazide-sensitive cotransporter (NCC), which is the primary mediator of salt reabsorption in animals. Inhibition of HSP90 activity accelerated NCC degradation via the ubiquitin-proteasome pathway, whereas HSP70/HSP90 organizer proteins stabilized NCC proteins (31). These findings support the notion that HSP90 machinery mediates the quality control of ER proteins. Accordingly, we found that HSP90 inhibition destabilized SCAP-SREBP proteins and accelerated their proteasome-dependent degradation (Fig. 3C).

Two distinct pathways have been reported for SCAP degradation. As detailed in the introduction, SREBP is cleaved by site 1 protease (S1P) and site 2 protease in the Golgi apparatus. It was reported that S1P cleavage is critical for Golgi to ER recycling of SCAP, with reduction in SREBP cleavage by S1P leading to SCAP degradation in the lysosome (26). On the other hand, it was also demonstrated in glioblastoma cells that N-glycosylation of SCAP is crucial for the stability of SCAP, with glucose depletion accelerating the proteasome-dependent degradation of SCAP (3). Our findings and those of others suggest that both proteasomal and lysosomal pathways take part in regulating SCAP degradation under different circumstances.

We found that HSP90 inhibition also led to SREBP degradation; however, in the absence of SCAP, 17-AAG had little effect on SREBP-1 precursor, whereas overexpression of SCAP restored the effect of 17-AAG on SREBP (Fig. 4, A and B). This indicates that 17-AAG-induced SREBP degradation is at least in part caused by SCAP decay, given that SREBP degrades rapidly in the absence of SCAP (27).

Although it is known that binding of cholesterol to SCAP and binding of 25-HC to Insig can both change the conformation of SCAP into which favors Insig binding and inhibits SCAP-Sec23-24 interaction (8), manipulation of sterols in the culture medium had no obvious effect on HSP90-SCAP-SREBP binding (Fig. 5A). These results are comprehensible considering the fact that the conformational changes induced by sterols are made on the transmembrane domain of SCAP (28, 29), whereas HSP90 interacts with the cytosolic WD40 domain of SCAP independently of the sterol-mediated conformational changes. In addition, the status of SREBP processing had little effect on 17-AAG-induced SCAP degradation (Fig. 5B). These findings support the notion that HSP90 regulates the functional stability of SCAP-SREBP complex rather than the response to sterol changes.

In addition to the maintenance of SCAP and SREBP protein complex stability, two studies also demonstrate the ability of HSP90 to facilitate protein transportation around ER and the Golgi apparatus. In mammalian cells, HSP90 was shown to modulate ER to Golgi transport of Rab GTase by forming a complex with guanine nucleotide dissociation inhibitor, which directs the recycling of Rab1 (32). In plants, HSP90 and its co-chaperones Hop/Sti1 interact with the ER-bound rice chitin receptor OsCERK1 and facilitate the ER to plasma membrane trafficking of OsCERK1 via a Sar1-dependent pathway (33). These studies show that HSP90 is indeed involved in the trafficking of certain membrane-bound proteins by forming a complex with the target. Herein, we presented clear evidence that HSP90 interacts with and stabilizes SCAP-SREBP proteins during their localization and transport around ER and the Golgi apparatus and even after the proteolytic processing of SREBP (Fig. 5, C–E). Further studies may uncover more details concerning the mechanism by which HSP90 regulates SCAP-SREBP trafficking.

Finally, HSP90 is highly expressed in various types of tumors and has long been a potential target for cancer therapy, because of its crucial role in the progression and survival of cancer cells (18). Given that the SREBP-SCAP system plays a fundamental role in life-sustaining lipid metabolism, our findings suggest HSP90 may be the link between tumorigenesis and lipid metabolism. This can be supported by our finding that increased HSP90 expression caused a significant increase in intracellular lipids level (Fig. 2G).

Recent studies reveal more connections between SCAP-SREBP and tumorigenesis. SCAP connected the increased glucose uptake and elevated lipogenesis in glioblastoma cells and knockdown or dominant negative of SCAP reduced tumor growth and prolonged survival of tumor-bearing mice (3). Inhibition of SCAP and SREBP has been shown to suppress glioblastoma tumor growth and increase the sensitivity of non-small cell lung cancer cells to chemical treatment (4, 5). These results are in agreement with a scenario in which increased HSP90 expression is associated with more stabilized SCAP-SREBP, consequently a more robust lipid biosynthesis, and ultimately a more favorable environment for tumorigenesis.

In conclusion, we have identified HSP90 as a new SREBP regulator that interacts with the SCAP-SREBP complex. HSP90 inhibition destabilized SCAP-SREBP proteins, down-regulated SREBP target gene expression, and decreased triglyceride and cholesterol levels in hepatoma cell lines and mouse livers. We elucidated the connection between HSP90 and lipid metabolism, one that may shed light on the mechanism linking HSP90 with tumorigenesis.

Experimental Procedures

Chemicals and Reagents—17-AAG was purchased from LKT Laboratories (St. Paul, MN). 17-DMAG was purchased from
FERMENTEK (Jerusalem, Israel). Protease inhibitor mixture, calpain inhibitor, cycloheximide, cholesterol, and oleic acid sodium salt were purchased from Nacalai Tesque (Kyoto, Japan). PMSF and 3× FLAG peptides were purchased from Sigma-Aldrich. Lipofectamine® RNAiMAX transfection reagent Lipofectamine® LTX with Plus™ reagent, and high capacity cDNA reverse transcription kits were purchased from Thermo Fisher Scientific (Waltham, MA). t-Imovalenic acid sodium salt, control siRNA-A, and HSP 90α/β siRNA (h) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Protein G-Sepharose 4 Fast Flow and HyClone FBS were purchased from GE Healthcare Life Sciences. MG132, DMEM, and DMEM/Ham’s F-12 were purchased from Wako (Osaka, Japan). ISOGEN was purchased from Nippon Gene (Tokyo, Japan).

Antibodies—Anti-FLAG M2 affinity gel, mouse IgG-agarose, monoclonal anti-FLAG (M2; catalog no. F1804), and anti-β-actin (AC-15; catalog no. A5451) antibodies and anti-Golgi 58K protein (58K-9; catalog no. G2404) mouse ascites fluid were purchased from Sigma-Aldrich. Monoclonal anti-SREBP1 (2A4; catalog no. sc-13551), anti-SREBP2 (1C6; catalog no. sc-13552), anti-HSP90 (F-8; catalog no. sc-13119), anti-HSP70 (3A3), polyclonal anti-SREBP1 (C-20; catalog no. sc-366), anti-SCAP (C-20; catalog no. sc-9675) antibodies, and normal mouse IgG control antibody were purchased from Santa Cruz Biotechnology. Peroxidase-conjugated affinity-purified donkey anti-mouse IgG, peroxidase-conjugated affinity-purified donkey anti-rabbit IgG, and peroxidase-conjugated affinity-purified donkey anti-goat IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Calnexin (C5C9; catalog no. 2679) rabbit mAb was purchased from Cell Signaling Technology (Danvers, MA).

Plasmid Constructs—The coding sequence of full-length (amino acid residues aa 2–1279), N-terminal domain (aa 2–731), and C-terminal domain of human SCAP (aa 732–1279) were cloned into p3×FLAG-CMV-7.1 vector (Sigma) using NotI and XbaI restriction enzyme sites to construct FLAG-fl-SCAP, FLAG-N-SCAP, and FLAG-C-SCAP plasmids, respectively. The coding sequence of full-length (aa 2–1177), N-terminal domain (aa 2–487), and C-terminal domain of human SREBP1a (aa 593–1177) were cloned into p3×FLAG-CMV-7.1 vector using NotI and XbaI restriction enzyme sites to construct FLAG-fl-SREBP1, FLAG-N-SREBP1, and FLAG-C-SREBP1 plasmids, respectively. The coding sequence of full-length (aa 2–1141), N-terminal domain (aa 2–481), and C-terminal domain of human SREBP2 (aa 555–1141) were cloned into p3×FLAG-CMV-7.1 vector using NotI and XbaI restriction enzyme sites to construct FLAG-fl-SREBP2, FLAG-N-SREBP2, and FLAG-C-SREBP2 plasmids, respectively. The coding sequence of human HSP90β (aa 1–724) and a synthesized oligonucleotide encoding two consecutive c-Myc peptides were cloned into a pcDNA3.1/Hygro (+) vector (Thermo Fischer Scientific) using NheI/NotI and NotI/XbaI sites, respectively, to construct Myc-HSP90 plasmid.

Cell Cultures—HEK293 cells, HepG2 cells, and mouse primary hepatocytes were cultured in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% (v/v) FBS. In some experiments, HepG2 cells were cultured in DMEM supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, 12.5 μM fluvastatin, and 5% (v/v) lipoprotein deficient serum in the absence or presence of 1 μg/ml 25-HC or 10 μg/ml cholesterol to manipulate SREBP processing. Chinese hamster cell lines CHO-7 and SRD-13A were cultured in DMEM/Ham’s F-12 supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) FBS, with 5 μg/ml cholesterol, 1 mM sodium mvalonate, and 20 μM sodium oleate added to the medium for SRD-13A to overcome SCAP deficiency. All cells were grown in a humidified incubator at 37°C and 5% CO₂.

Cell Fractionation—Separation of the ER and Golgi membranes were performed following our previous protocol (34) with minor modifications. HepG2 cells were seeded in 100-mm dishes (3×10⁵/dish) and cultured for 48 h. The cells were then harvested and resuspended in buffer A containing 50 mM Tris-HCl, pH 7.5, and 150 mM NaCl supplemented with 15% (w/v) sucrose. The cells were disrupted by passing through a 25-gauge needle 20 times. The cell suspension was centrifuged at 3,000 × g for 10 min, and the supernatant was collected. A discontinuous sucrose density gradient was prepared in a 2.2-ml polypropylene tube (Beckman Coulter, catalog no. 347357) by layering the following sucrose density solutions in buffer A (0.45 ml of 45% sucrose, 0.75 ml of 30% sucrose, 0.45 ml of cell supernatant in 15% sucrose, and 0.3 ml of 7.5% sucrose). The gradient solution was centrifuged in a Optima™ MAX-TL installed with a TLS-55 swing-bucket rotor (Beckman Coulter) with a program set as follows: 36,000 rpm for 2 h, 30,000 rpm for 1 h, 24,000 rpm for 1 h, 18,000 rpm for 1 h, 12,000 rpm for 1 h, and then slowdown without application of a break. The resulting solution was collected from top to bottom into 10 fractions (Fig. 5C) and analyzed by Western blotting. The presence of Calnexin and Golgi 58K protein was assessed as markers for the ER and Golgi apparatus, respectively. The ER (fractions 8–10) and Golgi (fractions 3 and 4) samples were collected for further analysis.

Immunoprecipitation—HEK293 cells were seeded in 100-mm dishes (3×10⁵/dish) on day 0. The cells were transfected with indicated plasmids by the calcium phosphate method on day 1. Culture medium was replaced by fresh medium on day 1. The cells were harvested on day 3 in PBS and lysed by passing through a 25-gauge needle 20 times in IP lysis buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 5% glycercol, and 0.5% Nonidet P-40. Cell lysates were preclarified by incubating with mouse IgG-agarose for 1 h at 4°C. Anti-FLAG IP was performed on the preclarified lysates using anti-FLAG affinity resin following the manufacturer’s protocol. The precipitated proteins were eluted by 3× FLAG peptides and collected for further analysis. HepG2, CHO-7, or SRD-13A cells were seeded in 100-mm dishes (3×10⁵/dish) and grown for 3 days. The cells were harvested in PBS and lysed by passing through a 25-gauge needle for 20 times in IP lysis buffer. In some experiment, HepG2 cells were prepared into total, ER, and Golgi fractions, and in such cases, the sucrose concentration in the fractions was diluted to ~5% (w/v) by IP lysis buffer prior to IP. Cell lysates or cell fractions were preclarified by incubating with protein G-Sepharose for 1 h at 4°C. Anti-HSP90 IP was performed on the preclarified lysates using protein G-Sepharose.
HSP90 Regulates SCAP and SREBP

Harose conjugated with anti-HSP90 (F-8) or control IgG antibodies following the manufacturer’s protocol. In all experiments, 10% of total cell lysates were saved as the input control. The immunoprecipitated samples were adjusted to equal volume to the input cell lysates, and equal volumes of input and immunoprecipitated samples were loaded to SDS-PAGE for immunoblotting.

**Immunoblotting**—Cells or mouse liver were lysed in radioimmune precipitation assay lysis buffer containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, and 1% Triton X-100 for 30 min on ice, and cell debris was removed by centrifugation at 12,000 x g and 4 °C for 10 min. Cell lysates were adjusted to appropriate concentration and treated with 6× Laemmli sample buffer containing 1× Tris-HCl, pH 6.8, 30% glycerol, 10% SDS, and 0.03% bromophenol blue. Protein samples were subjected to SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The membranes were immunoblotted with indicated antibodies and visualized by Amersham Biosciences ECL Western blotting detection reagent (GE Healthcare Life Sciences) or Immobilon Western chemiluminescent HRP substrate (Merck Millipore). Data acquisition and analysis were performed using an ImageQuant LAS 4000 system (GE Healthcare and Life Sciences).

**Silver Staining and Protein Mass Spectrometric Analysis**—Anti-FLAG immunoprecipitated protein samples obtained from HEK293 cells transfected with FLAG-C-SCAP expression plasmids or empty vector were subjected to SDS-PAGE and analyzed by silver staining using SilverQuest™ silver staining kit (Thermo Fisher Scientific Inc.) following the manufacturer’s protocol. The protein samples of FLAG-C-SCAP and control immunoprecipitates were digested with sequence grade trypsin, and the resulting peptides were subjected to electrospray ionization-quadrupole time of flight mass spectrometry. The peptide sequences acquired from MS analysis were searched using the Swiss-Prot protein sequence database under genus restriction of Homo sapiens using the in-house licensed Mascot searching program (version 2.1.03).

**17-AAG Inhibition and Small Interfering RNA Knockdown of HSP90**—HepG2 cells, CHO-7 cells, SRD-13A cells, and mouse primary hepatocytes were seeded in 6-well plates (5 × 10^5/well) on day 0. In some experiments, SRD-13A cells were transfected with FLAG-tagged full-length SCAP by the calcium phosphate method on day 1. On day 2, cells were cultured in medium containing indicated dosages and periods of 17-AAG to study the effect of HSP90 inhibition on SCAP and SREBP. In some experiments, HepG2 cells were treated with indicated periods of 17-AAG in the presence of 50 μM cycloheximide or 10 μM MG132 to determine protein half-life or to determine proteasome-dependence of SCAP and SREBP degradation, respectively. siRNA was employed to examine the effect of HSP90 knockdown. HepG2 cells were reverse-transfected with HSP90α/β siRNA or control siRNA-A using Lipofectamine® RNAiMAX transfection reagent following the manufacturer’s protocol, and the cells were harvested for immunoblot analysis 3 days after lipofection.

**Lentivirus Preparation and Transduction**—The coding sequence of human HSP90α (aa 1–724) was cloned into a lentiviral vector CSII-EF-MCS-IRES2-Venus (RIKEN, Japan, RDB04383) using NotI and BglII/BamHI restriction enzyme sites to construct HSP90 lentivirus. For virus packaging, HEK293T cells were cultured in 100-mm dishes at 2 × 10^6 overnight until reaching 70% confluence. The cells were transfected with HSP90 or empty lentivector along with packaging plasmids pCAG-HIVgp (RIKEN, RDB04394) encoding VSV-G protein and HIV-1 Gag and Pol, and pCMV-VSV-G-RSV-Rev (RIKEN, RDB04393) encoding VSV-G protein and RSV Rev by calcium phosphate method. To increase viral protein expression, 10 μM forskolin was applied to the cells 12 h after transfection. The culture supernatant containing viral particles was harvested 48 h after transfection and filtered through 0.45-μm sterile discs (Advantec, Tokyo, Japan; 25AS045AS). Viral titer was determined by transducing 2 × 10^6 HEK293T cells with 10-fold dilutions of virus supernatant. The percentage of Venus+ cells was calculated 48 h after transduction to determine the number of transducing units/ml of the virus.

For HSP90 overexpression experiments, HepG2 cells were seeded in 6-well plates at 5 × 10^5 and transduced with HSP90 or control lentivirus at a multiplicity of infection of 5 transducing units/cell in the presence of 4 μg/ml Polybrene 24 h after seeding. The culture medium containing virus and Polybrene was replaced by fresh medium 24 h after transduction. Transduction efficiency was over 80% as estimated by the percentage of Venus+ cells 48 h after transduction. The cells were harvested for further analysis 72 h after transduction.

**Real Time Quantitative PCR**—HepG2 cells or mouse liver tissues were homogenized in ISOGEN, and total RNA was extracted and reverse-transcribed to complementary DNA using high capacity cDNA reverse transcription kits following the manufacturer’s protocol. Real time quantitative PCR reactions were prepared using FastStart Universal SYBR Green Master (Roche Applied Science) following the manufacturer’s protocol. Data acquisition and analysis was conducted on an Applied Biosystems StepOnePlus™ real time PCR system (Thermo Fisher Scientific). Relative mRNA expressions in HepG2 cells and mouse liver tissues were normalized to 18S and 36B4, respectively. Primer sequences are listed in supplemental Table S2.

**In Vivo Evaluation of HSP90 Inhibition by 17-DMAG**—All animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of the University of Tokyo. Male 8-week-old C57/B6NCrl mice were purchased from Charles River Laboratories (Wilmington, MA) and maintained in the animal facility under controlled temperature and humidity conditions with a 12-h light/dark cycle. The mice were acclimatized for 7 days and then randomly divided into two groups (n = 5). The mice were intraperitoneally injected with 5 mg/kg body weight of 17-DMAG or an equal volume of PBS as a control three times at 12-h intervals. The mice were euthanized 4 h after the last i.p. injection, and the livers were excised for further experiments.

**Triglyceride and Cholesterol Measurements**—To determine triglyceride and cholesterol levels in HepG2 cells, properly treated cells were washed twice with ice-cold PBS (0.5 ml/well) in a 6-well plate. Intracellular lipids were dissolved by incubating the cells with 0.6 ml/well of hexane/chloroform (3:2, v/v) for 20 min on ice. The lipid-containing supernatant was collected
for measurements of triglyceride and cholesterol using kits purchased from Wako, and the cells were harvested to determine total protein content. The detail method for liver triglyceride and cholesterol measurements has been previously described (34). In brief, 100–150 mg of liver samples were homogenized in ice-cold chloroform/methanol (2:1, v/v) and incubated for 30 min at room temperature followed by adding 50 mM NaCl to separate organic phase. The lipid-containing organic phase was washed twice with 0.36M CaCl2/methanol (1:1, v/v) and then diluted with appropriate volume of chloroform. Triglyceride and cholesterol levels were measured using kits purchased from Wako.

**Statistical Analysis**—The data are presented as means ± S.D. unless stated otherwise. Significant difference was determined by computing p values between control and treatment groups using a two-tailed equal variance Student’s t test in which the null hypothesis was rejected at p < 0.05. Statistical analysis was performed using Microsoft Excel software.

**Author Contributions**—Y.-C. K. performed the experiments, analyzed data, and wrote the manuscript with input from J. I. and R. S. T. H. performed the animal experiments. T. S. and K. U. performed the mass spectrum analysis. M. S., J. I., and R. S. provided expertise and feedback. R. S. conceived and designed the experiments, wrote the manuscript, and secured funding.

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**Note Added in Proof**—There were several errors in the version of this manuscript that was published as a Paper in Press on December 21, 2016. In Fig. 1B, an incorrect immunoblot was used in the IP:FLAG, WB:SREBP2 panel. In Fig. 2F, an incorrect immunoblot was used in the N-SREBP1 panel. These errors have been corrected and do not affect the results or conclusions of the work.

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