

Crystal structure of Spot 14, a modulator of fatty acid synthesis

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Spot 14 (S14) is a protein that is abundantly expressed in lipogenic tissues and is regulated in a manner similar to other enzymes involved in fatty acid synthesis. Deletion of S14 in mice decreased lipid synthesis in lactating mammary tissue, but the mechanism of S14's action is unknown. Here we present the crystal structure of S14 to 2.65 Å and biochemical data showing that S14 can form heterodimers with MIG12. MIG12 modulates fatty acid synthesis by inducing the polymerization and activity of acetyl-CoA carboxylase, the first committed enzymatic reaction in the fatty acid synthesis pathway. Coexpression of S14 and MIG12 leads to heterodimers and reduced acetyl-CoA carboxylase polymerization and activity. The structure of S14 suggests a mechanism whereby heterodimer formation with MIG12 attenuates the ability of MIG12 to activate ACC.

lipogenesis | posttranslational | regulation

Fatty acid synthesis is a tightly regulated process that occurs at multiple levels within the cell. This regulation is essential for normal cellular function because fatty acids are required for phospholipid synthesis, triglyceride formation, and to serve as signaling molecules; however, excess fatty acid accumulation in cells can contribute to the pathogenesis of fatty liver disease, diabetes, cancer, and obesity (1, 2). The synthesis of fatty acids up to 16 carbons in length requires the action of only three enzymes, ATP citrate lyase, acetyl-CoA carboxylase (ACC), and fatty acid synthase. The most highly regulated of the three enzymes is ACC, the first committed enzyme in fatty acid synthesis.

ACC catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting and first committed step in de novo fatty acid biosynthesis. Two isoforms of ACC exist in mammals, ACC1 and ACC2, and both enzymes function to carboxylate acetyl-CoA to form malonyl-CoA (3–5). Mammalian ACCs are dimeric, multifunctional enzymes composed of four distinct domains: a biotin carboxylase (BC) domain, a biotin carboxyl carrier protein (BCCP) domain, the ACC central region, and a carboxyltransferase (CT) domain. Structures of the isolated BC (6–8), BCCP (9), and CT (10, 11) domains from various organisms show that the BC domain exists in both monomeric and dimeric forms, the BCCP domain is a monomer, and the CT domain exists as a dimer. The regulation of ACCs occurs at multiple levels: transcriptional regulation via sterol regulatory element binding protein (SREBP), liver X receptor, and carbohydrate responsive element binding protein (ChREBP) (12–14), phosphorylation/dephosphorylation (15, 16), and allosteric activation by citrate, which induces polymerization and increases the activity of ACC (1).

Recently, we reported the discovery that MIG12, a 183 amino acid protein, binds to ACC1 and ACC2, which induces polymerization and subsequently increases the enzymatic activity of the protein (17). The overexpression of MIG12 in mice resulted in high rates of fatty acid synthesis in liver and in the development of hepatic steatosis (17). Thus, the regulation of ACC by MIG12 can have important physiological consequences.

The only protein in the GenBank database that shares significant homology with MIG12 is a protein designated Spot 14 (S14).

S14 is a 150 amino acid protein that was initially discovered by Oppenheimer and colleagues (18) in a screen for proteins that were regulated by thyroid hormone in rat livers. Subsequent studies revealed that S14 is highly expressed in tissues with high rates of lipogenesis (19), and the gene is highly regulated at the transcriptional level by SREBP-1c (12, 20) and ChREBP (21), transcription factors that activate all genes involved in fatty acid synthesis (22). Although the molecular function of S14 is unknown, S14 knockout mice have reduced fatty acid synthesis in lactating mammary glands (23) but increased lipid synthesis in liver (24). However, in cultured rat primary hepatocytes, knockdown of both S14 and MIG12 using RNAi reduced fatty acid synthesis by ~65% (25).

Although S14 and MIG12 share 32% identity, S14 does not bind and activate ACC (17), and the biochemical function of S14 remains unknown. In the current study, we have determined the crystal structure of mouse S14 to 2.65-Å resolution. The structure of S14 reveals a helical protein arranged as a symmetric dimer. Cultured cell experiments indicate that S14 can form heterodimers with MIG12, suggesting a mechanism through which S14 could modulate ACC activity and subsequently rates of fatty acid synthesis via heterodimer formation with MIG12.

Results

A recombinant form of mouse S14, spanning residues 1–150, was used for structural studies. S14 exists as a dimer in solution according to size exclusion chromatography and analytical ultracentrifugation (*SI Text, Fig. S1*). Recombinant S14 was unable to activate or induce polymerization of ACC1 (17).

Structure of S14. The structure of S14 was determined by single wavelength anomalous diffraction phasing and refined to 2.65 Å (Table 1). The model spans residues 8–149, although residues 46–48 and 77–104 are poorly ordered and were not placed in the electron density. S14 crystallized in the space group *I*432 with a single monomer in the asymmetric unit. The biologically relevant dimer can be constructed using crystallographic symmetry.

The main body of the S14 monomer is composed of three antiparallel α -helices (Fig. 1A). Helices 1 and 2 are ~30 Å in length and helix 3 is ~60 Å in length. The increased length of helix 3 allows it to interact with an adjacent monomer. There-

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The authors declare no conflict of interest.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID code 3ONT).

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Table 1. Data collection and model refinement statistics

Data collection statistics	
Space group	I432
<i>a</i> , <i>b</i> , <i>c</i> , Å	144.9
Resolution, Å (final shell)	50–2.64 (2.69–2.64)
Reflections	
Total	261,643
Unique	7919
Completeness, %	99.8 (100.0)
Average multiplicity	9.1 (7.5)
<i>R</i> _{sym} *, %	6.3 (55.2)
Model refinement statistics	
Resolution, Å	39–2.65
No. of reflections	7512
<i>R</i> _{work} , %	22.3
<i>R</i> _{free} , %	24.9
rms deviations from target values	
Bond lengths, Å	0.013
Bond angles, °	1.39

Values in parentheses pertain to the outermost shell of data.

$$*R_{\text{sym}} = \sum_{h,i} |I_{h,i} - \langle I_h \rangle| / \sum_{h,i} I_{h,i}$$

by, an antiparallel 4-helix bundle is created by packing the C terminus of helix 3 against the main body of the other monomer to form the homodimer (Fig. 1*B*). The S14 dimer is $\sim 25 \times 30 \times 75$ Å with the monomers related by 2-fold symmetry. The total surface area buried at the dimer interface is $\sim 2,650$ Å² with helix 3 burying $\sim 1,400$ Å² through its interaction with the neighboring monomer (Fig. 2*A*).

Sequence Conservation Between S14 and MIG12. Analysis of the sequence conservation between S14 and MIG12 revealed that the dimer interface of S14 is highly conserved between S14 and MIG12, whereas two regions of lowest conservation, termed box A and B, are not involved in dimerization (Fig. 2*B* and *C*).

Interaction of S14 and MIG12 in Cultured Cells. To determine whether S14 directly interacted with MIG12, the proteins were overexpressed in Chinese hamster ovary-K1 (CHO-K1) cells (Fig. 3). In one set of experiments, S14 contained a FLAG tag and in the second set of experiments MIG12 contained a FLAG tag. Cell lysates were incubated with an anti-FLAG antibody to precipitate either S14 or MIG12, and supernatants and pellets were subjected to SDS-PAGE. S14, MIG12, and ACC1 were visualized by immunoblot analysis using antibodies that recognize ACC1,

S14, and MIG12 as described in the *Materials and Methods*. As shown in Fig. 3, both S14 and MIG12 were able to coimmunoprecipitate each other as well as ACC1, although the efficiency of ACC1 coimmunoprecipitation with S14 was markedly weaker than with MIG12 and likely represents S14's interaction with the MIG12:ACC complex.

To confirm that a direct interaction between S14 and MIG12 exists, we analyzed the interaction of S14 and MIG12 by Blue Native PAGE (Fig. 4*A*). Overexpression of either S14 or MIG12 alone in CHO-K1 cells resulted in the formation of homodimers of the overexpressed protein; however, the coexpression of MIG12 and S14 resulted in the formation of heterodimers in the cell (Fig. 4*A*). To determine whether the quantity of S14 changed the relative amounts of MIG12 that heterodimerized with S14, CHO-K1 cells were transfected with MIG12 and increasing amounts of S14 and cell lysate proteins were separated by Blue Native PAGE for immunoblot analysis. As shown in Fig. 4*B*, MIG12 incorporation into heterodimer complexes increased as a function of S14 concentration.

S14 Reduces the Activity of MIG12 in Cultured Cells. To determine if S14 altered the function of MIG12, we monitored the polymerization and ACC activity in the presence of MIG12 and increasing amounts of S14 (Fig. 5*A* and *C*). Increasing S14 levels resulted in reduced ACC polymerization (Fig. 5*A*) and less MIG12 incorporation into ACC polymers (Fig. 5*B*). Consistent with the reduced polymerization of ACC, the activation of partially purified ACC also decreased with increasing levels of S14 (Fig. 5*C*).

S14 Knockdown Increases ACC Polymerization and Activity in Vivo. To determine whether S14 participates in the regulation of ACC in vivo, we knocked down S14 in livers of living mice by injecting S14 oligos or S14 oligos containing a mismatch (MM) formulated into lipidoid nanoparticles (Fig. 6). One week after injection of S14 siRNA into mice, livers were harvested using a freeze clamp technique. Cytosolic proteins prepared from livers were analyzed by Blue Native PAGE, and immunoblot analysis was carried out for S14 and MIG12. The siRNA directed against S14 reduced the mRNA levels of S14 by $\sim 90\%$ and the S14 protein was below the detection limits of the antibody (Fig. 6*A*). In mice injected with a control MM siRNA, MIG12 was present primarily as a heterodimer with S14. In contrast, most S14 normally is present as a homodimer based on the sizes of the complexes in the Blue Native PAGE. Reduction of S14 by siRNA knockdown resulted in the formation of MIG12 homodimers (Fig. S2), which resulted in increased MIG12-induced ACC1 polymerization and activity (Fig. 6*B* and *C*). In a separate study, male mice were injected with the S14 siRNA and after 5 d they were injected with tritiated water to measure in vivo rates of hepatic fatty synthesis. As shown in Fig. 6*D*, the knockdown of S14 resulted in a 30% reduction in rates of hepatic fatty acid synthesis.

Discussion

The structure of S14 reveals that it exists as a homodimer, and analysis of the residues involved in dimerization reveals that the dimer interface is conserved or highly similar between S14 and MIG12 (Fig. 2*C*). Helix 3 is unable to dimerize in isolation, and dimerization requires helix 3 from one monomer and helix 1 and 2 from another (26, 27). This is consistent with the crystal structure of S14 where the C terminus of helix 3 packs against helix 1 and 2 of the other monomer and is the major region of interaction within the dimer. Sequence conservation between S14 and MIG12 is highest across helix 3 (50% overall identity and 57% identity for residues at the dimer interface). Nonconserved residues in MIG12 at the dimer interface either pack against conserved residues or are solvent accessible (Fig. 2*B*). Conservation of the dimer interface in S14 and MIG12 suggested that S14 and MIG12 form heterodimers. The coimmunoprecipitation and

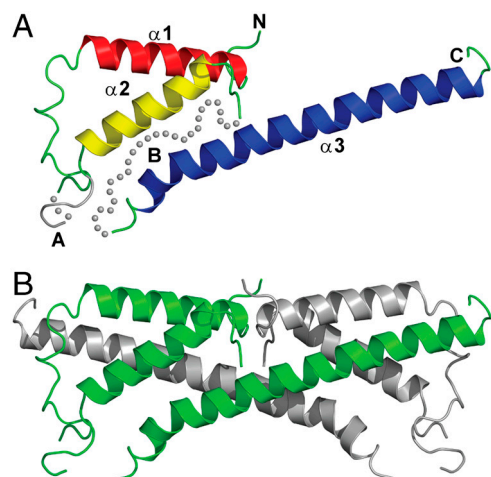


Fig. 1. Structure of S14. (A) The structure of the S14 monomer reveals three antiparallel α -helices. The regions of lowest homology to MIG12 (box A and B) are shown in gray. Disordered regions in the structure are indicated with spheres. (B) The S14 homodimer creates two antiparallel 4-helix bundles in a symmetric arrangement. Each monomer is colored either green or gray.

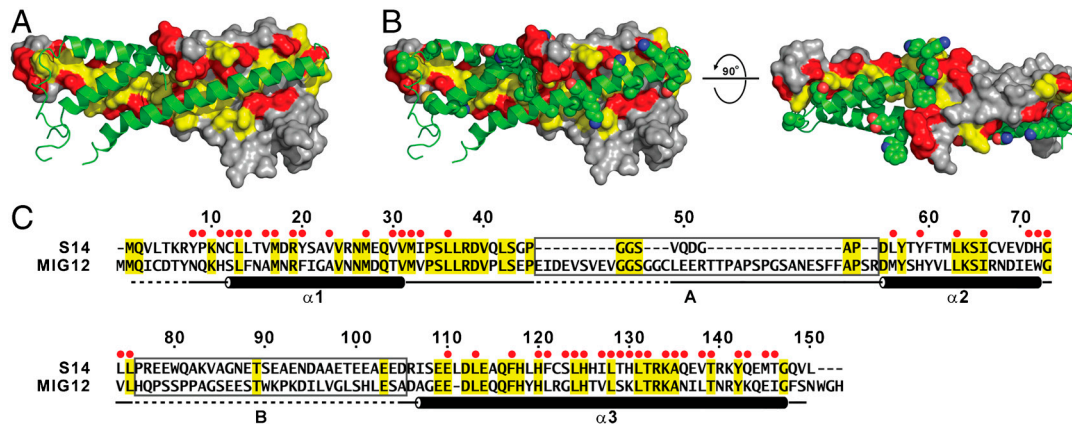


Fig. 2. The S14 dimer interface. (A) The buried surface of a S14 monomer within the S14 homodimer is shown in red (nonconserved residues) and yellow (conserved residues). (B) Residues at the dimer interface of a S14:MIG12 heterodimer. S14 is shown in surface representation as in A. The nonconserved residues at the dimer interface, changed to the corresponding residues of MIG12 (green, shown as spheres), interact with conserved regions of S14 or are surface exposed and do not create unfavorable interactions or steric clashes. (C) Sequence alignment of S14 and MIG12. Conserved residues are shaded yellow, red circles denote residues at the dimer interface, and the regions of lowest homology are boxed. The secondary structure of S14 is shown below the sequence.

Blue Native PAGE studies presented here demonstrate that S14 can heterodimerize with MIG12. We have previously shown that recombinant MIG12 exists predominantly as a homodimer in solution (17) and that preformed homodimers of S14 and MIG12 are unable to associate (preliminary observations); therefore, it is likely that heterodimer formation must precede homodimerization of either S14 or MIG12.

S14 knockdown studies performed in mice show that S14 normally exists both as a S14 homodimer and as a heterodimer with MIG12. The majority of MIG12 exists as a heterodimer with S14 whereas the preponderance of S14 exists as a homodimer in mouse liver (Fig. S2). We have measured approximate concentra-

tions of S14 and MIG12 in mouse liver using standard curves with known amounts of purified recombinant S14 and MIG12. The S14 protein concentrations in mouse liver appear to be ~10-fold higher than that of MIG12, and most MIG12 in liver normally exists as a heterodimer with S14 (preliminary observation). Heterodimer formation between S14 and MIG12 reduces MIG12's interaction with ACC, decreasing polymerization and activation of ACC, which adds another level of complexity to the regulation of ACC. Additional studies with recombinant S14:MIG12 heterodimers will be required to determine the extent to which they bind and/or change the activity of ACC.

The interaction of MIG12 with ACC1 and ACC2 is likely mediated in part by residues that are conserved between S14 and MIG12 and does not include the regions of lowest homology (box A and B, Fig. 2C). A limited number of amino acid substitutions might suffice to convert S14 into an ACC1 binding protein.

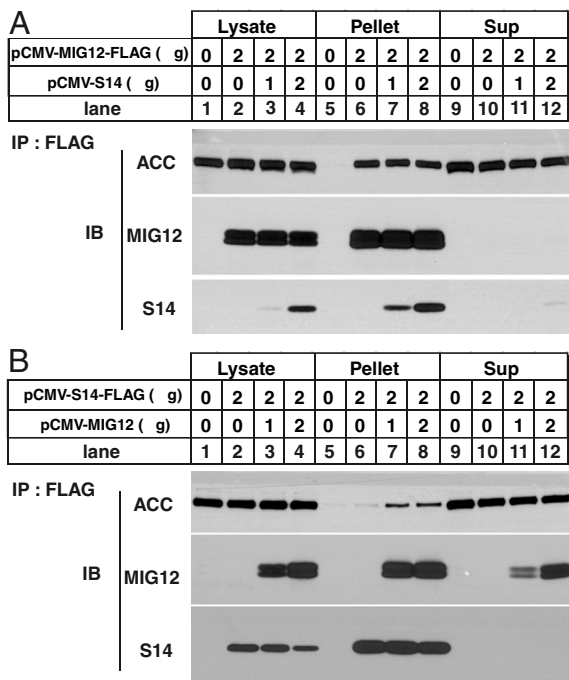


Fig. 3. Interaction of S14 and MIG12. CHO-K1 cells were transfected with the indicated amount of S14 and MIG12. (A) pCMV-FLAG-MIG12 and pCMV-S14 were cotransfected into CHO-K1 cells. After 24 h, cells were harvested and MIG12 was immunoprecipitated using an anti-FLAG antibody. Proteins from the whole cell lysate, pellet, and supernatant were separated by SDS-PAGE and analyzed by immunoblot using antibodies against mouse MIG12, S14, or ACC1 as described in *Materials and Methods*. (B) pCMV-FLAG-S14 and pCMV-MIG12 were cotransfected and analyzed as in A.

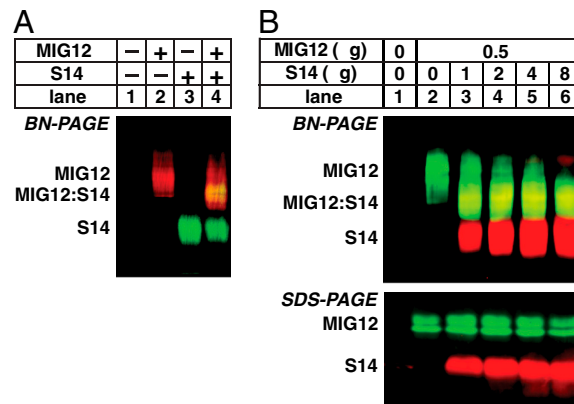


Fig. 4. S14 and MIG12 form heterodimers. (A) CHO-K1 cells were transfected with pCMV-MIG12 (1 μ g) or pCMV-S14 (5 μ g) alone, or with both plasmids. After 24 h, cells were harvested and cell lysates were prepared and separated on Blue Native PAGE (BN-PAGE) for analysis by immunoblot using rabbit polyclonal anti-MIG12 and mouse monoclonal anti-S14 antibodies. IRDye800-conjugated anti-mouse antibody and IRDye680-conjugated anti-rabbit antibody were used as the secondary antibodies. Immunoreactive bands were visualized using the LI-COR Odyssey infrared imaging system (MIG12, red; S14, green; S14:MIG12 heterodimer, yellow). (B) CHO-K1 cells were cotransfected with pCMV-MIG12 (0.5 μ g) and the indicated amounts of pCMV-S14. After 24 h, cells were harvested and cell lysates were separated on BN-PAGE or SDS-PAGE and analyzed by immunoblot analysis as described in A. IRDye800-conjugated anti-rabbit antibody and IRDye680-conjugated anti-mouse antibody were used as the secondary antibodies (MIG12, green; S14, red; S14:MIG12 heterodimer, yellow).

heterodimers will be required to understand the regulation of ACCs by these proteins.

Materials and Methods

Materials. [^{14}C]KHCO₃ (50 mCi/mmol) was obtained from American Radiolabeled Chemicals Inc. PEG 8000, acetyl-CoA sodium salt, ATP, Igepal CA-630, and KHCO₃ were purchased from Sigma.

Purification of S14. Mouse S14 was expressed as a GST fusion in BL21 cells (Novagen) and purified by glutathione affinity chromatography. The GST tag was removed by tobacco etch virus protease cleavage, and S14 was further purified by ion exchange followed by size exclusion chromatography. Selenomethionine containing S14 was produced in BL21 cells (29) and purified under reducing conditions.

Crystallization and Structure Determination. Crystals of S14 were formed by mixing equal volumes of S14 (10 mg/mL in 10 mM Tris pH 7.5, 50 mM NaCl, 0.01% (wt/vol) Na₂S₂O₅, 1 mM DTT) with 100 mM MES pH 6.0, 15 mM MgCl₂, 5% (wt/vol) PEG 3000, and 30% (vol/vol) PEG 200. Cubic crystals, belonging to the space group I432 with unit cell dimensions of $a = b = c = 144.9 \text{ \AA}$, suitable for diffraction experiments, grew to dimensions of $\sim 0.15 \text{ mm} \times 0.15 \text{ mm} \times 0.10 \text{ mm}$ within 3 d. Selenomethionine S14 crystals were grown using similar conditions.

Crystals were transferred through a solution containing 100 mM MES pH 6.0, 15 mM MgCl₂, 5% (wt/vol) PEG 3000, 35% (vol/vol) PEG 200, and 20% (vol/vol) glycerol before being flash-frozen in liquid nitrogen prior to data collection at cryogenic temperature ($\sim 100 \text{ K}$). A two-wavelength anomalous diffraction experiment was performed on a selenomethionine derivatized crystal at the Advanced Photon Source (Argonne National Laboratory) synchrotron at the Structural Biology Center beamline 19ID. For structure solution, diffraction intensities, collected at the high-energy remote wavelength (0.9537 \AA) to 2.65- \AA resolution, were used in HKL3000 (30) to perform automated structure solution and initial model building.

Model Building and Refinement. Once the initial model was obtained, the model was further improved by iterations of manual model building using the program O (31) followed by refinement against a maximum likelihood target function using REFMAC5 (32). Finally, ordered solvent molecules were added to the model and the model was refined using translation libration screw-motion parameters to obtain a final $R = 22.3\%$ and $R_{\text{free}} = 24.9\%$. Additional model statistics are shown in Table 1. The refined model and structure factor amplitudes are available in the Protein Data Bank (PDB ID code 3ONT).

Immunoprecipitation. CHO K-1 cells (ATCC CCL-61) were plated at a density of $7 \times 10^5/10\text{-cm}$ plate in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 5% fetal calf serum, 100 units/mL penicillin G, and 100 $\mu\text{g/mL}$ streptomycin sulfate on day 0. On day 2, cells were transfected with various amounts of the indicated plasmids using Eugene 6 transfection reagent (Roche). On day 3, cells were rinsed with ice cold PBS and lysed with 20 mM Hepes pH 7.6, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, protease inhibitors (Roche), and phosphatase inhibitors (Roche). The crude cell lysate was centrifuged at $19,000 \times g$ for 20 min, and the supernatant was preincubated with 50 μL of Protein A/G agarose beads (Santa Cruz Biotechnology) for 30 min. The cleared supernatant was mixed with 50 μL of anti-FLAG M2 agarose beads (Sigma) and incubated with rotation for 2 h at 4°C. The protein bound beads were washed 5 times with ice cold PBS supplemented with 0.05% Tween-20 and eluted with 200 μL of 100 $\mu\text{g/mL}$ of FLAG peptide (Sigma) in PBS. The eluted proteins were subjected to SDS-PAGE, and immunoblot analysis was performed using anti-mouse MIG12 (17) or S14 (Roche) antibodies.

Partial Purification of ACC from Mouse Liver and CHO-K1 Cells. ACC was purified from mouse liver as described previously (17) with slight modifications. Mice were anesthetized by injecting pentobarbital (80 mg/kg), and livers were harvested by the freeze clamp method. Liver slices (0.2 g) were homogenized in 1 mL of 20 mM Hepes pH 7.6, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 150 mM NaF, 5 μM Compound C, protease inhibitors, and phos-

phatase inhibitors (Roche). The homogenate was centrifuged at $3,500 \times g$ for 10 min, and the supernatant was re-centrifuged at $100,000 \times g$ for 45 min. Proteins in the supernatant were precipitated in 2.5% PEG 8000 at $10,000 \times g$ for 15 min followed by a second round of precipitation in 5.5% PEG 8000. The pellet was washed once with distilled water and dissolved in 20 mM Hepes pH 7.6, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 50 mM NaF, 5 μM Compound C, and protease inhibitors.

For CHO-K1 cells, cells were washed once with 10 mL of ice cold PBS and resuspended in 400 μL of 20 mM Hepes pH 7.6, 250 mM sucrose, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.8 mg/mL digitonin, 150 mM NaF, 5 μM Compound C, protease inhibitors, and phosphatase inhibitors. Cells were lysed by passage through a 27-gauge needle and centrifuged at $19,000 \times g$ for 20 min. Proteins in the supernatant were precipitated in 5.5% PEG 8000 at $19,000 \times g$ for 5 min. The pellet was washed once with distilled water and dissolved in 20 mM Hepes pH 7.6, 250 mM sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM DTT, 50 mM NaF, 5 μM Compound C, and protease inhibitors.

In Vitro ACC Activity Assay. ACC activity was measured as described previously (17).

In Vivo RNAi in Mice. siRNA oligos against mouse S14 were designed and tested for activity in cultured primary hepatocytes as described (33). The most active oligos and one containing a nucleotide mismatch for a control were synthesized and formulated into lipidoid nanoparticles as described (33) and delivered via tail vein injection (5 mg/kg body weight) into four C57BL/6J. Mice were fed a fat-free/high carbohydrate diet from the day of injection.

In Vivo Fatty Acid Synthesis in Mice. MM or S14 siRNA in lipidoid formulations were delivered via tail vein injection into 129S6/SvEv male mice at a dose of 5 mg/kg (7 mice for MM, 10 mice for S14 siRNA). Five days after siRNA administration, mice were injected intraperitoneally with ^3H -labeled water (50 mCi), and rates of hepatic fatty acid synthesis were determined as described (34).

Analysis of ACC Polymerization and Dimerization of S14 and MIG12 Using Blue Native Gels. Blue Native gels were prepared as described (17). Cytosolic proteins from mouse liver or CHO-K1 cells were separated using two forms of nondenaturing Blue Native PAGE: 3.5–10% for analysis of ACC and 14% for analysis of S14 and MIG12. Proteins were transferred to 0.45 μM PVDF membrane (GE Healthcare Life Sciences). Coomassie G-250 was removed from the membranes by sequential washing with methanol, water, and PBST. The membranes were incubated with blocking solution containing 5% non-fat dried milk and 5% newborn calf serum in PBST or LI-COR blocking buffer for 30 min. Immunoblot analyses were performed using rabbit polyclonal antibodies against rat ACC1, mouse MIG12 (17), and a mouse monoclonal antibody against S14 (Roche). Horseradish peroxidase linked anti-rabbit IgG (GE Healthcare) and IRDye 800-conjugated anti-rabbit antibody (LI-COR) were used as secondary antibodies to visualize ACC or MIG12. To detect S14, the membrane bound with primary antibody was incubated with biotin conjugated anti-mouse antibody (PIERCE) and then IRDye 680-conjugated streptavidin (LI-COR). Antibody bound bands were detected by Supersignal substrate system (Pierce) or LI-COR Odyssey infrared imaging system as described previously (17).

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