Control of lipid metabolism by phosphorylation-dependent degradation of the SREBP family of transcription factors by SCF^{Fbw7}

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Summary

The sterol regulatory element binding protein (SREBP) family of transcription factors controls cholesterol and lipid metabolism. The nuclear forms of these proteins are rapidly degraded by the ubiquitin-proteasome pathway, but the signals and factors required for this are unknown. Here, we identify a phosphodegron in SREBP1a that serves as a recognition motif for the SCF^{Fbw7} ubiquitin ligase. Fbw7 interacts with nuclear SREBP1a and enhances its ubiquitination and degradation in a manner dependent on the phosphorylation of T426 and S430 by GSK-3. Fbw7 also degrades nuclear SREBP1c and SREBP2, and inactivation of endogenous Fbw7 results in stabilization of nuclear SREBP1 and -2, enhanced expression of SREBP target genes, enhanced synthesis of cholesterol and fatty acids, and enhanced receptor-mediated uptake of LDL. Thus, our results suggest that Fbw7 may be a major regulator of lipid metabolism through control of the phosphorylation-dependent degradation of the SREBP family of transcription factors.

Introduction

Members of the sterol regulatory element binding protein (SREBP) family of transcription factors control cholesterol and lipid metabolism and play critical roles during adipocyte differentiation (Brown and Goldstein, 1999; Osborne, 2000). In addition, SREBP1c is an important regulator of insulin-dependent gene expression (Foretz et al., 1999; Shimomura et al., 1998). Two genes, *srebf1* and *srebf2*, encode three different SREBP proteins, SREBP1a, SREBP1c, and SREBP2 (Edwards et al., 2000; Osborne, 2000). SREBPs are synthesized as large precursor proteins that are inserted into the nuclear and endoplasmic reticulum membranes and are transcriptionally inactive. In sterol-depleted cells, SREBP cleavage-activating protein (SCAP) escorts SREBP to the Golgi apparatus where SREBP is processed sequentially by two membrane-associated proteases that release the NH2-terminal domain (Rawson et al., 1997; Sakai et al., 1998). This transcriptionally active fragment of SREBP is translocated to the nucleus and binds to the promoters of SREBP target genes. Most of these genes are involved in the control of lipid biosynthesis and metabolism. In sterol-loaded cells, the SCAP/SREBP complex is trapped in the ER as a result of sterol-induced binding of SCAP to Insigs, which are resident proteins of the ER membrane (Yang et al., 2002). Therefore, the cleavage of SREBP is prevented and transcription of the lipid biosynthetic target genes declines. Many transcription factors, particularly those involved in the control of cell growth, are unstable proteins targeted for degradation by the ubiquitin-proteasome system (Conaway et al., 2002; Muratani and Tansey, 2003). The nuclear, transcriptionally active fragments of SREBPs are unstable and degraded by the proteasome system in a ubiquitination-dependent manner (Hirano et al., 2001). We have previously demonstrated that the ubiquitination and degradation of SREBPs can be regulated by coactivator-mediated acetylation (Giandomenico et al., 2003). In addition, it has been suggested that nuclear SREBPs are, at least in part, ubiquitinated and degraded as a functional consequence of their transcriptional activity (Sundqvist and Ericsson, 2003). The factors regulating the ubiquitination and degradation of SREBPs have, however, not been identified.

Polyubiquitination is the rate-limiting step in protein degradation and involves a three-step cascade of ubiquitin-transfer reactions (Glickman and Ciechanover, 2002). First, the ubiquitin-activating enzyme (E1) activates ubiquitin and transfers it to the ubiquitin-conjugating enzyme (E2). The E2 acts together with a specificity-determining component, E3, to transfer ubiquitin to the target protein. E3s are adaptors that link the ubiquitination machinery of the E2 to specific substrates. The largest and most versatile class of E3s is the Skp1-Cul1-F box protein ubiquitin ligases (SCFs). SCFs are modular complexes that consist in mammalian cells of Skp1, Cul1, Rbx1, and one of a family of F box proteins (Cardozo and Pagano, 2004; Jin et al., 2004). Distinct regions of the F box protein confer the ability to bind substrates, whereas the F box motif docks with Skp1, which in turn binds to the Cullin scaffold. Many F box proteins recognize and bind phosphorylated Ser and Thr residues in substrate proteins. In Fbw7 (also known as hCdc4), WD40 repeats mediate recognition of phosphorylated substrates, and Fbw7 targets phosphorylated cyclin E, c-Myc, and c-Jun for proteolysis (Koepp et al., 2001; Nateri et al., 2004; Strohmaier et al., 2001; Welcker et al., 2004; Yada et al., 2004). Fbw7 is

mutated in breast, endometrial, ovarian, and colon cancer and functions as a tumor suppressor whose loss causes genetic instability (Ekholm-Reed et al., 2004; Rajagopalan et al., 2004; Spruck et al., 2002; Strohmaier et al., 2001). Deletion of Fbw7 in the mouse causes embryonic lethality with defects in cardiovascular development and increased cyclin E and Notch protein in the embryo and placenta (Tetzlaff et al., 2004; Tsunematsu et al., 2004). In the current study, we identify two residues, T426 and S430, in SREBP1a that are phosphorylated in the nuclear form of the protein. Mutation of either of these residues greatly stabilizes nuclear SREBP1a. The sequence surrounding T426 and S430 conforms to the phospho binding motif for Fbw7, termed the Cdc4 phosphodegron, or CPD, found in cyclin E and c-Myc. We show that Fbw7 interacts with nuclear SREBP1a and regulates its ubiquitination and degradation in a manner dependent on the phosphorylation of T426 and S430. Consequently, expression of Fbw7 inhibits SREBP-dependent transcription, and this effect is dependent on T426 and S430. Fbw7 also degrades nuclear SREBP1c and SREBP2, suggesting that Fbw7 controls the stability of all members of the SREBP family of transcription factors. Furthermore, inactivation of endogenous Fbw7 results in stabilization of nuclear SREBP1 and -2, enhanced expression of endogenous SREBP target genes, enhanced synthesis of cholesterol and fatty acids, and enhanced uptake of LDL. Thus, our results indicate that Fbw7 is a major regulator of lipid metabolism by controlling the phosphorylation-dependent ubiquitination and degradation of the SREBP family of transcription factors.

Results

SCF^{Fbw7} negatively regulates the stability and function of SREBP

Many proteins regulated by ubiquitin-mediated degradation are recognized by E3 ligases of the multisubunit RING finger type. Most of these complexes contain a Cullin (Cul) protein, which functions as a molecular scaffold and interacts with both the adaptor subunit and the RING finger protein. Importantly, different adaptor proteins interact with specific Cul proteins. Since Cullins recruit specific families of E3 ligases, dominant-negative (DN) Cullins can be used to identify which family of ligases that interact with a specific substrate. If the expression of a specific DN Cullin increases the steady-state abundance of a cotransfected protein, this indicates that the E3 belongs to the family of ligases that interact with that specific Cullin. To identify the E3 complex that regulates the stability of nuclear SREBP1a, Myc-SREBP1a was expressed in 293T cells, either alone or together with DN Cul1-5. As illustrated in Figure 1A, the levels of SREBP1a were low in the transfected 293T cells and specifically enhanced in cells expressing DN-Cul1 (compare lanes 1 and 2 in Figure 1A), indicating that the degradation of SREBP1a is regulated by a SCF complex. Similarly, we found that SREBP1a was specifically stabilized in cells expressing DN-Ubc3/hCdc34 (Figure S1), an E2 ligase often associated with SCF complexes. To analyze if SCF complexes regulated the function of endogenous SREBP, U2OS cells were transfected with a SREBP-responsive promoter reporter gene in the absence or presence of DN Cul1-5 (Figure 1B). As a result of sterol-regulated processing of endogenous SREBP, transcription from the promoter reporter gene was low in cells incubated in the presence of sterols and induced in cells incubated in the absence of sterols. Importantly, expression of DN-Cul1 enhanced transcription from the promoter reporter gene, indicating that endogenous SREBPs are regulated by a Cul1-containing SCF complex.

There are two main families of F box proteins: the Fbw family, containing WD40 repeats, and the Fbl family, containing leucine-rich repeats. To test if the stability of SREBP1a was regulated by any of these proteins, nuclear SREBP1a was expressed in 293T cells, either alone or in the presence of individual members of these families of F box proteins. As illustrated in Figure 1C, coexpression of the F box protein Fbw7 decreased the levels of SREBP1a. In contrast, the other F box proteins did not decrease the expression of SREBP1a, and some even increased the expression of SREBP1a. Fbw7 also induced the degradation of nuclear SREBP1c and SREBP2 (Figure S1), suggesting that Fbw7 regulates the stability of all members of the SREBP family of proteins. Expression of an Fbw7 protein containing a mutation in its F box or expression of the WD40 domain alone did not induce the degradation of SREBP1a, indicating that both domains are important for Fbw7-mediated degradation (Figure 1D). Rather, the nonfunctional Fbw7 proteins worked in a DN manner, stabilizing SREBP1a (compare lanes 1, 3, and 4 in Figure 1D). The Fbw7-mediated degradation of SREBP1a was inhibited by the proteasome inhibitor MG-132 (Figure 1E), indicating that the process is dependent on the ubiquitin-proteasome system. There are three different isoforms of Fbw7: α, β, and γ. Both the α and γ isoforms are localized in the nucleus, whereas the β isoform is cytoplasmic. To determine if the different isoforms of Fbw7 have different specificity for SREBP, 293T cells were transfected with nuclear SREBP1a either alone or in the presence of increasing amounts of the individual isoforms of Fbw7. The α and γ isoforms of Fbw7 greatly enhanced the degradation of SREBP1a, while coexpression of the β isoform had a significantly lower effect, if any, on the turnover of SREBP1 (Figure 1F). Expression of Fbw7α and -γ inhibited the expression of a SREBP-dependent promoter reporter gene, whereas Fbw7β and the nonfunctional mutants of Fbw7α were unable to do so (Figure 1G), supporting the hypothesis that Fbw7α and -γ regulate the stability and function of endogenous SREBPs. Together, these results demonstrate that the F box protein Fbw7 regulates the turnover and function of nuclear SREBP1a, -1c, and -2.

Fbw7-mediated degradation of SREBP1a is dependent on T426 and S430

The sequence surrounding T426 and S430 in SREBP1a conforms to the CPD found in cyclin E and c-Myc (Figure 2A), suggesting that this region of SREBP1a contains an Fbw7 binding motif. Mutation of T426, S430, or both residues to alanine in the nuclear form of SREBP1a drastically enhanced the steady-state levels of the protein when expressed in 293T cells (Figure 2B), suggesting that both T426 and S430 are required for efficient degradation of nuclear SREBP1a. This hypothesis was supported by our observation that mutation of T426 and S430, either individually or in combination, blocked Fbw7-mediated degradation of SREBP1a (Figure 2C). To confirm that the reduced abundance of SREBP1a in response to Fbw7 expression resulted from increased proteolysis, we measured the half-life of nuclear SREBP1a, either wild-type or the T426A/S430A mutant, in the absence or presence of coexpressed

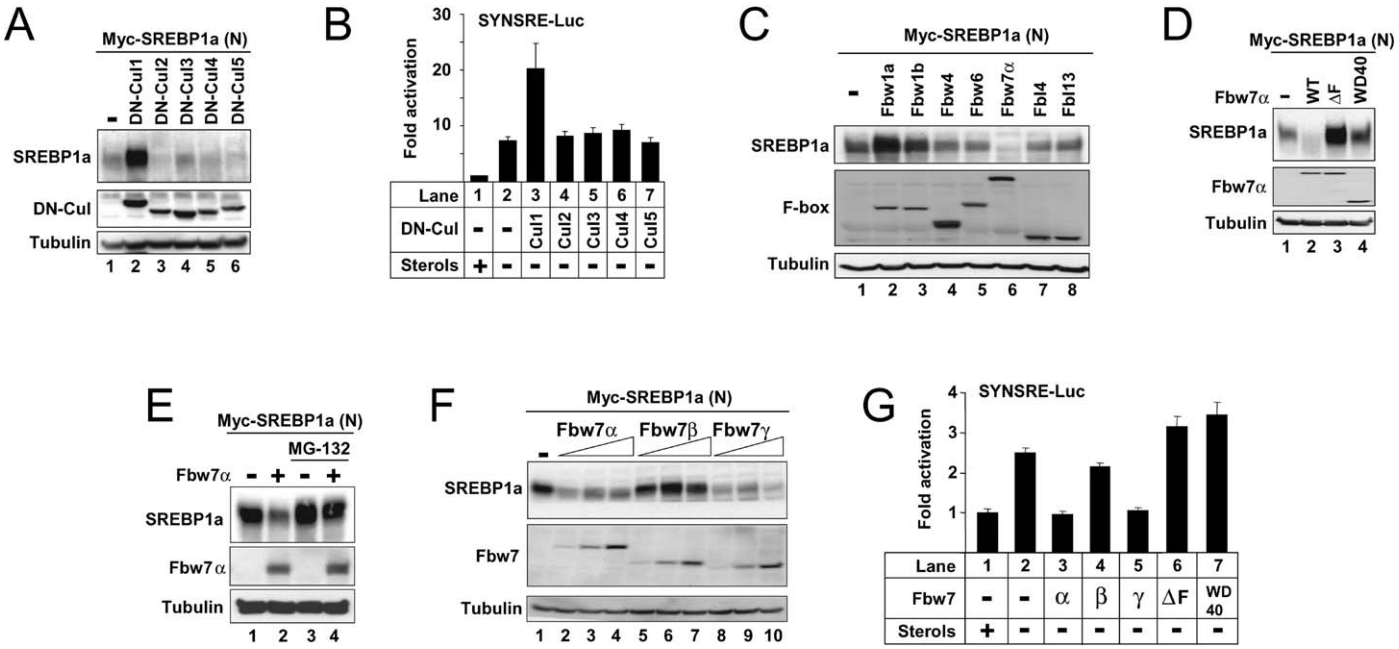

Figure 1. SCF^{Fbw7} negatively regulates the stability and function of SREBP1a

A) 293T cells were transfected with a vector expressing Myc-tagged nuclear (N) SREBP1a in the absence or presence of the indicated Flag-tagged DN-Cullins. The levels of SREBP1a, DN-Cullins, and α-tubulin in cell lysates were determined by Western blotting.

B) U2OS cells were transfected with SYNSRE-luc in the absence or presence of the indicated DN-Cullins, and the cells were grown in the absence or presence of sterols. Thirty-six hours after transfection, the luciferase activity was measured.

C) 293T cells were transfected with Myc-SREBP1a in the absence or presence of the indicated GST-tagged F box proteins. The levels of SREBP1a, F box proteins, and α-tubulin in cell lysates were detected by Western blotting.

D) 293T cells were transfected with Myc-SREBP1a in the absence or presence of Flag-Fbw7α, either wild-type or the indicated mutants. The levels of SREBP1a, Fbw7α, and α-tubulin in cell lysates were detected by Western blotting.

E) 293T cells were transfected with Myc-SREBP1a in the absence or presence of GST-Fbw7α and treated with vehicle alone (DMSO) or MG-132 (50 μM) for 6 hr prior to lysis. The levels of SREBP1a, Fbw7α, and α-tubulin in cell lysates were detected by Western blotting.

F) 293T cells were transfected with Myc-SREBP1a in the absence or presence of increasing amounts of GST-tagged Fbw7α, -β, or -γ. The levels of SREBP1a, Fbw7, and α-tubulin in cell lysates were detected by Western blotting.

G) 293T cells were transfected with SYNSRE-luc in the absence or presence of Fbw7α, -β, and -γ or the indicated mutants of Fbw7α, and the cells were grown in the absence or presence of sterols. Thirty-six hours after transfection, the luciferase activity was measured.

Fbw7. Expression of Fbw7 enhanced the turnover of wild-type SREBP1a, whereas the T426A/S430A mutant was stable and insensitive to Fbw7 expression (Figure 2D). The transcriptional activities of the T426A, S430A, and T426A/S430A mutants were enhanced compared to the wild-type protein and were insensitive to Fbw7 expression (Figure 2E), further supporting the role of T426 and S430 in regulating the stability and function of SREBP1a. Our data predict that Fbw7 functions to recruit the SCF complex to nuclear SREBP1 and promote its ubiquitination. To test this idea, we determined whether Fbw7 could induce the ubiquitination of nuclear SREBP1a when the two proteins were expressed in 293T cells. As illustrated in Figure 2F, expression of Fbw7α stimulated the ubiquitination of wild-type SREBP1a, whereas it was unable to enhance the ubiquitination of the T426A/S430A mutant.

SREBPs interact with Fbw7 in a phosphorylation-dependent manner

To determine whether Fbw7 and SREBP1a associate in vivo, we immunoprecipitated Fbw7 from cells expressing nuclear SREBP1a and Fbw7. The cells were also transfected with DN-Cul1 to prevent Fbw7-dependent degradation of SREBP1a. Wild-type SREBP1a and Fbw7α interacted efficiently, whereas mutation of T426 or S430 in SREBP1a blocked its interaction with Fbw7α (Figure 3A). Fbw7α also interacted with SREBP1c and SREBP2 (Figure S2). T426 and S430 correspond to S432 and S436 in SREBP2 (Figure 2A). Interestingly, the interaction between SREBP2 and Fbw7, as well as Fbw7-mediated degradation of SREBP2, was lost when S432 or S436 were mutated (Figure S2). The interactions between Fbw7 and c-Myc and cyclin E are dependent on phosphorylation of conserved Thr and Ser residues in the CPD motifs of these proteins. To determine if T426 or S430 in SREBP1a was phosphorylated in vivo, we generated phosphorylation-specific antibodies directed against these residues. When expressed in 293T cells, nuclear SREBP1a was recognized by both antibodies in a phosphorylation-dependent manner (Figure S3), indicating that both residues are phosphorylated in vivo. When the antibodies were used to analyze the phosphorylation of endogenous SREBP1, we found that nuclear SREBP1 phosphorylated on T426 and S430 accumulated in HeLa, HepG2, and U2OS cells treated with the proteasome inhibitor MG-132 (Figure 3B and data not

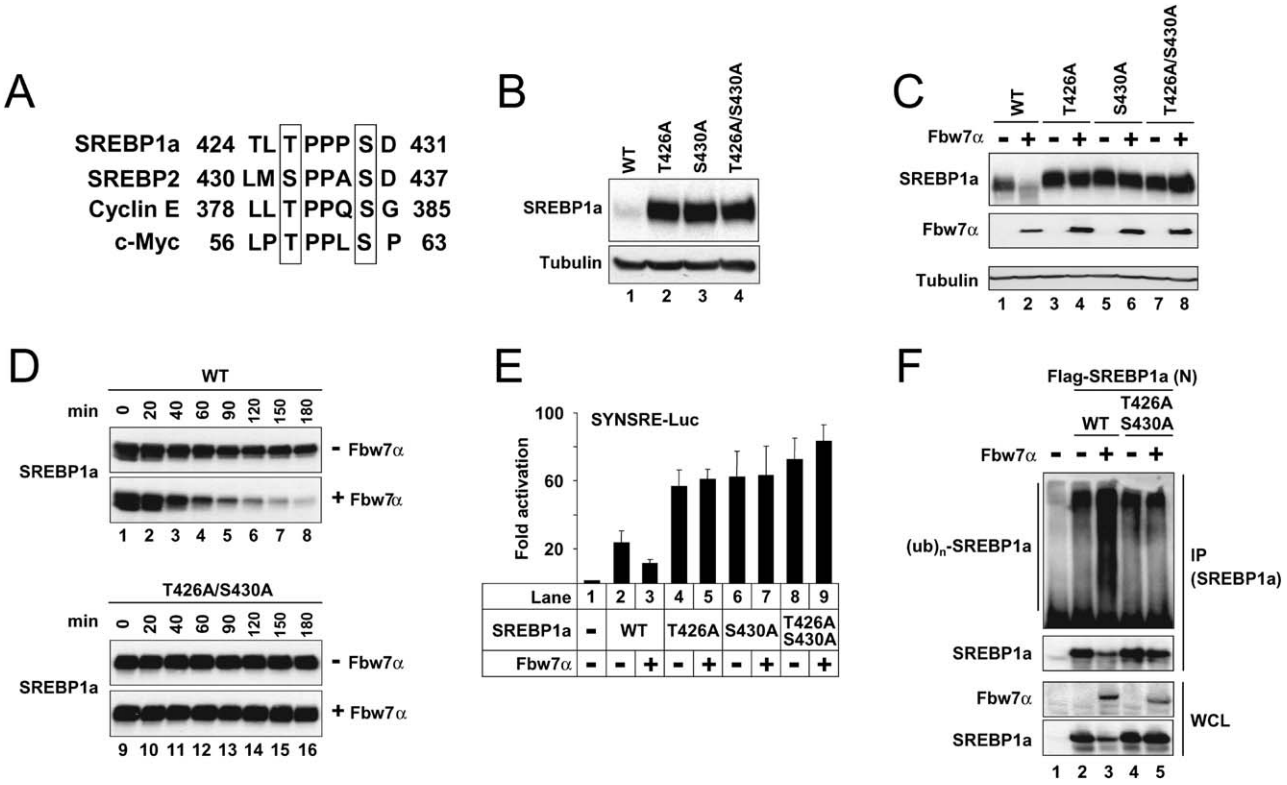

Figure 2. Fbw7-mediated degradation of SREBP1a is dependent on T426 and S430

A) Sequence alignment of the Cdc4 phosphodegron (CPD) in SREBP1a, SREBP2, c-Myc, and cyclin E.

B) 293T cells were transfected with Myc-SREBP1a, either wild-type or the indicated point mutants. The levels of SREBP1a and α-tubulin in cell lysates were determined by Western blotting.

C) 293T cells were transfected with Myc-SREBP1a, either wild-type or the indicated point mutants, in the absence or presence of Flag-Fbw7α. The levels of SREBP1a, Fbw7α, and α-tubulin in cell lysates were determined by Western blotting.

D) 293T cells were transfected with Myc-SREBP1a, either wild-type or the T426A/S430A mutant, in the absence or presence of Flag-Fbw7α. Thirty-six hours after transfection, the cells were either lysed directly or incubated in the presence of cyclohexamide (100 μg/ml) for the indicated time period in order to determine SREBP turnover. The levels of SREBP1a were determined by Western blotting.

E) 293T cells were transfected with SYNSRE-luc in the absence or presence of SREBP1a, either wild-type or the indicated point mutants, in the absence or presence of Fbw7α. Thirty-six hours after transfection, the luciferase activity was measured.

F) 293T cells were transfected with Flag-SREBP1a, either wild-type or the T426A/S430A mutant, and HA-ubiquitin in the absence or presence of GST-Fbw7α. SREBP1a was immunoprecipitated (IP) from whole-cell lysates with anti-Flag antibodies and separated by SDS-PAGE. Ubiquitination of SREBP1a and the amount of SREBP1a in the immunoprecipitates, and the levels of SREBP1a and Fbw7α in whole-cell lysates (WCL), were determined by Western blotting.

shown), indicating that nuclear SREBP1 phosphorylated on these residues is rapidly turned over by proteasome-mediated degradation. We detected no phosphorylation of T426 or S430 in the membrane-associated precursor form of SREBP1. This result is in agreement with our observation that Fbw7 is unable to enhance the degradation of the precursor form of SREBP1a (data not shown). Thus, our data indicate that nuclear SREBP1 is phosphorylated on T426 and S430, suggesting that the phosphorylation of these residues is important for Fbw7 binding and Fbw7-dependent degradation of SREBP1. To further define the role of T426 and S430 phosphorylation in mediating Fbw7 binding, we used synthetic peptides corresponding to the CPD motif in SREBP1a in peptide pull-down assays with Fbw7α. The binding of Fbw7α to the CPD peptide was strongly dependent upon T426 phosphorylation (Figure 3C). Interestingly, phosphorylation of S430 was not required for Fbw7 binding in vitro, although Fbw7 bound to the doubly phosphorylated peptide more avidly (compare lanes 3 and 5 in Figure 3C). In contrast, mutation of S430 blocked both the interaction between SREBP1a and Fbw7 and Fbw7-mediated degradation of SREBP1a in vivo (Figures 3A and 2C). Similar results have also been reported for the CPD in c-Myc; phosphorylation of T58 is required for interaction with Fbw7, whereas both T58 and S62 are required for Fbw7-mediated turnover of c-Myc (Welcker et al., 2004; Yada et al., 2004). In c-Myc, T58 phosphorylation requires S62 phosphorylation, and it will be interesting to analyze if the phosphorylation of T426 and S430 in SREBP1a is regulated in a similar manner. Similar results to those shown in Figure 3C were also obtained when peptides corresponding to the CPD motif in SREBP2 were used in peptide pull-down assays with Fbw7α (Figure S4), indicating that phosphorylation of S432 and S436 in SREBP2 is critical for its interaction with Fbw7. Taken together, our data indicate that phosphorylation of the CPD motifs in SREBP1 and SREBP2 regulates their interactions with Fbw7 and suggest that phos-

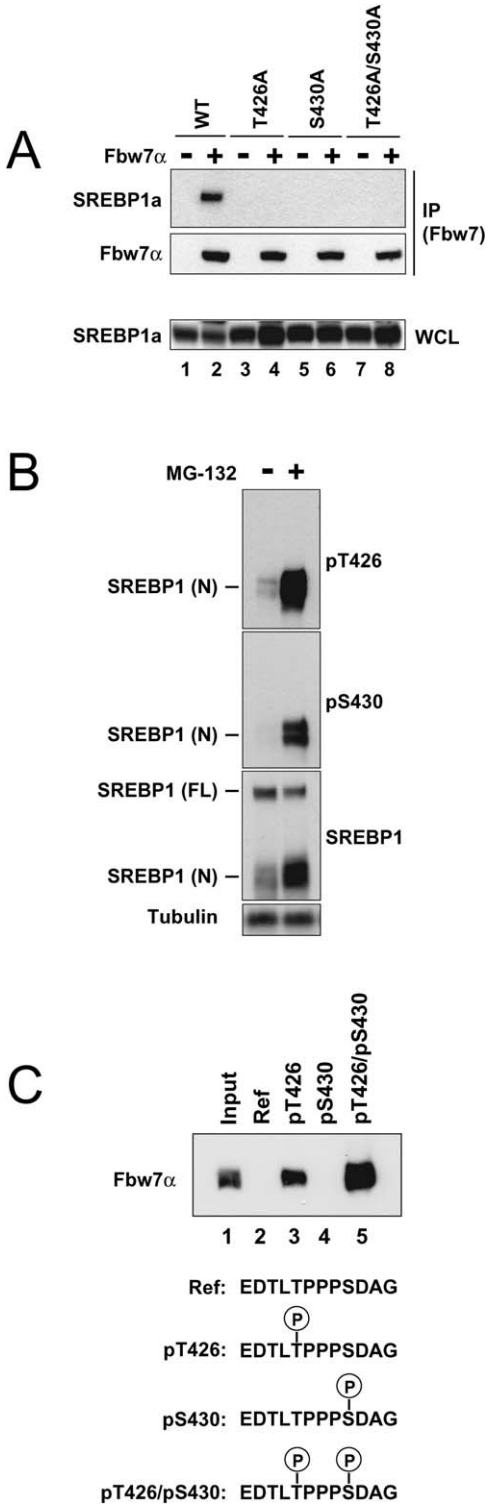

Figure 3. SREBP1a and Fbw7 interact in a phosphorylation-dependent manner

A) 293T cells were transfected with DN-Cul1 and Myc-SREBP1a, either wild-type or the indicated point mutants, in the absence or presence of Flag-Fbw7α. Cell lysates were immunoprecipitated with anti-Flag antibodies. Immunoprecipitated SREBP1a and Fbw7α and the levels of SREBP1a in cell lysates were determined by Western blotting.

B) HeLa cells were treated with vehicle alone (DMSO) or MG-132 (50 μM) for 4 hr. The levels of SREBP1 and α-tubulin and the phosphorylation of SREBP1 on T426 (pT426) and S430 (pS430) were determined by Western blotting.

C) Flag-Fbw7α was expressed in 293T cells, and the cell lysates were used in peptide pull-down assays, using four separate peptides corresponding to residues 422–433 of human SREBP1a, either unphosphorylated (Ref) or the same peptide phosphorylated on T426 (pT426), S430 (pS430), or both residues (pT426/pS430). The bound proteins were subjected to SDS/PAGE and Western blotting using 20% of input as control.

phorylation is required for Fbw7-driven turnover of the nuclear forms of both proteins.

GSK-3 phosphorylates the CPD motif in SREBP1

There is substantial evidence that GSK-3β regulates c-Myc stability and is the primary c-Myc T58 kinase. In addition, the CPD in cyclin E is also phosphorylated by GSK-3β. Recombinant GSK-3β phosphorylated both T426 and S430 in recombinant SREBP1a (Figure S5), indicating that GSK-3β could regulate the function of the CPD motif in SREBP1a. To test this hypothesis, recombinant SREBP1a, either wild-type or the T426A/S430A mutant, was incubated with recombinant GSK-3β, and the interaction between the phosphorylated SREBP proteins and Fbw7α were tested. As illustrated in Figure 4A, GSK3-mediated phosphorylation of wild-type SREBP1a induced a 4-fold increase in its interaction with Fbw7α (compare lanes 1 and 2), whereas the interaction between Fbw7α and the T426A/S430A mutant was unaffected (compare lanes 3 and 4), indicating that phosphorylation of T426 and S430 in SREBP1a is critical for its interaction with Fbw7. To test whether endogenous GSK-3 could phosphorylate the CPD motif in endogenous SREBP1, HeLa cells were treated with lithium, a pharmacologic GSK-3 inhibitor. Inhibition of GSK-3 reduced the phosphorylation of both T426 and S430 (Figure 4B), indicating that both residues are phosphorylated by GSK-3 in vivo. This hypothesis was supported by our observation that the phosphorylation of T426 and S430 was reduced when nuclear SREBP1a was cotransfected with a peptide corresponding to the axin GSK-3-interaction domain (GID) that binds to and inhibits GSK-3 (Figure 4C). In contrast, a control GID peptide bearing a point mutation that prevents GSK-3 interaction did not affect the phosphorylation of SREBP1a. Furthermore, siRNA-mediated inactivation of GSK-3β in HeLa and U2OS cells reduced the phosphorylation of T426 and S430 in endogenous SREBP1 (Figure 4D), confirming that GSK-3β targets the CPD motif in SREBP1. Importantly, inhibition of GSK-3 by either lithium treatment or expression of the wild-type GID prevented Fbw7-driven SREBP1a elimination in 293 cells (Figure 4E). Growth factors, including insulin and PDGF, negatively regulate the kinase activity of GSK-3 through Akt-mediated phosphorylation of Ser-9 in GSK-3. Both insulin and PDGF have been reported to enhance the levels of nuclear SREBP1 (Demoulin et al., 2004; Hegarty et al., 2005), and insulin signaling is a major regulator of lipid metabolism. Thus, we speculated that insulin-dependent inhibition of GSK-3 could attenuate the phosphorylation of T426 and/or S430 in SREBP1 and thereby block its Fbw7-mediated degradation. As illustrated in Figure 4F, a short treatment of HepG2 cells with insulin enhanced the inhibitory phosphorylation of GSK-3. Consequently, the phosphorylation of SREBP1 on both T426 and S430 was reduced in response to insulin treatment, resulting in the accumulation of nuclear SREBP1. We did not ob-

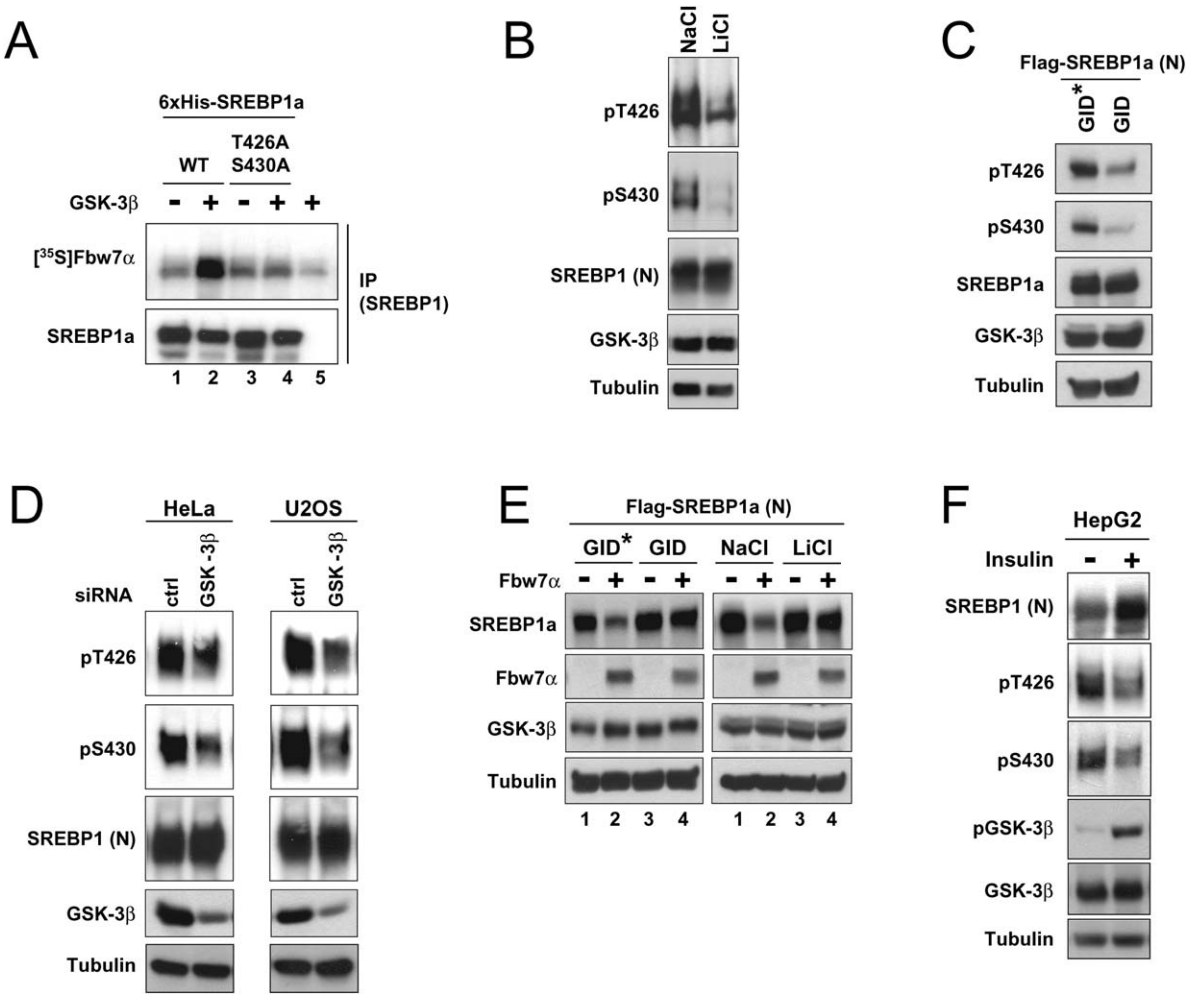

Figure 4. GSK-3 phosphorylates the CPD motif in SREBP1

A) 6xHis-SREBP1a, either wild-type or the T426A/S430A mutant, was incubated in the absence or presence of recombinant GSK-3β. The phosphorylated proteins were used for pull-down assays with in vitro translated [^{35}S]Fbw7α. No SREBP protein was added to lane 5. Following immunoprecipitation of SREBP1a and extensive washing, the bound proteins were subjected to SDS-PAGE, and the amount of [^{35}S]Fbw7α was determined by phosphoimage analysis. The amount of immunoprecipitated 6xHis-SREBP1a was determined by Western blotting.

B) HeLa cells were treated with NaCl or LiCl for 4 hr. The expression of SREBP1, GSK-3β, and α-tubulin and the phosphorylation of SREBP1 on T426 (pT426) and S430 (pS430) were determined by Western blotting.

C) 293 cells were transfected with Flag-SREBP1a in the presence of the axin GSK-interacting domain (GID) or an inactive GID mutant (GID*). The expression of SREBP1, GSK-3β, and α-tubulin and the phosphorylation of SREBP1 on T426 (p426) and S430 (pS430) were determined by Western blotting.

D) U2OS (left) and HeLa (right) cells were transfected with control or GSK-3β siRNA (20 nM) for 48 hr. The expression of SREBP1, GSK-3β, and α-tubulin and the phosphorylation of SREBP1 on T426 (pT426) and S430 (pS430) were determined by Western blotting.

E) 293 cells were transfected with Flag-SREBP1a in the absence or presence of GST-Fbw7α. In the right panel, cells were treated with either NaCl or LiCl (25 mM) 6 hr prior to lysis to inhibit endogenous GSK-3. In the left panel, cells were cotransfected with the axin GSK-interacting domain (GID) or an inactive GID mutant (GID*). The expression of SREBP1, GSK-3β, Fbw7α, and α-tubulin in cell lysates was determined by Western blotting.

F) HepG2 cells were placed in media containing 0.5% FBS for 18 hr and either left untreated (lane 1) or treated with 100 nM insulin (lane 2) for 1 hr. The expression of nuclear SREBP1, GSK-3β, and α-tubulin and the phosphorylation of SREBP1 on T426 (pT426) and S430 (pS430) and of GSK-3β on S9 (pGSK-3) were determined by Western blotting.

serve any induction of SREBP1a or SREBP1c mRNA in response to the short insulin treatment (data not shown). Thus, our results suggest that inactivation of GSK-3 in response to insulin signaling attenuates the phosphorylation of the CPD motif in mature SREBP1, thereby inhibiting Fbw7-mediated degradation. Taken together, our data indicate that Fbw7-driven SREBP1 turnover requires endogenous GSK-3 activity and support a model in which Fbw7-dependent degradation of SREBP1 requires phosphorylation of SREBP1 on T426 and S430 by GSK-3.

Regulation of endogenous SREBP by endogenous Fbw7

In order to determine if endogenous Fbw7 regulates the degradation of endogenous SREBP, we used siRNA targeting the common region of Fbw7. Because antibodies that recognize endogenous Fbw7 are not available, we confirmed the efficacy

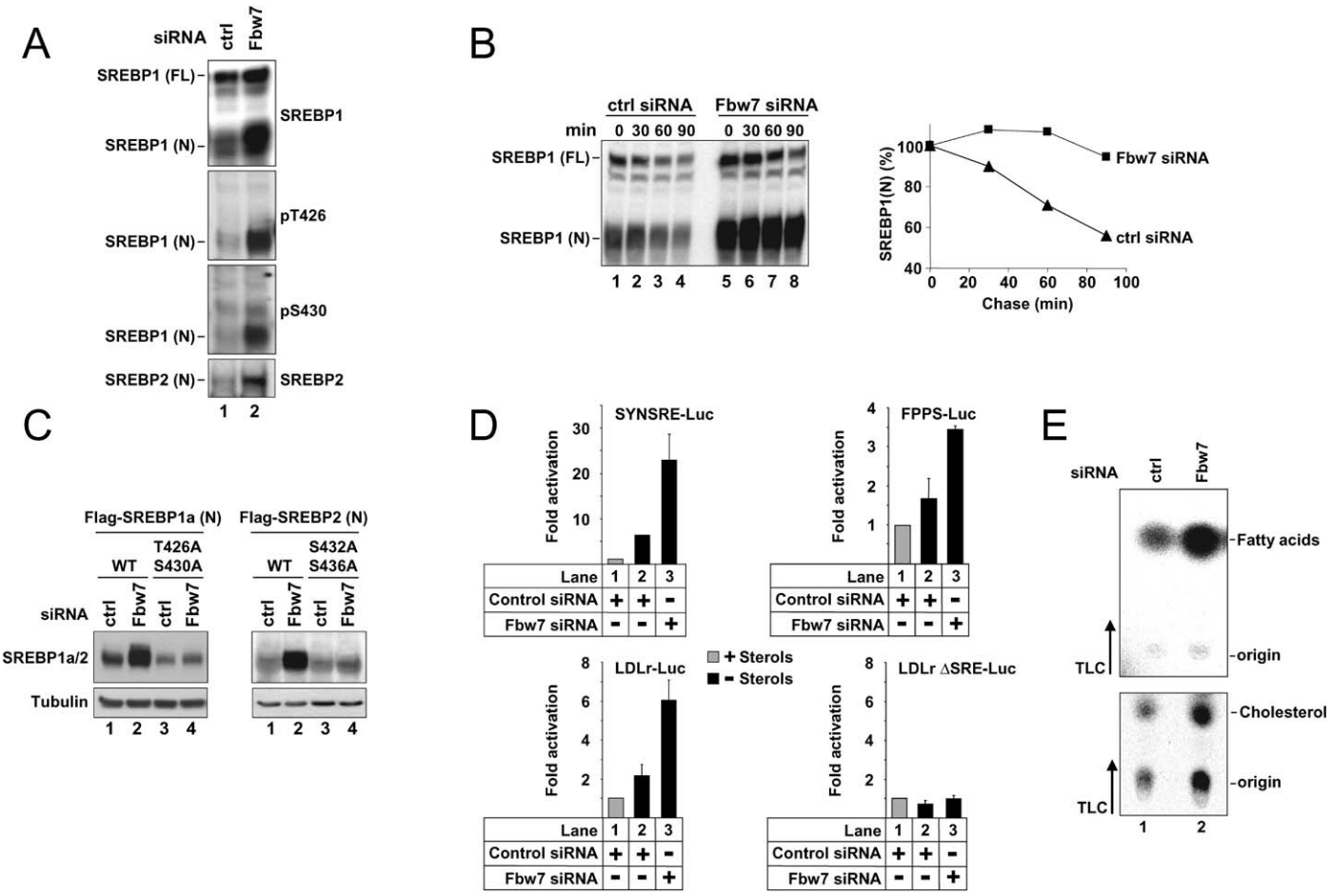

Figure 5. Regulation of endogenous SREBP by endogenous Fbw7

A) U2OS cells were transfected with control or Fbw7 siRNA (20 nM) for 48 hr. The expression of SREBP1 and SREBP2 and the phosphorylation of SREBP1 on T426 (pT426) and S430 (pS430) were determined by Western blotting. The migration of the precursor (FL) and nuclear (N) forms of SREBP1 is indicated.

B) U2OS cells were transfected with control or Fbw7 siRNA (20 nM). Forty-eight hours after transfection, cells were treated with cycloheximide for the indicated times. The levels of SREBP1 in cell lysates were detected by Western blotting. The relative amount of nuclear SREBP1 at each time point is plotted as percent of the amount at the start of the assay (right panel).

C) 293 cells were transfected with Flag-SREBP1a (left) or Flag-SREBP2 (right), either wild-type (0.5 μg) or the indicated mutant (0.05 μg), together with control or Fbw7-directed siRNA. The levels of SREBP1a, SREBP2, and α-tubulin in cell lysates were detected by Western blotting.

D) U2OS cells were transfected with the indicated promoter reporter constructs in the presence of either control or Fbw7 siRNA, and the cells were grown in the absence or presence of sterols. Thirty-six hours after transfection, the luciferase activity was measured.

E) U2OS cells were transfected with either control or Fbw7 siRNA. Thirty-six hours after transfection, cells were placed in fresh media supplemented with [^{14}C]acetate. Fatty acids (upper) and cholesterol (lower) were extracted and resolved by thin-layer chromatography. Radioactive products were visualized by phosphoimage analysis.

of the Fbw7 siRNA by demonstrating that the siRNA decreased expression of transfected Flag-tagged Fbw7, using Western blotting, and of endogenous Fbw7 mRNA, using semiquantitative PCR (Figure S6). The levels of nuclear SREBP1 were enhanced in U2OS cells treated with the Fbw7 siRNA, whereas no effect was observed on the precursor form of the protein (Figure 5A). In agreement with our hypothesis, the SREBP1 protein that accumulated in response to inactivation of Fbw7 was phosphorylated on T426 and S430. Nuclear SREBP2 also accumulated in cells treated with Fbw7 siRNA (Figure 5A), suggesting that Fbw7 regulates the stability of all SREBP proteins. To confirm that the increased levels of nuclear SREBP1 resulted from decreased proteolysis, we measured the half-life of SREBP1 in U2OS cells treated with control or Fbw7-directed siRNA. As illustrated in Figure 5B, nuclear SREBP1 was rapidly degraded in control cells, whereas siRNA-mediated inactivation of Fbw7 delayed the turnover of SREBP1. Our data suggest that the stabilization of SREBP1 in response to inactivation of Fbw7 should be dependent on T426 and S430. To test this hypothesis, 293 cells expressing nuclear SREBP1a, either wild-type or the T426A/S430A mutant, were treated with either control or Fbw7 siRNA. Inactivation of Fbw7 enhanced the expression of wild-type SREBP1a, whereas the expression of the T426A/S430A mutant was unaffected (Figure 5C, left), providing further support for a role of T426 and S430 in regulating Fbw7-mediated ubiquitination and degradation of SREBP1. Similar results were obtained when the experiment was repeated with wild-type SREBP2 and the S432A/S436A mutant

(Figure 5C, right). Inactivation of Fbw7 in U2OS cells enhanced the expression of SREBP-dependent promoter reporter genes (Figure 5D), supporting the hypothesis that Fbw7-mediated degradation of nuclear SREBPs affects the function of these proteins in vivo. Importantly, the effect of the Fbw7 siRNA was dependent on SREBP, since mutation of the SREBP binding site in the LDL receptor promoter blocked the induction of this promoter reporter in response to Fbw7 siRNA (Figure 5D, lower right panel).

Members of the SREBP family of transcription factors regulate cholesterol and lipid metabolism, and our data indicate that inactivation of Fbw7 results in stabilization of transcriptionally active SREBP1 and enhanced expression of SREBP target genes. Thus, we speculated that inactivation of endogenous Fbw7 should enhance lipid synthesis. To test this hypothesis, U2OS cells were treated with control or Fbw7 siRNA, and the synthesis of cholesterol and fatty acids was monitored in the transfected cells. In agreement with our hypothesis, the synthesis of both cholesterol and fatty acids was enhanced up to 3-fold in cells treated with the Fbw7 siRNA (Figure 5E). Taken together, these results suggest that endogenous Fbw7 negatively regulates the stability of nuclear SREBP, thereby regulating the expression of SREBP target genes and lipid synthesis.

Stabilization of nuclear SREBP in Fbw7$^{-/-}$ cells

Fbw7-deficient mice have been generated, and these animals die in utero at embryonic day 10.5 as a result of impaired vascular development. Attempts to isolate mouse embryonic fibroblasts from such immature embryos have not been successful. However, Fbw7-deficient HCT116 colon carcinoma cells have been generated (Rajagopalan et al., 2004). The steady-state levels of nuclear SREBP1 were increased in the Fbw7$^{-/-}$ HCT116 cells when compared to the parental cells, whereas the levels of the precursor form of SREBP1 were unaffected (Figure 6A). In agreement with our earlier observations, the levels of nuclear SREBP2 were also enhanced in Fbw7$^{-/-}$ cells. Semiquantitative RT-PCR analysis revealed that the expression of the LDL receptor and HMG-CoA synthase genes, both of which are positively regulated by SREBP, were increased significantly in Fbw7$^{-/-}$ cells (Figure 6B), suggesting that stabilization of nuclear SREBP results in enhanced expression of endogenous target genes. In order to analyze the contribution of individual Fbw7 isoforms to the turnover of SREBP1, Fbw7$^{-/-}$ cells were transduced with retroviruses expressing either Fbw7α, -β, or -γ, followed by selection of clones expressing similar levels of the individual Fbw7 isoforms (Figure S7). Expression of nuclear Fbw7α and -γ in Fbw7$^{-/-}$ cells prevented the accumulation of nuclear SREBP1, whereas the cytoplasmic β isoform had no effect (Figure 6C). Interestingly, the same result was obtained when the abundance of cyclin E was analyzed (Figure 6C, middle panel). These data are in agreement with our earlier results (Figures 1F and 1G) and indicate that the α and γ isoforms of Fbw7 targets nuclear SREBP1 for degradation, whereas Fbw7β is unable to do so. This hypothesis was strengthened when the expression of endogenous SREBP target genes was analyzed in the different Fbw7 clones by semiquantitative RT-PCR. Expression of Fbw7α and -γ in Fbw7$^{-/-}$ cells decreased the expression of SREBP target genes, whereas the expression of Fbw7β had only a limited effect (Figure 6D).

Fbw7 negatively regulates lipid synthesis and metabolism

In agreement with our hypothesis, the synthesis of both cholesterol and fatty acids was enhanced dramatically (8- to 10-fold) in Fbw7$^{-/-}$ cells (Figure 7A). However, SREBPs do not only regulate lipid synthesis but also lipid metabolism, since activation of the SREBP pathway results in enhanced expression of the LDL receptor gene and, thereby, increased clearance of LDL cholesterol from the circulation. Our earlier data demonstrated that the expression of the LDL receptor gene was enhanced in Fbw7$^{-/-}$ cells. In agreement with these observations, we found that the amount of LDL receptor protein was enhanced in Fbw7$^{-/-}$ cells (Figure 7B). To confirm that this activation translated into functional LDL receptor protein, we performed LDL uptake assays in Fbw7$^{+/+}$ and Fbw7$^{-/-}$ cells, using fluorescently labeled LDL. The amount of fluorescent dye that accumulates in lysosomes reflects the cell surface LDL receptor activity. There was a remarkable increase in the intensity of intracellular fluorescence in Fbw7$^{-/-}$ cells when compared with Fbw7$^{+/+}$ cells (Figure 7C), indicating a substantial increase in LDL receptor activity in a majority of the Fbw7$^{-/-}$ cells. The liver plays an important role in the regulation of whole-body lipid metabolism, and Fbw7α is expressed in most human tissues, including liver. To determine if Fbw7 could affect SREBP function and lipid metabolism in liver cells, we used siRNA to suppress endogenous Fbw7 in HepG2 cells. Mature SREBP1 and -2 accumulated in HepG2 cells treated with Fbw7 siRNA (Figure 7D). Consequently, the expression of the SREBP-dependent HMG-CoA synthase and LDL receptor promoter reporter genes were enhanced in response to Fbw7 inactivation (Figure 7E). Inactivation of Fbw7 in HepG2 cells also resulted in a 3-fold induction of cholesterol synthesis (data not shown) as well as an increase in the levels of LDL receptor protein (Figure 7D). Importantly, receptor-mediated uptake of LDL in HepG2 cells was enhanced in response to inactivation of Fbw7 (Figure 7F), suggesting that Fbw7 regulates SREBP function and lipid metabolism also in liver cells.

In summary, our data demonstrate that Fbw7 negatively regulates the stability and function of nuclear SREBP1 and -2 by promoting their ubiquitination and proteasome-mediated degradation through a phosphorylation-dependent mechanism. Consequently, Fbw7 regulates the expression of SREBP target genes and, thereby, the synthesis of cholesterol and fatty acids, as well as receptor-mediated uptake of LDL. In addition, our results suggest that these processes are regulated by insulin signaling.

Discussion

Members of the SREBP family of transcription factors control lipid synthesis and metabolism. The transcriptionally active forms of these proteins are rapidly degraded by the ubiquitin-proteasome pathway. We now demonstrate that nuclear SREBP1 and -2 are degraded by an Fbw7-dependent pathway. Fbw7 had previously been found to promote the degradation of cyclin E and c-Myc after phosphorylation within their CPD motifs. We demonstrate that the nuclear forms of SREBP1 and -2 are targeted for degradation by Fbw7 subsequent to phosphorylation within domains that are highly related to the CPD motifs found in cyclin E and c-Myc. We identify two residues in nuclear SREBP1a, T426 and S430, that are phosphory-

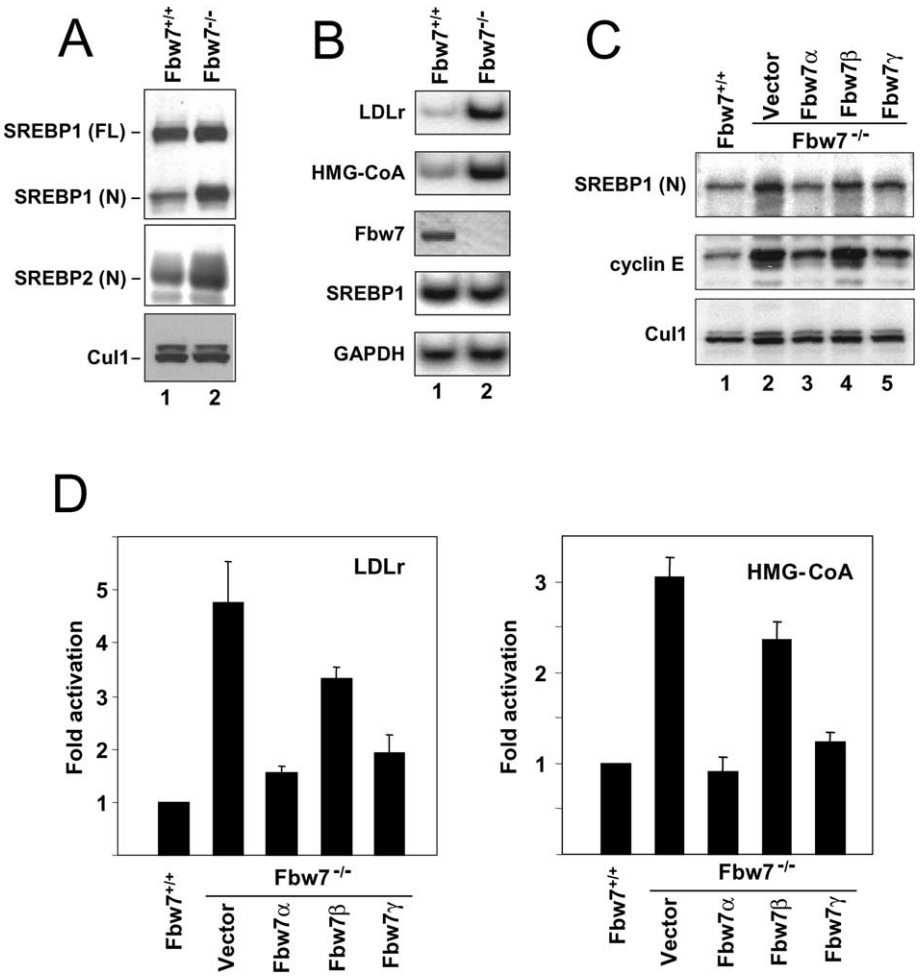

Figure 6. Stabilization of nuclear SREBP in Fbw7$^{-/-}$ cells

A) Total cell lysates were prepared from Fbw7$^{+/+}$ and Fbw7$^{-/-}$ HCT116 cells and analyzed by SDS-PAGE. The levels of SREBP1, SREBP2, and Cul1 in cell lysates were detected by Western blotting. The migration of the precursor (FL) and nuclear (N) forms of SREBP1 is indicated.

B) RNA was isolated from Fbw7$^{+/+}$ and Fbw7$^{-/-}$ cells, and the abundance of transcripts derived from the indicated genes was determined by RT-PCR.

C) Total cell lysates were prepared from Fbw7$^{+/+}$ (lane 1) and Fbw7$^{-/-}$ (lane 2) cells or from Fbw7$^{-/-}$ cells reconstituted with the indicated Fbw7 isoforms (lanes 3–5). The levels of nuclear SREBP1, cyclin E, and Cul1 in cell lysates were detected by Western blotting.

D) RNA was isolated from Fbw7$^{+/+}$ (lane 1) and Fbw7$^{-/-}$ (lane 2) cells or from Fbw7$^{-/-}$ cells reconstituted with the indicated Fbw7 isoforms (lanes 3–5). Total RNA was used to determine the expression of the low-density lipoprotein receptor (LDLr, left panel) and 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CoA, right panel) genes by semi-quantitative RT-PCR. The level of GAPDH mRNA in each sample was used for normalization. The results are presented as fold induction over the mRNA levels found in Fbw7$^{+/+}$ cells. The results represent the average ±SD of one representative experiment performed in triplicate.

lated by GSK-3, both in vitro and in vivo. Mutation of either of these residues blocked the interaction between nuclear SREBP1a and Fbw7 and inhibited Fbw7-mediated degradation of SREBP1a. In addition, inactivation of endogenous GSK-3 reduced the phosphorylation of both T426 and S430 and attenuated Fbw7-mediated degradation of nuclear SREBP1a. The precursor form of SREBP1 was not phosphorylated on T426 or S430 and was not sensitive to Fbw7-dependent degradation. Our results are in agreement with the recent observation that SREBP1c (also known as ADD1) is phosphorylated by GSK-3, both in vitro and in vivo (Kim et al., 2004). Although the authors did not identify the phosphorylated residues, it was demonstrated that GSK-3 inhibited SREBP-dependent transcription. The SREBP1a and SREBP1c proteins originate from a single gene as a result of alternative splicing and use of alternative promoters, and the two proteins only differ in the length of the amino terminal transactivation domain (Osborne, 2000). Thus, the sequence surrounding T426 and S430 is identical in both proteins. We have found that SREBP1c is phosphorylated on residues corresponding to T426 and S430 and that SREBP1c is degraded in response to Fbw7 expression (Figures S1 and S2 and data not shown). Thus, Fbw7-mediated degradation of SREBP1c could, at least in part, explain the negative effect of GSK-3 on the transcriptional activity of SREBP1c. In support of this hypothesis, we found that the phosphorylation of T426 and S430 was decreased and that nuclear SREBP1 accumulated when GSK-3 was inhibited in response to insulin signaling. Thus, our results suggest that inactivation of GSK-3 in response to insulin signaling attenuates the phosphorylation of the CPD motif in SREBP1, thereby blocking Fbw7-mediated degradation of nuclear SREBP1. However, it is also possible that GSK-3 phosphorylates other residues in SREBP1 and that these modifications have effects on the transcriptional and/or DNA binding activities of SREBP1. Mutation of the residues corresponding to T426 and S430 in SREBP2, S432 and S436, blocked the interaction between SREBP2 and Fbw7 as well as Fbw7-mediated degradation of SREBP2, suggesting that phosphorylation of these residues is critical for Fbw7-mediated degradation of SREBP2.

The stability and function of SREBP1 were negatively affected in response to Fbw7 expression, and Fbw7-mediated degradation of SREBP1 was impaired by mutation of the Fbw7 F box or by pharmacological inhibition of proteasome function. The membrane-associated precursor form of SREBP1 was not phosphorylated on T426 or S430, and Fbw7 did not affect the degradation of this form of the protein, indicating that Fbw7-mediated degradation is specific for the nuclear form of SREBP1. The role of endogenous Fbw7 in the degradation of

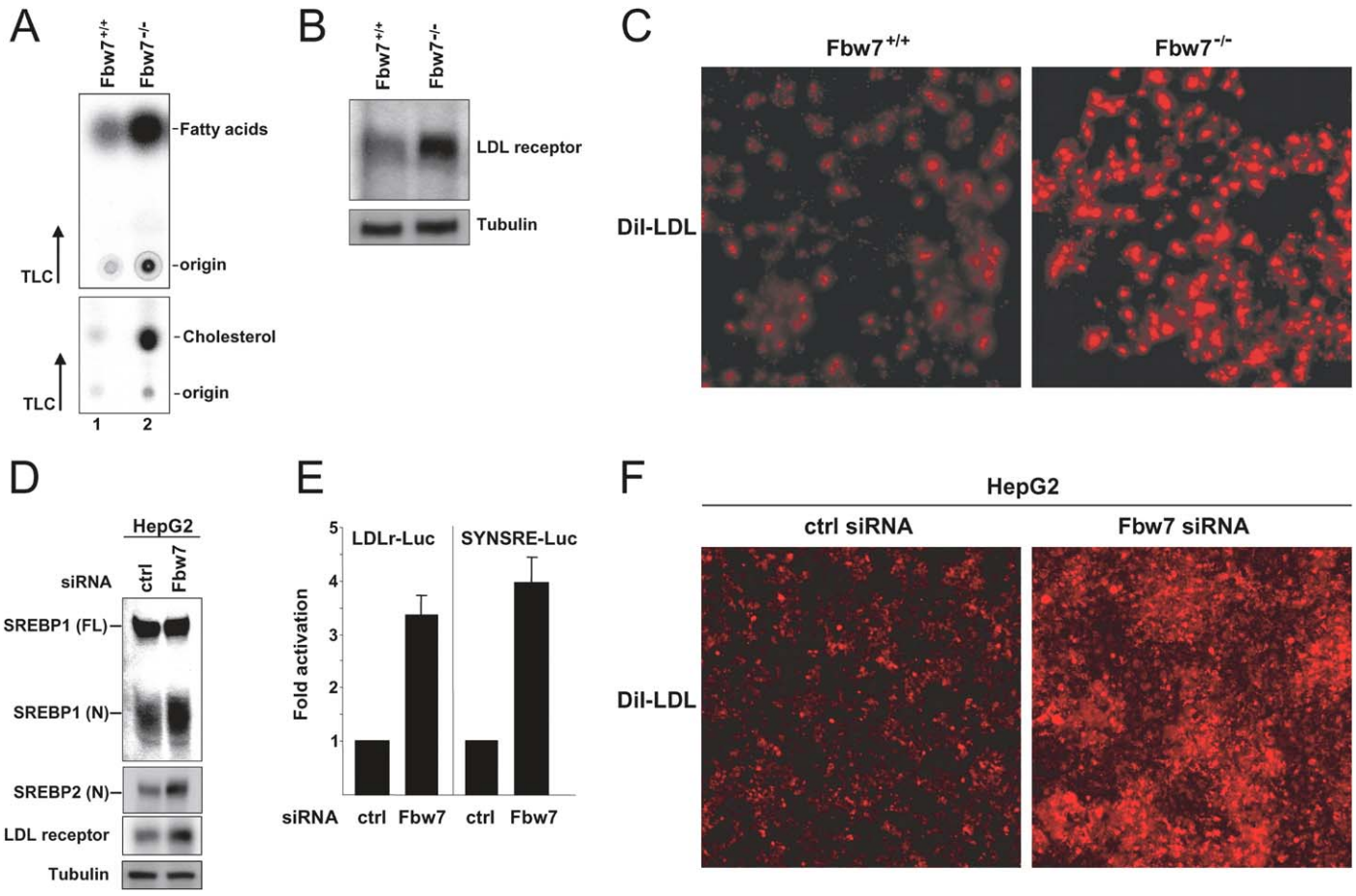

Figure 7. Fbw7 negatively regulates lipid synthesis and metabolism

A) Fbw7$^{+/+}$ (lane 1) and Fbw7$^{-/-}$ (lane 2) cells were placed in fresh media supplemented with [^{14}C]acetate for 2 hr. Fatty acids (upper) and cholesterol (lower) were extracted and resolved by thin-layer chromatography. Radioactive products were visualized by phosphoimage analysis.

B) Total cell lysates were prepared from Fbw7$^{+/+}$ (lane 1) and Fbw7$^{-/-}$ (lane 2) cells. The levels of LDL receptor and α-tubulin were detected by Western blotting.

C) Fbw7$^{+/+}$ (left) and Fbw7$^{-/-}$ (right) cells were placed in fresh media supplemented with fluorescently labeled LDL (DiI-LDL) for 4 hr. The cells were washed, fixated, and examined by fluorescence microscopy. Photographs were taken using identical exposure times and camera settings.

D) HepG2 cells were transfected with control or Fbw7 siRNA (20 nM) for 48 hr. The expression of SREBP1, SREBP2, LDL receptor, and α-tubulin were determined by Western blotting. The migration of the precursor (FL) and nuclear (N) forms of SREBP1 is indicated.

E) HepG2 cells were transfected with the indicated promoter reporter constructs in the presence of either control or Fbw7 siRNA (20 nM). Thirty-six hours after transfection, the luciferase activity was measured.

F) HepG2 cells were transfected with control or Fbw7 siRNA (20 nM) for 48 hr. Receptor-mediated uptake of DiI-LDL was analyzed as described in (**C**).

endogenous SREBP1 and -2 was studied by siRNA-mediated silencing of Fbw7 as well as a parallel approach employing Fbw7-deficient cells. Inhibition of Fbw7 by either approach enhanced the abundance of nuclear SREBP1 and -2 and enhanced the expression of SREBP target genes. Importantly, inhibition of Fbw7 resulted in enhanced synthesis of both cholesterol and fatty acids, suggesting that Fbw7-mediated degradation of nuclear SREBP is physiologically relevant. This hypothesis was supported by our observation that receptor-mediated uptake of LDL was enhanced in cells lacking Fbw7, demonstrating that Fbw7 controls both lipid synthesis and metabolism.

The human Fbw7 locus encodes three protein isoforms, Fbw7α, Fbw7β, and Fbw7γ (Jin et al., 2004). By reconstitution of Fbw7-deficient cells with the individual Fbw7 proteins, we have demonstrated that expression of Fbw7α and Fbw7γ reversed both the accumulation of nuclear SREBP1 and the activation of SREBP target genes seen in Fbw7$^{-/-}$ cells, whereas Fbw7β was inactive. We also found a similar isoform specificity following coexpression of nuclear SREBP1a and the three different Fbw7 proteins. Both Fbw7α and Fbw7γ are nuclear, whereas Fbw7β is localized to the cytoplasm, supporting our observation that Fbw7-mediated degradation of SREBPs is restricted to the nuclear forms of the proteins.

The *fbw7* gene is found within a chromosomal region that is mutated in many human cancers, and Fbw7 mutations have been found in cancer cells. The fact that SREBPs, c-Myc, and cyclin E are all Fbw7 substrates could reflect coordinate regulation of these proteins in normal cells. In addition, SREBP1, cyclin E, and c-Myc are phosphorylated by GSK-3 within their CPD motifs, and all proteins become resistant to Fbw7-driven turnover when GSK-3 is inhibited. Thus, SREBPs could share

elements within the signal transduction and proteolytic pathways with key regulators of cell growth and division. Members of the SREBP family of transcription factors regulate lipid synthesis, and the synthesis of membrane lipids is critical for cell growth and proliferation. Thus, loss of Fbw7 in cancers may deregulate membrane lipid synthesis, thereby promoting cell growth. It will, therefore, be important to analyze the phosphorylation of T426/S430 and the interaction between SREBP1 and Fbw7 in response to extracellular signals known to regulate cell growth and proliferation. We hope that the phosphorylation-specific anti-SREBP1 antibodies described in the current study will be helpful in these efforts. We have demonstrated that GSK-3 phosphorylates both T426 and S430 in vitro and in vivo and that inhibition of endogenous GSK-3 attenuates Fbw7-mediated degradation of SREBP1. However, it is possible that other kinases will also phosphorylate T426 and/or S430, depending on the cell type and extracellular signal. A more extensive analysis of the kinases targeting the CPD motifs in SREBPs is, therefore, warranted. In conclusion, our results demonstrate that Fbw7 controls the stability and function of all members of the SREBP family of proteins by inducing their phosphorylation-dependent ubiquitination. Cardiovascular disease is highly correlated with elevated levels of cholesterol in the circulation. The most common treatment for elevated cholesterol levels in humans is a group of drugs called statins. These compounds block cholesterol synthesis and, therefore, activate SREBPs. Activation of SREBPs results in enhanced expression of the LDL receptor gene and, thereby, increased clearance of LDL cholesterol from the circulation (Brown and Goldstein, 1999). We have demonstrated that inactivation of Fbw7 enhances the expression of SREBP target genes, including the LDL receptor gene, and that this results in enhanced synthesis of cholesterol and fatty acids as well as enhanced receptor-mediated uptake of LDL. Thus, the phosphorylation of the CPD motifs in SREBP1 and -2 and their interaction with Fbw7 may be attractive targets when developing new cholesterol-lowering therapies.

Experimental procedures

Cell culture

All tissue culture media and antibiotics were obtained from Invitrogen and Sigma. HEK293T, HEK293, HepG2, U2OS, and HeLa cells were from ATCC. Fbw7$^{+/+}$ and Fbw7$^{-/-}$ HCT116 cells were provided by B. Vogelstein (Rajagopalan et al., 2004) and were used at a low passage number.

Reagents and antibodies

Anti-Flag antibody (M5), cycloheximide, and standard chemicals were from Sigma. Monoclonal anti-Myc (9E10), anti-SREBP1 (E4 and 2A4), anti-tubulin (TU-02), anti-GST (B-14), anti-cyclin E (C-19), anti-LDL receptor (C7), goat anti-SREBP2 (N-19), and rabbit anti-SREBP1 (H-160) antibodies were from Santa Cruz. Anti-Cul1 antibody was from Zymed. Anti-GSK-3β was from Cell Signaling, and recombinant GSK-3β was from NEB.

Generation of phosphorylation-specific SREBP1 antibodies

Synthetic phosphopeptides corresponding to residues 423–429 (T426 phosphorylated) and 427–433 (S430 phosphorylated) in human SREBP1a were coupled to keyhole limpet haemocyanin before being injected into rabbits. The phosphopeptides and the corresponding nonphosphorylated peptides, as well as phospho-Ser (anti-pS430) and phospho-Thr (anti-pT426), were coupled to Sulpholink (Pierce) and used as affinity matrixes to purify the individual antibodies from rabbit sera.

Plasmids, retroviruses, DNA transfections, and viral infection

The expression vectors for Flag- and Myc-tagged SREBP1a, SREBP1c, and SREBP2 (amino acid residues 2–490, 2–466, and 2–485, respectively) have been described (Sundqvist and Ericsson, 2003). Point mutants in SREBP1a and SREBP2 were generated by site-directed mutagenesis (QuickChange, Stratagene). The HMG-CoA synthase (SYNSRE-luc), farnesyldiphosphate synthase (FPPS-luc), and LDL receptor (LDLr-luc and LDLrΔSRE-luc) promoter reporter constructs have been described (Dooley et al., 1998; Ericsson et al., 1996; Sanchez et al., 1995). All other expression vectors have been described previously (Koepp et al., 2001; Welcker et al., 2004). Transient transfections were performed using the MBS transfection kit (Stratagene). The Fbw7 (Fbw7.2), GSK-3β, and control siRNA were from Ambion and have been described (Welcker et al., 2004; Welcker et al., 2003). MSCV-based retroviruses expressing various Fbw7 isoforms were generated using Gateway cloning vectors (Invitrogen). pMSCV-Fbw7α, β, and γ or empty vector was transfected into retrovirus packaging cells (Phoenix) using calcium phosphate-based transfection procedures. Two days after transfection, viruses were harvested and used to infect HCT116 cells lacking Fbw7 as indicated. Cells were selected with puromycin (1 μg/ml) for 2 weeks. Individual colonies were picked and analyzed by RT-PCR to detect Fbw7 expression.

Immunoprecipitations and immunoblotting

Cells were lysed in buffer A (50 mM HEPES [pH 7.2], 150 mM NaCl, 1 mM EDTA, 20 mM NaF, 2 mM sodium orthovanadate, 10 mM β-glycerophosphate, 1% (w/v) Triton X-100, 10% (w/v) glycerol, 1 mM PMSF, 10 mM sodium butyrate, 1% aprotinin, 0.1% SDS, and 0.5% sodium deoxycholate) and cleared by centrifugation. For coimmunoprecipitations, cell lysates were prepared in the absence of SDS and sodium deoxycholate. For ubiquitination assays, cell lysates were prepared under denaturing conditions and processed as described (Sundqvist and Ericsson, 2003). Cell lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Millipore). To ensure that equal amounts of protein were loaded in each well, the levels of α-tubulin or Cul1 in the samples were estimated by Western blotting. Proteins were in vitro translated using the T7 TNT kit (Promega) and ^{35}S-labeled methionine and cysteine and incubated with 6xHis-SREBP1a followed by immunoprecipitation of SREBP1a. The samples were washed extensively and resolved by SDS-PAGE.

Determination of protein half-life

Cells were treated with cycloheximide to stop protein synthesis and incubated for the indicated times. Total cell lysates were prepared and the proteins separated by SDS-PAGE and transferred to nitrocellulose membranes. SREBP1 was visualized by Western blotting followed by quantitation on a CCD camera (Fuji) and image analysis software (Aida Image Analyzer 3.10).

Peptide pull-down assays

Four separate peptides corresponding to residues 422–433 of human SREBP1a or the same peptide phosphorylated on T426, S430, or both residues were synthesized using Fmoc chemistry. The peptides were coupled to Sulpholink and used in peptide pull-down assays with cell lysates from 293T cells expressing Flag-Fbw7α in buffer A. The immobilized peptides were washed three times in buffer A, and the bound proteins were subjected to SDS/PAGE and Western blotting using 20% of input as controls.

Luciferase and β-galactosidase assays

Cells were transiently transfected with the indicated promoter reporter genes in the absence or presence of expression vectors for SREBP1a, either wild-type or the indicated mutants. Where indicated, the media was supplemented with cholesterol and 25-hydroxycholesterol (50 and 5.0 μg/ml, respectively) to suppress the activation of endogenous SREBPs. After 36 hr, luciferase activities were determined in duplicate samples as described by the manufacturer (Promega). The pCH110 vector encoding the β-galactosidase gene under the control of the SV40 promoter (Amersham Biosciences) was used as an internal control for transfection efficiency. Luciferase values (relative light units [RLU]) were calculated by dividing the luciferase activity by the β-galactosidase activity. The data represent the average ±SD of three independent experiments performed in duplicates.

Analysis of lipid synthesis

Cells were placed in fresh media supplemented with [^{14}C]acetate (10 μCi/ml) and incubated for 2 hr. The cells were washed, trypsinized, and collected by centrifugation. The pelleted cells were hydrolyzed in 60% (w/v) KOH in methanol, and the nonpolar lipids (cholesterol) were extracted in hexane. Following addition of 1 M HCl, polar lipids (fatty acids) were extracted in chloroform:methanol (2:1). The lipids were resolved by thin-layer chromatography (Silica gel 60, Merck). The radioactive products were identified by comparison with unlabeled standards, visualized with iodine vapor.

LDL uptake assays

Cells grown on coverslips were incubated with fluorescently labeled LDL (Dil-LDL) for 4 hr, washed, and fixed in 2% paraformaldehyde in PBS for 20 min at room temperature. The cells were subsequently permeabilized in 0.2% Triton X-100 in PBS, washed in PBS, and incubated in 10 mM glycine in PBS. Nuclei were stained with DAPI. Intracellular fluorescent dye was detected by microscopy using Axioplan-2 Zeiss (Jena, Germany) rhodamine filters with a Hamamatsu ORCA CCD digital camera and identical exposure times and camera settings.

RT-PCR assays

RNA was extracted with Trizol reagent (Invitrogen). mRNA was subjected to RT with oligo dT, followed by PCR with target-specific primers. The PCR reactions, using Invitrogen High-Fidelity DNA polymerase, were optimized for the individual target genes. The PCR programs and primer sequences for the human LDL receptor, HMG-CoA synthase, Fbw7α, Fbw7β, Fbw7γ, SREBP1, β-actin, and GAPDH genes are available on request.

Supplemental Data

Supplemental Data include seven figures and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/1/6/379/DC1/.

Acknowledgments

We thank B. Vogelstein and B.E. Clurman for reagents and Ulla Engström for technical support. This work was supported by grants from the Swedish Research Council and the Novo Nordisk Foundation (to J.E.) and National Institutes of Health Grant AG11085 (to J.W.H.). A.S. was supported in part by a grant from the Magnus Bergvalls Stiftelse. J.J. was supported in part by Department of Defense Breast Cancer grant DAMD 17-02-1-0284. J.E. is a Research Fellow of the Royal Swedish Academy of Sciences through a grant from the Knut and Alice Wallenberg Foundation.

Received: December 13, 2004
Revised: April 8, 2005
Accepted: April 27, 2005
Published: June 7, 2005

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