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Inhibition of mTOR complex 2 induces GSK3/FBXW7-dependent degradation of sterol regulatory element-binding protein 1 (SREBP1) and suppresses lipogenesis in cancer cells

Shaohua Li[1,2], **You-Take Oh**[1], **Ping Yue**[1], **Fadlo R. Khuri**[1], and **Shi-Yong Sun**[1]

[1]Department of Hematology and Medical Oncology, Emory University School of Medicine and Winship Cancer Institute, Atlanta, GA, USA

[2]Beijing Institute of Basic Medical Sciences, Beijing, P. R. China

Abstract

Cancer cells feature increased *de novo* lipogenesis. Sterol regulatory element-binding protein 1 (SREBP1), when presented in its mature form (mSREBP1), enhances lipogenesis through increasing transcription of several of its target genes. Mammalian target of rapamycin (mTOR) complexes, mTORC1 and mTORC2, are master regulators of cellular survival, growth and metabolism. A role for mTORC1 in the regulation of SREBP1 activity has been suggested; however the connection between mTORC2 and SREBP1 has not been clearly established and hence is the focus of this study. mTOR kinase inhibitors (e.g., INK128), which inhibit both mTORC1 and mTORC2, decreased mSREBP1 levels in various cancer cell lines. Knockdown of rictor, but not raptor, also decreased mSREBP1. Consistently, reduced mSREBP1 levels were detected in cells deficient in rictor or Sin1 compared to parent or rictor-deficient cells with re-expression of ectopic rictor. Hence it is mTORC2 inhibition that causes mSREBP1 reduction. As a result, expression of the mSREBP1 target genes acetyl-CoA carboxylase and fatty acid synthase was suppressed, accompanied with suppressed lipogenesis in cells exposed to INK128. Moreover, mSREBP1 stability was reduced in cells treated with INK128 or rictor knockdown. Inhibition of proteasome, GSK3 or the E3 ubiquitin ligase, FBXW7, prevented mSREBP1 reduction induced by mTORC2 inhibition. Thus mTORC2 inhibition clearly facilitates GSK3-dependent, FBXW7-mediated mSREBP1 degradation, leading to mSREBP1 reduction. Accordingly, we conclude that mTORC2 positively regulates mSREBP1 stability and lipogenesis. Our findings reveal a novel biological function of mTORC2 in the regulation of lipogenesis and warrant further study in this direction.

Keywords

mTORC2; mTOR kinase inhibitors; SREBP1; GSK3; FBXW7; degradation

Correspondence: Shi-Yong Sun, Emory University School of Medicine and Winship Cancer Institute, 1365-C Clifton Road NE, C3088, Atlanta, GA 30322. Phone: (404) 778-2170; Fax: (404) 778-5520; ; Email: ssun@emory.edu

Conflict of interest

The authors declare no conflict of interest.

Introduction

It has been shown that neoplastic tissues and tumor cells are able to synthesize lipid [1]. Consistently, the expression of sterol regulatory element-binding proteins (SREBPs), a family of master regulators of lipogenesis, and several SREBP target genes such as acetyl coenzyme-A carboxylase (ACC), fatty acid synthase (FASN) and low density lipoprotein receptor are elevated in tumor cells [2, 3]. Accordingly, targeting SREBPs and lipogenesis in cancer cells inhibits the growth and survival of cancer cells [4–7]. Hence SREBPs and lipogenesis have emerged as potential cancer therapeutic targets.

There are three mammalian SREBP isoforms (SREBP1a, SREBP1c, and SREBP2) encoded by two genes, SREBF1 and SREBF2. SREBP1a and SREBP1c are generated by alternative splicing and vary in their expression levels across different tissues, with SREBP1a being the most abundant isoform in most cultured cell lines. These isoforms have distinct, but overlapping functions in the regulation of lipogenesis. SREBP1 mainly regulates fatty acid, phospholipid and triacylglycerol synthesis, while SREBP2 primarily controls cholesterol synthesis via positive regulation of the expression of cholesterol-synthesis genes [2, 8].

SREBP1 is synthesized as an inactive precursor that is attached to the membrane of the endoplasmic reticulum, where it is associated with the SREBP-cleavage activating protein (SCAP). Following cleavage in the Golgi, the released mature form of the N-terminal protein (mSREBP1), which contains the DNA-binding and transcriptional activation domains, translocates to the nucleus and activates transcription by binding to the sterol response element (SRE) of their target genes [3, 8]. Regulation of SREBP1 levels at transcriptional, post-transcriptional or post translational level (i.e., gene expression, protein maturation or protein stability) can cause a change in lipid homeostasis. It is known that sterols inhibit the cleavage of the precursor SREBP1 and the mSREBP1 is rapidly catabolized, primarily due to GSK3-dependent and FBXW7-mediated protein ubiquitinylation and proteasomal degradation, thereby resulting in suppressed transcription [8, 9].

The mammalian target of rapamycin (mTOR) regulates a variety of biological functions essential for maintaining cancer cell survival and growth by forming two complexes through direct interaction with different partner proteins: raptor (mTOR complex 1; mTORC1) and rictor (mTOR complex 2; mTORC2). mTORC1 is well known to regulate many key cellular processes including cell growth and metabolism primarily via regulating cap-dependent protein translation initiation. However less is known about the biological functions of mTORC2 except for acting as an Akt S473 kinase and regulating cell skeleton [10, 11]. A few studies have implicated the role of mTORC1 in positive regulation of SREBP activity at multiple levels including gene expression, protein processing and nuclear translocation [12–19]. However, the involvement of mTORC2 in the regulation of SREBPs and lipid metabolism has not been well studied. Two recent reports using hepatic rictor-knockout mice suggest that mTORC2 may positively regulate hepatic SREBPs levels and lipogenesis without defining the underlying mechanisms [20, 21].

Our recent studies have shown that mTORC2 positively regulates the stability of cyclin D1 by suppressing GSK3-dependent and SCF E3 ubiquitin ligase-mediated protein degradation [22]. Given that mSREBP1 undergoes GSK3/FBXW7-dependent degradation [23–25], we hypothesized that mTORC2 might positively regulate lipogenesis via stabilizing mSREBP1 by suppressing GSK3/FBXW7-mediated degradation. In this report, we have indeed demonstrated that mSREBP1 is a key cellular target of mTORC2. mTORC2 stabilizes mSREBP1 through suppressing GSK3-dependent and FBXW7-mediated protein degradation and hence positively regulates lipogenesis. Accordingly, inhibition of mTORC2 (e.g., with an mTOR kinase inhibitor) triggers mSREBP1 degradation, leading to suppressed transcription of its target genes and lipogenesis. Hence, our findings provide the first evidence to connect the mTORC2 to regulation of mSREBP1 and lipid metabolism in cancer as well other cells, revealing a novel biological function of the mTORC2.

Results

mTOR kinase inhibitors decrease mSREBP1 levels in cancer cells

We treated 8 different types of cancer cell lines with different concentrations of the mTOR kinase inhibitor, INK128, for 8 h and then prepared whole-cell protein lysates for Western blotting to detect SREBP1 and other proteins. In every tested cell line treated with 20 nM and 100 nM INK128, we observed concentration-dependent reduction of mSREBP1 with minimal or almost no effect on the levels of precursor SREBP1. In parallel, we detected decreased levels of p-Akt and p-S6 (Fig. 1A), indicating effective suppression of both mTORC1 and mTORC2 signaling by INK128 under the tested conditions. Reduction of mSREBP1 by INK128 occurred at 4 h and was sustained up to 24 h in a fashion similar to the suppression of Akt and S6 phosphorylation (Fig. 1B). Hence it is clear that INK128 effectively decreases mSREBP1 levels, while suppressing both mTORC1 and mTORC2 signaling. We repeated this observation with another mTOR kinase inhibitor, AZD8055. In comparison, rapamycin decreased mSREBP1 levels in H157 and HCT116 cells, but not in H1299 cells (Fig. 1C).

Genetic suppression of rictor and Sin1, but not raptor expression, decreases mSREBP1 levels

To determine the involvement of mTORCs in the positive regulation of mSREBP1 levels, we compared the effects of knockdown of rictor and raptor on mSREBP1 levels. We detected reduced levels of mSREBP1 in A549-shRictor cells, in which rictor was stably silenced, but not in A549-shRaptor cells with stable knock down of raptor (Fig. 2A). In agreement, transient knockdown of rictor, but not raptor, with small interfering RNA (siRNA) transfection reduced mSREBP1 levels (Fig. 2B). Moreover, we detected reduced levels of mSREBP1 in rictor knockout (KO) murine embryonic fibroblasts (MEFs) in comparison with wild-type (WT) and rictor-KO-rictor MEFs, in which rictor is re-expressed (Fig. 2C), indicating that rictor deficiency reduces mSREBP1 levels and rictor re-expression can rescue mSREBP1 reduction. Consistently, knockout of Sin1, another essential component of mTORC2, also decreased mSREBP1 levels (Fig. 2C). Collectively, these results robustly show an mTORC2-dependent positive regulation of mSREBP1 levels. Under the tested conditions (e.g., knockdown or knockout of rictor or knockout of Sin1), we observed

minimal reduction of precursor SREBP1 levels, indicating that mTORC2 primarily regulates mSREBP1 levels.

Inhibition of mTORC2 destabilizes mSREBP1 protein through promoting its proteasomal degradation

We next determined the underlying mechanism by which mTORC2 inhibition decreases SREBP1 levels. Given that mSREBP1 is known to be an unstable protein regulated by degradation [24, 25], we compared the effects of INK126 on mSREBP1 levels in the absence and presence of the proteasome inhibitor, MG132. We detected mSREBP1 reduction in both H157 and H1299 cells exposed to INK128. The presence of MG132 not only increased the basal levels of mSREBP1, but also prevented mSREBP1 reduction induced by INK128 (Fig. 3A), suggesting that INK128 decreased mSREBP1 levels through proteasome-mediated degradation. Moreover, we compared the stabilities of mSREBP1 protein between DMSO and INK128-treated cells and found that mSREBP1 protein levels were reduced much more rapidly in INK128-treated cells than in DMSO-treated cells, demonstrating that INK128 destabilizes mSREBP1 protein (Fig. 3B). We also detected faster reduction of mSREBP1 protein in A549-shRictor cells than in A549-pLKO.1 and shRaptor cells (Fig. 3C), indicating that knockdown of rictor, but not raptor, facilitates mSREBP1 degradation. Consistently, mSREBP1 protein in rictor-KO MEFs was degraded more rapidly than that in WT MEFs (Fig. 3D), further supporting the notion that mTORC2 is associated with positive regulation of mSREBP1 stability. Taking these data together, it is obvious that inhibition of mTORC2 with either INK128 or genetic rictor depletion initiates proteasomal degradation of mSREBP1.

INK128 induces mSREBP1 reduction in part through a GSK3-dependent mechanism

Since GSK3 is known to be involved in mSREBP1 degradation [23, 25], we next determined whether GSK3 is involved in mSREBP1 degradation induced by mTORC2 inhibition. INK128 decreased the levels of mSREBP1 in the absence of the GSK3 inhibitor CHIR99021 or SB216763 in both H157 and H1299 cells. The presence of CHIR99021 or SB216763 increased basal levels of mSREBP1 and partially rescued mSREBP1 reduction caused by INK128 (Fig. 4A). In agreement, genetic inhibition of GSK3 via siRNA-mediated gene knockdown also increased basal levels of mSREBP1 and in part prevented mSREBP1 reduction induced by INK128 in these cell lines (Fig. 4B). These data hence suggest that INK128 induces mSREBP1 reduction or degradation at least in part through a GSK3-dependent mechanism.

mTORC2 inhibition induces FBXW7-mediated mSREBP1 degradation

It has been suggested that the E3 ubiquitin ligase, FBXW7, mediates GSK3-dependent mSREBP1 degradation [23, 25]. We then investigated whether FBXW7 is involved in mTORC2 inhibition-induced mSREBP1 degradation. We first studied the effect of FBXW7 silencing in H1299 cells on INK128-induced mSREBP1 reduction and found that INK128 decreased mSREBP1 levels in control siRNA- and non-transfected cells, but not in cells transfected with FBXW7 (Fig. 5A). Furthermore, we noticed that FBXW7 knockdown substantially elevated basal levels of mSREBP1 (Fig. 5A). The knockdown efficiency of FBXW7 was confirmed with reverse transcription-PCR (RT-PCR) (Fig. 5B) due to lack of a

specific FBXW7 antibody suitable for use in Western blotting analysis. In agreement, both INK128 and AZD8055 decreased mSREBP1 levels in HCT116 parental cells, but not in HCT116 cells deficient in FBXW7 (FBXW7-KO) (Fig. 5C). Again we observed elevated basal levels of mSREBP1 in HCT116/FBXW7-KO cells (Fig. 5C). Moreover, we examined the effect of FBXW7 deficiency on rictor knockdown-induced reduction of mSREBP1 and found that rictor knockdown decreased mSREBP1 levels in HCT116 parental cells, but not in FBXW7-KO HCT116 cells (Fig. 5D). These data together provide strong support for the involvement of FBXW7 in mediating mSREBP1 degradation induced by mTORC2 inhibition.

mTORC2 inhibition induces mSREBP1 degradation independent of Akt inhibition

Considering that mTORC2 functions as an Akt S473 kinase, which suppresses GSK3 activity through phosphorylation, we further studied whether Akt inhibition is involved in mediating mSREBP1 degradation triggered by mTORC2 inhibition. To this end, we compared the effects of INK128 with other PI3K (BKM120) and Akt (API-1, MK2206 and perifosine) inhibitors on altering mSREBP1 levels. As presented in Fig. 6A, INK128 decreased mSREBP1 levels in every tested cell line as we demonstrated above. Under the tested conditions, INK128 reduced the levels of p-Akt and p-PRAS40, indicating that it effectively inhibits Akt signaling. BKM120 reduced mSREBP1 levels in H157 and A549 cells, but not in H1299 cells. However, it suppressed the phosphorylation of Akt and PRAS40 in H157 and H1299 cells, indicating that it inhibits Akt signaling only in H157 and H1299 cells. AP-1 weakly suppressed Akt and PRAS40 phosphorylation in the tested cell lines accompanied with no (A549) or even increased (H157 and H1299) effect on mSREBP1 levels. Both MK2206 and perifosine effectively suppressed Akt and PRAS40 phosphorylation in all the tested cell lines as INK128 did, but decreased mSREBP1 levels only in H157 cells. Rather, we observed increased mSREBP1 in A549 and H1299 cells exposed to MK2206. Interestingly, we found that both INK128 and BKM120 had no effects on decreasing p-GSK3 levels in any of the tested cell lines, whereas AP-1, MK2206 and perifosine decreased p-GSK3 levels in H157 cells, but not in A549 and H1299 cells, indicating that suppression of Akt does not necessarily lead to inhibition of GSK3 phosphorylation.

Moreover, we compared the effects of rictor and Akt knockdown on mSREPB levels and found that both Akt II (specifically for Akt1) or Akt I (specifically for Akt1 and Akt2) siRNA knockdown had limited effect on decreasing mSREBP1 levels in comparison with rictor knockdown, which substantially reduced mSREBP1 levels (Fig. 6B). Hence it is clear that Akt has limited effect on the regulation of mSREBP levels in our tested cancer cells.

Inhibition of mTORC2 suppresses the expression of SREBP1 target genes and lipogenesis

Finally, we determined the impact of mSREBP1 degradation induced by mTORC2 inhibition on the expression of SREBP1 target genes and on lipogenesis. We found that INK128 treatment effectively decreased the levels of both ACC and FASN, two well-known SREBP1-regulated proteins, in the tested cancer cell lines in both dose-and time-dependent manners (Figs. 7A and B). We also detected reduced levels of both ACC and FASN in MEFS deficient in either rictor (rictor-KO) or Sin 1(Sin1-KO). When rictor was re-

introduced into rictor-KO MEFs, both ACC and FASN levels were restored back to normal levels (Fig. 7C). Moreover, we detected reduced levels of *FASN* and *ACACA* (encoding ACC) gene expression at the mRNA level in INK128-treated cancer cells (Fig. 7D), indicating that INK128 treatment suppresses transcription of SREBP1 target genes. Collectively, we conclude that inhibition of mTORC2 suppresses the expression of SREBP1 target genes at both mRNA and protein levels. In addition, we examined the effect of INK128 on the lipogenesis of cancer cells. With red oil O staining, we detected far fewer cells positive for lipid droplets in INK128-treated H1299 cells than in DMSO-treated cells (Fig. 7E), suggesting that INK128 significantly inhibits the formation of lipid droplets or lipogenesis.

Discussion

In agreement with recent finding from two studies using hepatic rictor-deficient mice showing that mTORC2 is required for hepatic SREBP1 activation and lipogenesis [20, 21], the current study using both pharmacological and genetic approaches has demonstrated that mTORC2 plays an essential role in the positive regulation of SREBP1 activation and lipogenesis in mammalian cells including human cancer cells. Our conclusions are primarily based on the following findings: 1) mTOR kinase inhibitors that inhibit both mTORC1 and mTORC2 strongly decreased levels of mSREBP1 with minimal effect on precursor SREBP1 levels in various types of cancer cells (Fig. 1); 2) knockdown of rictor, but not raptor, in different cancer cell lines substantially decreased mSREBP1 levels (Fig. 2); 3) both rictor-KO and Sin1-KO MEFS exhibited reduced levels of mSREBP1 in comparison with wild-type MEFs and re-expression of rictor in rictor-KO MEFs restored mSREBP1 levels (Fig. 2); and 4) inhibition of mTORC2 with both mTOR kinase inhibitors and deficiency with rictor or Sin1 suppressed the expression of ACC and FASN, which are encoded by two well-known SREBP1 target genes, and reduced lipid droplet formation (Fig. 7). Hence, it is clear that, in addition to mTORC1, mTORC2 also plays a critical role in promoting SREBP1 activation and lipogenesis.

Rapamycin is generally thought to be weak or inactive against mTORC2. In this study, we found that rapamycin also decreased mSREBP1 levels, as did INK128 or AZD6055, in some cancer cell lines such as H157 and HCT116. We recently reported that acute or short-term treatment of certain cancer cell lines (e.g., H157) with rapamycin disrupted the assembly of not only mTORC1, but also mTOCR2, despite increasing Akt phosphorylation, demonstrating that rapamycin inhibits mTORC2 in addition to mTORC1 in at least some cancer cell lines [26]. Therefore, it is not surprising to see mSREBP1 reduction induced by rapamycin in some cell lines.

The current study has resulted in some important findings towards defining the mechanism by which mTORC2 positively regulates SREBP1 activation. We have shown that inhibition of mTORC2 triggers proteasomal degradation of mSREBP1, as demonstrated by the decreased stability of mSREBP1 upon INK128 treatment, rictor knockdown and rictor knockout and the rescue of INK128-induced mSREBP2 reduction by proteasomal inhibition with MG132 (Fig. 3). Moreover, we have further shown that mTORC2 inhibition-induced mSREBP1 degradation is mediated by the E3 ubiquitin ligase FBXW7 and is at least in part

dependent on GSK3, since inhibition of GSK3 with both chemical inhibitors and genetic knockdown in part rescued INK128-induced mSREBP1 reduction (Fig. 4) and knockdown or knockout of FBXW prevented mSREBP1 reduction induced by INK128 or rictor knockdown (Fig. 5). Hence, we conclude that mTORC2 activates SREBP1 by stabilizing mSREBP1 through suppressing its GSK3-dependent and FBXW7-mediated degradation. We noted that inhibition of GSK3 with either chemical inhibitors or gene knockdown only in part rescued the INK128-induced decrease in mSREBP1 levels (Fig. 4). Whether this implies an incomplete inhibition of GSK3 activity or that an additional kinase is involved in priming phosphorylation of mSREBP1 for FBXW7-mediated degradation needs further investigation.

GSK3 is known to be inactivated by Akt through phosphorylation. Given that mTORC2 functions as an Akt S473 kinase, it is reasonable to question the involvement of Akt in mTORC2-dependent stabilization of mSREBP1. Previous studies have generated conflicting results regarding the involvement of Akt in regulation of mTORC2-dependent lipogenesis: one study found that constitutive Akt activation in mTORC2-deficient hepatocytes restored lipogenesis suppressed by mTORC2 deficiency [20], while another observed that enforced Akt activation failed to activate lipogenesis in the absence of mTORC2 or in liver specific rictor-KO mice [21]. In both studies, the involvement of Akt in mTORC2 activation of SREBP1 was not investigated. In our study, we found that inhibition of Akt with different inhibitors (e.g., PI3K and Akt inhibitor) did not mimic the ability of INK128 to decrease mSREBP1 levels across the different tested cell lines. Moreover, knockdown of Akt also did not mimic the effect of rictor knockdown in decreasing mSREBP1 levels (Fig. 6). Therefore, we favor a model in which mTORC2 positively regulates mSREBP stability independent of Akt.

It has been shown that mTORC2 does not affect GSK3 activity although it directly activates Akt, since Sin1 knockout induced suppression of mTORC2, which abolished Akt S473 phosphorylation without affecting GSK3 phosphorylation [27]. In our previous study, we showed that different mTOR kinase inhibitors, at the typical tested concentration ranges (e.g., 100 nM), suppressed Akt S473 phosphorylation without inhibiting GSK3 phosphorylation [22]. Consistently, another study also reported that AZD8055 did not suppress GSK3 phosphorylation in rhabdomyosarcoma cells [28]. These data indicate that inhibition of mTORC2 does not necessarily lead to GSK3 activation. In our current study, we did not see INK128 suppression of GSK3 phosphorylation either, although it potently inhibited Akt S473 phosphorylation and decreased mSREBP1 levels. Therefore, we believe that mTORC2 inhibition-induced GSK3-dependent mSREBP1 degradation is unlikely to be secondary to Akt inhibition. Considering the importance of constitutive GSK3 activity in maintaining the biological activities of mTOR kinase inhibitors including the induction of cyclin D1 and Mcl-1 degradation (our unpublished data) and growth inhibition [22], we suggest that the presence of constitutive GSK3 activity may be sufficient for mSREBP1 to undergo FBXW7-mediated degradation upon mTORC2 inhibition. We are currently investigating the mechanism(s) by which mTORC2 negatively regulates GSK3-dependent and FBXW7-mediated protein degradation.

An increased rate of lipid synthesis or lipogenesis in cancerous tissues has long been recognized as an important aspect of the rewired metabolism of transformed cells [3].

Accordingly, targeting SREBPs and lipogenesis in cancer cells inhibits their growth and survival [4–7]. Some agents have been shown to exert their anticancer activity via suppression of SREBPs and lipogenesis [7, 29–31]. mTOR kinase inhibitors have been recently developed as potential anticancer agents and are currently tested in clinical trials [32]. Therefore it is likely that the anticancer activity of the mTOR kinase inhibitors is attributed at least in part to their ability to inhibit SREBP1 activity and lipogenesis. In addition to cancer, other diseases such as coronary artery disease, non-alcoholic fatty liver disease, obesity, type II diabetes, immunity disorders and neuronal diseases are also associated with altered SREBPs expression and lipid homeostasis [8, 14, 33]. Thus, the potential application of mTOR kinase inhibitors in the treatment of these diseases should also be considered and investigated.

In summary, our current study has demonstrated that mTORC2 positively regulates SREBP1 activity and lipogenesis through suppressing GSK3-dependent and FBXW7-mediated degradation of mSREBP1 and subsequent expression of SREBP1 target genes involved in regulation of lipogenesis, hence connecting mTORC2 to the positive regulation of lipid metabolism, a novel biological function of mTORC2. Accordingly, mTOR, particularly mTORC2, inhibitors may have potential application in the treatment of certain diseases associated with abnormal lipid metabolism such as cancer, diabetes and obesity.

Materials and Methods

Reagents

All mTOR kinase inhibitors, the GSK3 inhibitor SB216763, the proteasome inhibitor MG132, the protein synthesis inhibitor cycloheximide (CHX) and the antibodies against GSK3α/β, phospho-GSK3α/β (Ser21/9), phospho-Akt (Ser473), Akt, rictor and raptor were the same as described previously [22]. The GSK3 inhibitor CHIR99021 was purchased from LC laboratories (Woburn, MA). BKM120 was supplied by Novartis Pharmaceuticals Corporation (East Hanover, NJ). API-1 (NSC177233) was obtained from the National Cancer Institute (Bethesda, MD). MK2206 was purchased from Active Biochem (Maplewood, NJ). Perifosine was supplied by Keryx Biopharmaceuticals, Inc (New York, NY). Oil Red O was purchased from Sigma Chemical Co. (St. Louis, MO). SREBP1 (sc-13551), FASN (sc-55580) and α-tubulin (sc-23948) antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). ACC antibody (#3662) was purchased from Cell Signaling Technology, Inc. (Beverly, MA). Polyclonal Sin1 antibody (A300-910A) was purchased from Bethyl Laboratories, Inc. (Montgomery, TX).

Cell lines and cell culture

Human lung cancer cell lines (A549, H1299, H157 and Calu-1) used in this study, A549-pLKO.1, A549-shRaptor and A549-shRictor stable cell lines were described in our previous papers [22, 34]. BCPAP human thyroid cancer cell line was authenticated and provided by Dr. R. Schweppes (University of Colorado School of Medicine, Aurora, CO). LOXIMVI (human melanoma) and HeLa (human cervical cancer) cells were obtained from Dr. P. Giannakakou (Weill Medical College of Cornell University, New York, NY) and Dr. R. Lotan (M.D. Anderson Cancer Center, Houston, TX), respectively. Immortalized WT and rictor-KO MEFs [35] were provided by M. A. Magnuson (Vanderbilt University Medical

Center, Nashville, TN). Rictor-KO MEFs with re-expression of myc-rictor (Rictor-KO/rictor) or matched vector (Rictor-KO/vector) [36] were provided by Dr. D. D. Sarbassov (M.D. Anderson Cancer Center, Houston, TX). WT and Sin1-KO MEFs [27] were provided by Dr. B. Su (Yale University School of Medicine, New Haven, CT). HCT116-FBXW7-WT and HCT116-FBXW7-KO cell lines were provided by Dr. B. Vogelstein (Johns Hopkins University School of Medicine, Baltimore, MA). Except for A549, H157 and BCPAP cells, other cell lines have not been authenticated. These cell lines were cultured in RPMI 1640, DMEM or McCoy's medium containing 5% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Protein preparation and Western blot analysis

Cells were seeded into 10-cm dishes and cultured until 70–80% confluence. The cells were then switched to 0.5% FCS conditions (to minimize the suppressive effect of lipid sterol in serum on mSREBP production) overnight and then treated with tested agents. Whole-cell protein lysates were prepared with lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride, 2.0% Igepal CA-630, 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 1 mM EDTA and 1% Triton X-100). Western blot analysis was performed as described previously [22].

Gene knockdown by siRNA

The non-silencing (control), rictor, raptor, GSK3α/β and FBXW7 siRNAs were the same as described previously [22]. Akt siRNA I (#6211) that targets Akt1 and Akt2 and siRNA II (#6510) that targets Akt1 were purchased from Cell Signaling (Beverly, MA). Transfection of these siRNA duplexes was conducted in 6-well plates using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's manual.

RT-PCR and real-time PCR (qPCR)

Total cellular RNA was isolated using TRIzol regents. 2 μg of total RNA was then used as templates for the reverse transcription reaction using the Easy Transcript kit (abm; Richmond, BC, Canada) according to the manufacturer's instructions. RT-PCR for detection of FBXW7 was the same as described previously [22]. Detection of SREBP1 target genes including *FASN* and *ACACA* was conducted by qPCR with the iTaq Universal SYBR Green Supermix (Bio-Rad) on a 7500 Fast Real-time PCR System (Life Technologies/Applied Biosystems; Grand Island, NY) following the manufacturer's instructions. The primers for these genes are as follows: *FASN* 5′-AACTCCAAGGACACAGTCACCAT-3′ (forward) and 5′-CAGCTGCTCCACGAACTCAA-3′ (reverse); *ACACA* 5′-GGATGGGCGGAATGGTCTCTTT-3′ (forward) and 5′-GCCAGCCTGTCGTCCTCAATGTC-3′ (reverse); and *Actin* 5′-CTCTTCCAGCCTTCCTTCCT-3′ (forward) and 5′-AGCACTGTGTTGGCGTACAG-3′ (reverse).

Oil red O staining

Cells on coverslips were washed with PBS, followed by fixing the cells with 10% formalin at room temperature for 1 h. After removing formalin and washing cells with distilled water

twice and 60% isopropanol (for 5 min at RT), the cells were then dried at room temperature. The dried cell were then stained with 0.21% Oil red O working solution at 50 °C for 20 min. Oil red O solution was then removed and the cells were then immediately washed with 60% isopropanol once for 5 seconds and then distilled water for 4 times [37]. Images were then acquired under the microscope and lipid droplet-positive cells were counted.

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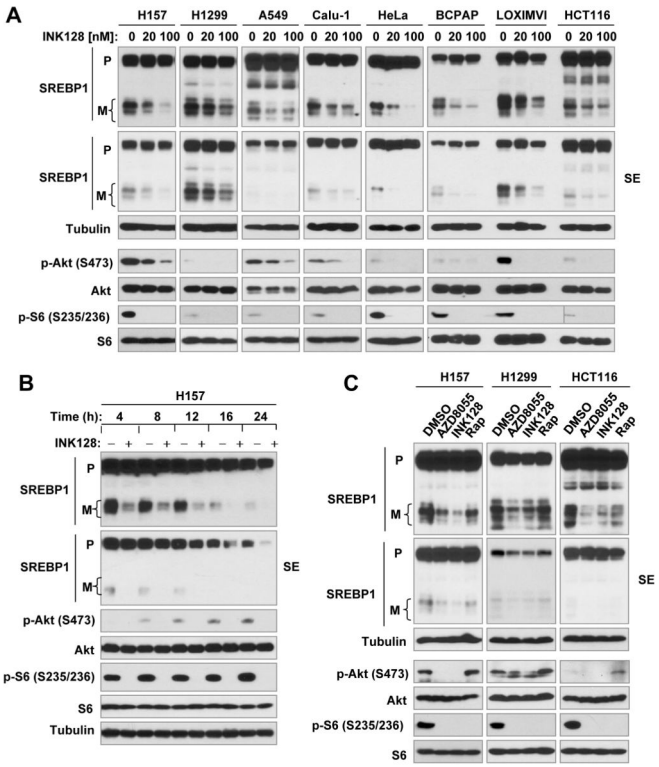

Fig. 1. mTOR kinase inhibitors reduce mSREBP1 levels in cancer cells

A, The various cell lines as indicated were treated with different concentrations of INK128 for 8 h. *B*, H157 cells were treated with 100 nM INK128 for different times as indicated. *C*, The given cell lines were treated with 100 nM INK128, 100 nM AZD8055 or 10 nM rapamycin (Rap) for 12 h. After the aforementioned treatments, whole-cell protein lysates were prepared from these cell lines and subjected to Western blotting for detection of the indicated proteins. SE, short exposure; P, precursor; M, mature form.

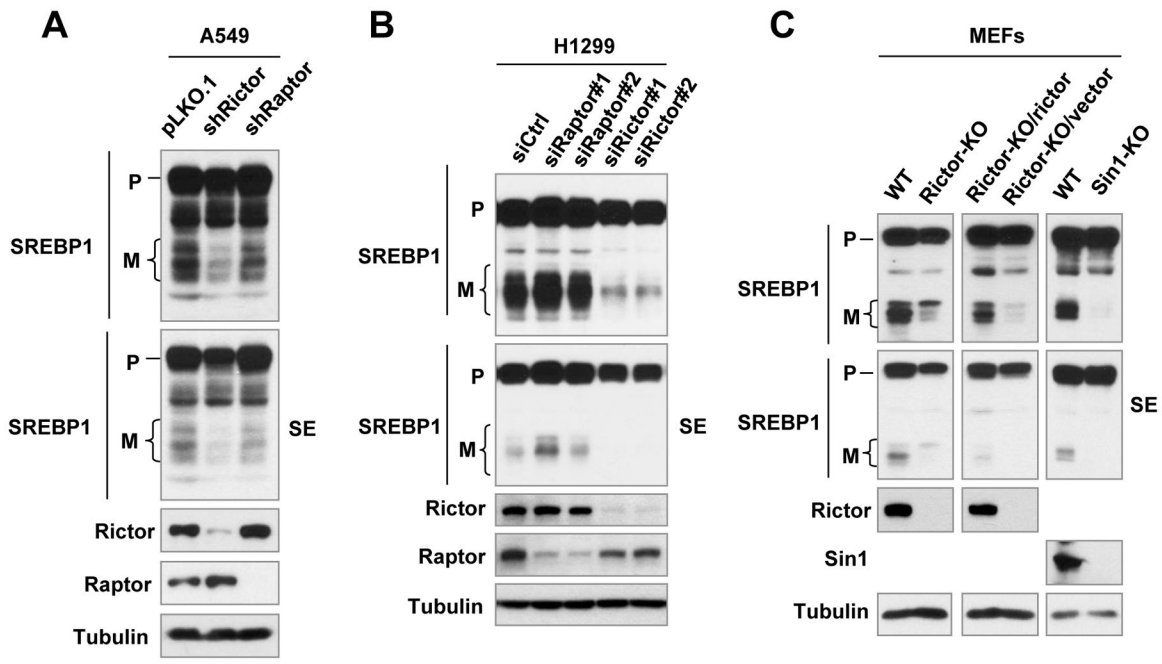

Fig. 2. Genetic inhibition of rictor or Sin1, but not raptor, expression reduces mSREBP1 levels

Whole-cell protein lysates were prepared from the indicated A549 transfectants (*A*), H1299 cells transiently transfected with different siRNAs as indicated for 48 h (B) or MEFs (C) and were then subjected to Western blotting to detect the indicated proteins. SE, short exposure; P, precursor; M, mature form.

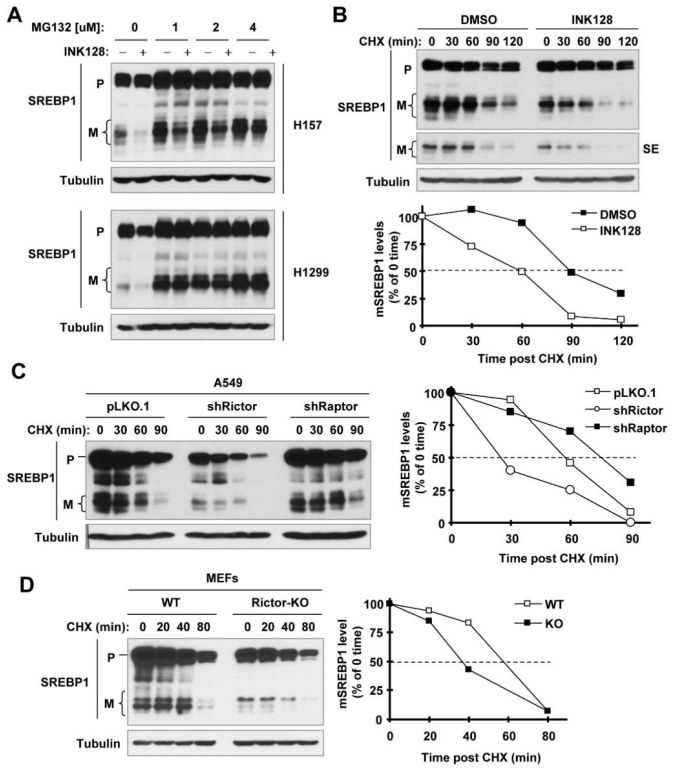

Fig. 3. INK128 treatment (*A* and *B*) or genetic rictor suppression (*C* and *D*) promotes mSREBP1 degradation (*A*) and decreases its stability (*B–D*)

A, The indicated cell lines were pre-treated with the indicated concentrations of MG132 for 30 min and then co-treated with DMSO or 100 nM INK128 for an additional 4 h. The cells were then harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. *B*, H1299 cells were treated with DMSO or 100 nM INK128 for 4 h. The cells were then washed with PBS 3 times and re-fed with fresh medium containing 10 μg/ml CHX. At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. *C* and *D*, The indicated cell lines were exposed to 10 μg/ml CHX. At the indicated times, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Protein levels in *B–D* were quantified with NIH Image J Software and were normalized to tubulin. SE, short exposure; P, precursor; M, mature form.

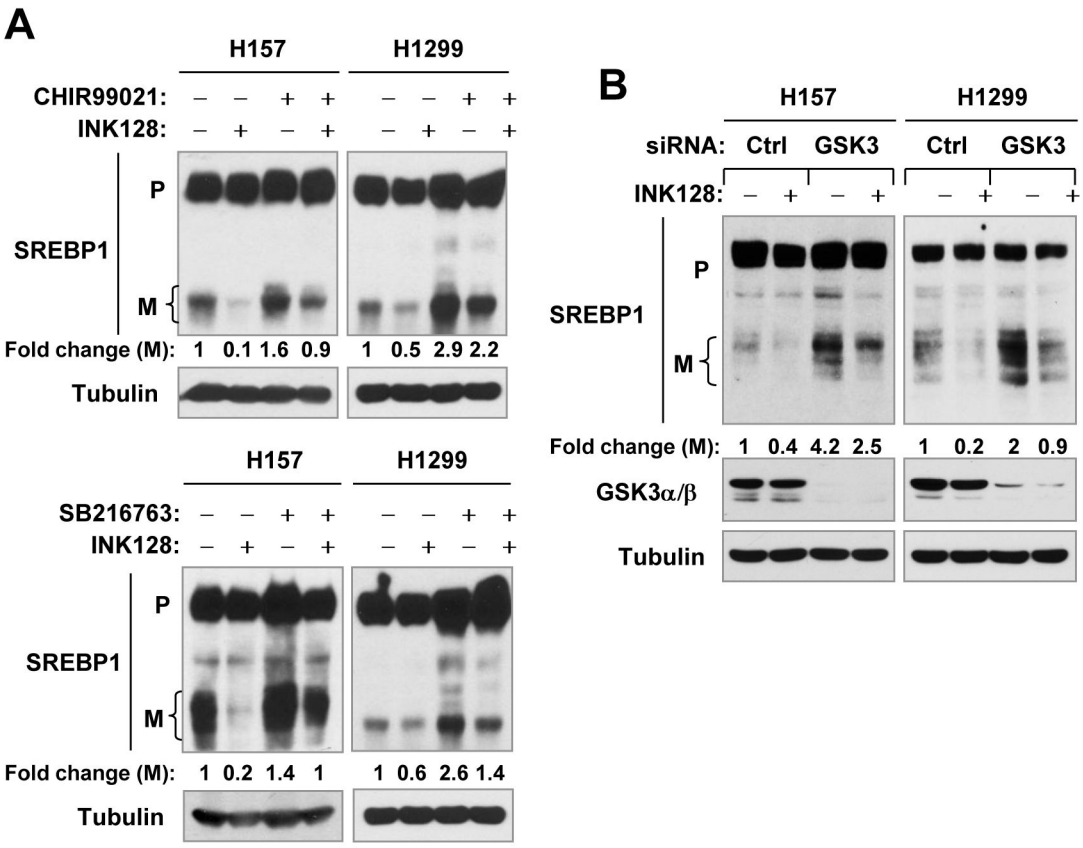

Fig. 4. Inhibition of GSK3 with SB216763 or CHIR99021 (*A*) and siRNA knockdown (*B*) in part rescues mSREBP1 reduction induced by INK128

A, The indicated cell lines were pretreated with 10 μM SB216763 or CHIR99021 for 30 min and then co-treated with 100 nM INK128 for an additional 5 h. *B*, H157 or H1299 cells were transfected with the indicated siRNAs and after 48 h were exposed to 100 nM INK128 for an additional 8 h. After the aforementioned treatments, whole-cell protein lysates were prepared from these cells for Western blotting to detect the given proteins. P, precursor; M, mature form.

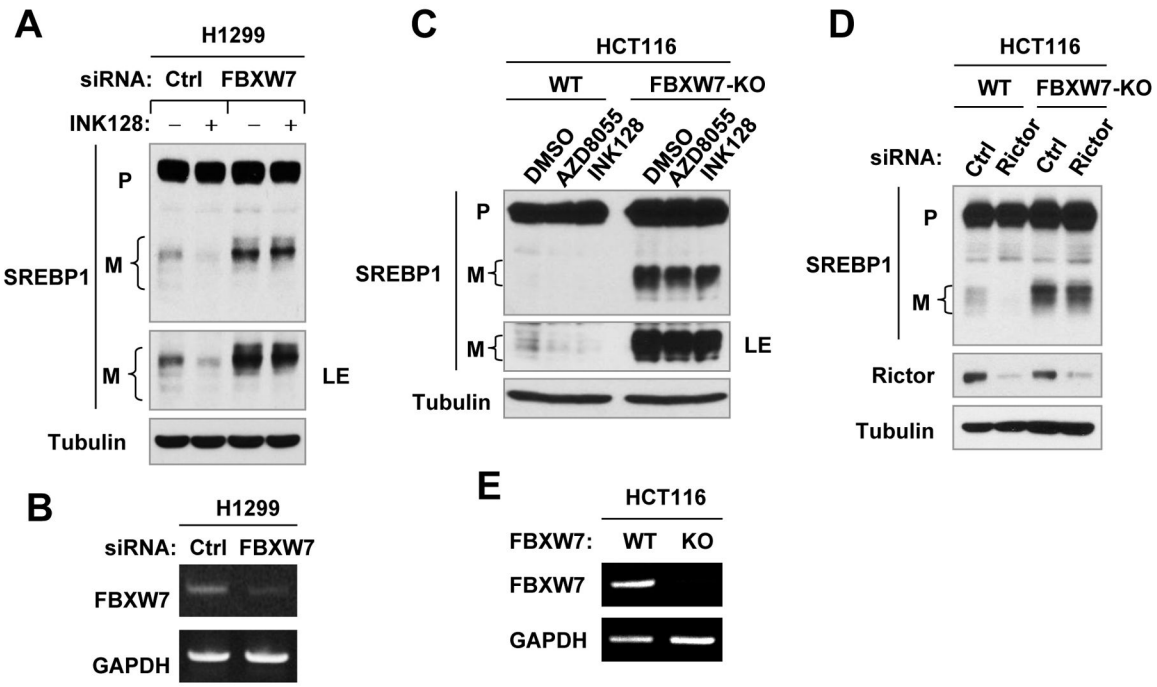

Fig. 5. Knockdown (*A* and *B*) or knockout (*C*–*E*) of FBXW7 rescues mSREBP1 reduction induced by mTOR kinase inhibitors (*A* and C) or rictor silencing (*D*)

A, H1299 cells were transfected with control (Ctrl) or FBXW7 siRNA and after 48 h were exposed to 100 nM INK128 for an additional 8 h. *B*, FBXW7 knockdown efficiency in *A* was evaluated with RT-PCR. *C*, WT and FBXW7-KO HCT116 cell lines were treated with 100 nM of the indicated mTOR kinase inhibitors for 8 h. *D*, WT and FBXW7-KO HCT116 cells were transfected with the given siRNAs for 48 h. After these treatments, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blotting. *E*, FBXW7 knockout in HCT116 cells was confirmed with RT-PCR. LE, longer exposure; P, precursor; M, mature form.

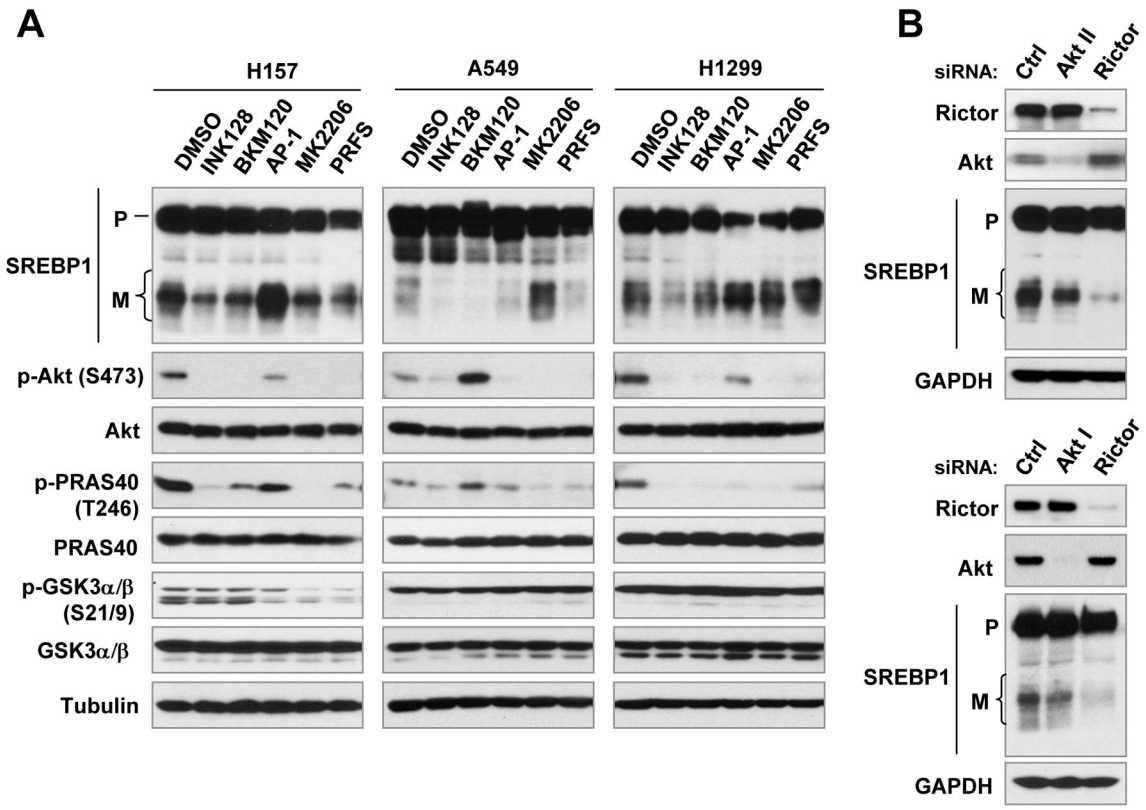

Fig. 6. Comparing the effects of INK128 with other PI3K or AKT inhibitors (*A*) and the effects of rictor with Akt knockdown (*B*) on decreasing mSREBP1 levels in cancer cells

A, The indicated cell lines were treated with DMSO, 100 nM INK128, 1 μM BKM120 or 5 μM of API-1, MK2206 and perifosine (PRFS) for 8 h. *B*, H1299 cells were transfected with the indicated siRNAs for 48 h. After the aforementioned treatment or transfection, the cells were harvested for preparation of whole-cell protein lysates and subsequent Western blotting. P, precursor; M, mature form.

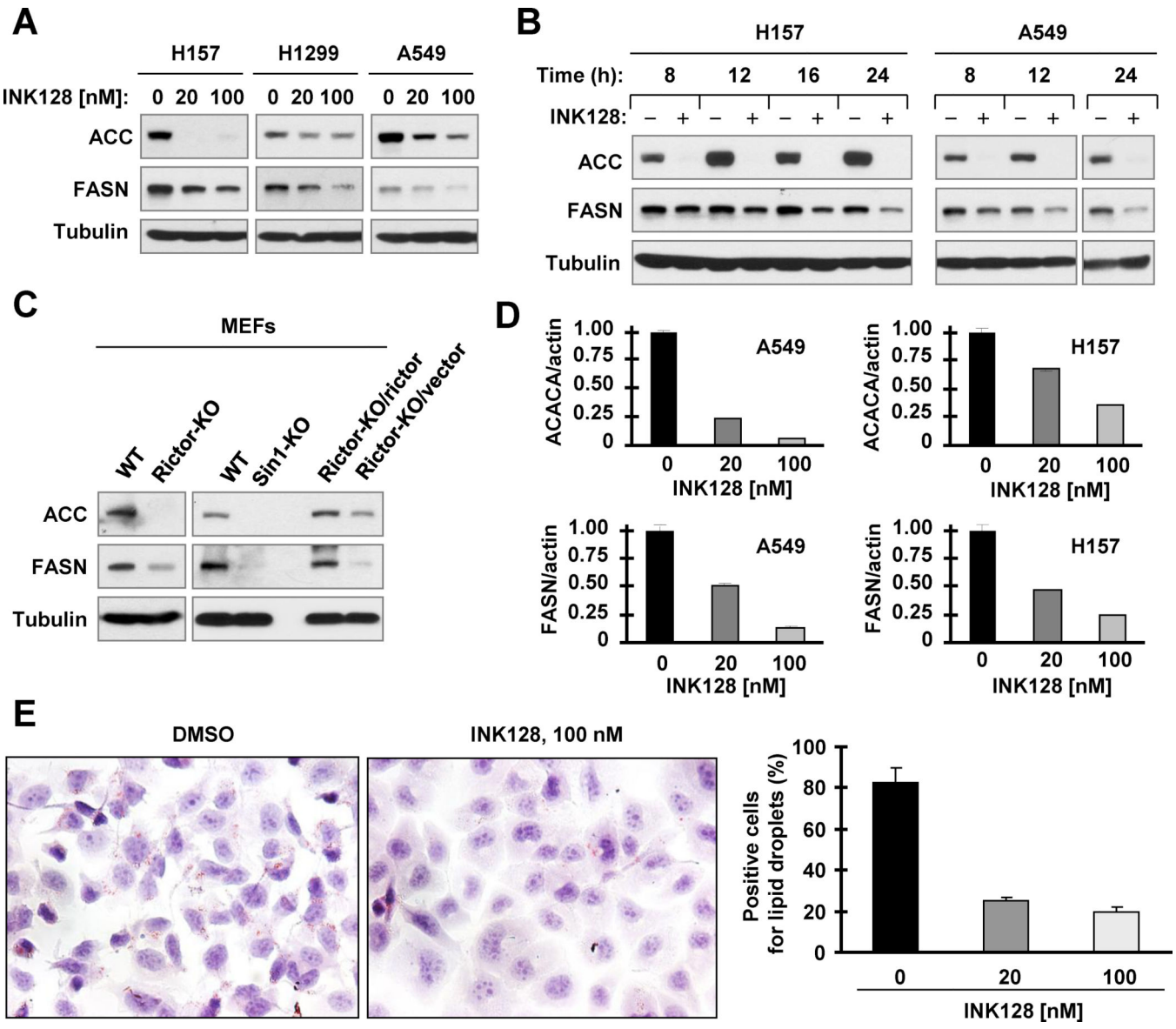

Fig. 7. INK128 treatment (*A*, *B*, *D* and E) or genetic depletion of rictor or sin1 (*C*) suppresses the expression of SREBP1 target genes at both the protein (*A–C*) and mRNA (*D*) level and inhibits the formation of lipid droplets (*E*)

A–C, Whole-cell protein lysates were prepared from the given cell lines treated with the indicated concentrations of INK128 for 8 h (*A*), exposed to 100 nM INK128 for different times as indicated (*B*), or deficient in the indicated genes (*C*) and then subjected to Western blotting to detect the indicated proteins. *D*, The given cell lines were treated with the indicated concentrations of INK128 for 8 h. Total cellular RNA were then prepared from these cells to detect the expression of the given genes with qPCR. *E*, H1299 cells were exposed to 100 nM INK128 for 48 h and then subjected to Red Oil O assay for detection of lipid droplets.