Cancer Research

Destabilization of Fatty Acid Synthase by Acetylation Inhibits *De Novo* Lipogenesis and Tumor Cell Growth

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Abstract

Fatty acid synthase (FASN) is the terminal enzyme in *de novo* lipogenesis and plays a key role in cell proliferation. Pharmacologic inhibitors of FASN are being evaluated in clinical trials for treatment of cancer, obesity, and other diseases. Here, we report a previously unknown mechanism of FASN regulation involving its acetylation by KAT8 and its deacetylation by HDAC3. FASN acetylation promoted its degradation via the ubiquitin–proteasome pathway. FASN acetylation enhanced its association with the E3 ubiquitin ligase TRIM21. Acetylation destabilized FASN and resulted in decreased *de novo* lipogenesis and tumor cell growth. FASN acetylation was frequently reduced in human hepatocellular carcinoma samples, which correlated with increased HDAC3 expression and FASN protein levels. Our results suggest opportunities to target FASN acetylation as an anticancer strategy. *Cancer Res; 76(23); 6924–36. ©2016 AACR.*

Introduction

In *de novo* lipogenesis, fatty acid synthase (FASN) catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH as a reducing equivalent. FASN expression is commonly low in normal and nonproliferating cells, which typically import lipids from the extracellular milieu. In contrast, actively proliferating cells, especially tumor cells, have increased demands for lipids, which is highly dependent on *de novo* synthesis. Accordingly, FASN is frequently upregulated in many types of tumors, such as breast, prostate, liver, colon, and ovarian cancers (1–6). Moreover, increased FASN expression is linked to chemoresistance, tumor metastasis, and poorer prognosis in numerous types of cancers (7–10). These observations suggest that FASN plays an important role in affecting tumor metabolism, and FASN-catalyzed *de novo* lipid synthesis could be a rational therapeutic target for cancer. Extensive efforts have been invested into the development of FASN inhibitors for treating cancer, as well as nonproliferative disease, such as obesity. For instance, cerulenin and its semisynthetic derivate C75, both of which inhibit the condensation reaction of fatty acid synthesis, can induce apoptosis in breast cancer cells and delay tumor progression in a xenograft model of ovarian cancer (11, 12); orlistat, which inhibits the release of palmitate (13), can induce apoptosis, inhibit cell proliferation, and suppress tumor growth in prostate cancer cells (14). However, many current FASN inhibitors exhibit strong toxicity in preclinical and clinical trials (15), underscoring the importance of identifying new strategies to target FASN.

Regulation of FASN has been studied extensively, and its regulation at the level of transcription has been the primary focus to date. Human *FASN* gene expression is controlled by multiple transcription factors, including upstream stimulatory factor (USF), sterol-regulatory element–binding protein-1 (SREBP-1), and carbohydrate-responsive element–binding protein (16–18). It has been reported that hypoxia, which is a common microenvironment inside solid tumors, induces SREBP-1 expression and increases *FASN* transcription in cultured human breast cancer cells (19). Insulin, an important metabolism and energy regulator, triggers *FASN* mRNA expression via USF and SREBP-1 recruitment to *FASN* promoter in *Drosophila* SL2 cells and mouse 3T3L1 adipocytes (20–22). Moreover, *FASN* mRNA expression is

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upregulated by EGF in cultured human prostate cancer cells (23) and in the liver of rats fed a carbohydrate/protein diet (24). In addition, *FASN* mRNA expression can also be downregulated in mouse livers by glucagon, an antagonistic hormone of insulin (25). Furthermore, polyunsaturated fatty acids and leptin can suppress *FASN* mRNA expression via impairing the recruitment of SREBP-1 and other nuclear factors to *FASN* promoter in cultured breast cancer cells (26) and in rat hepatocytes and adipocytes (27). In contrast to the transcriptional regulation of *FASN*, much less is known about its posttranslational regulation.

As an evolutionarily conserved protein posttranslational modification, acetylation regulates a wide range of biological processes, gene transcription and metabolism, in particular (28, 29). Several acetylome studies have identified more than 15,000 acetylation sites on >4,500 proteins, including FASN (29–32). In this study, we investigate the regulatory mechanism and functional consequence of FASN acetylation.

Materials and Methods

In vivo ubiquitylation assay

In vivo ubiquitylation assay was performed as described previously (33, 34). Briefly, cells were harvested at 36 hours after transfection and then lysed in 1% SDS (0.5 mmol/L Tris-HCl, pH 7.5, 0.5 mmol/L EDTA, 1.0 mmol/L DTT) with protease and phosphatase inhibitor cocktail, and then immunoprecipitated in 0.1% SDS by dilution. Analyses of ubiquitylation were determined by Western blot analysis.

Cell culture

HEK293T, HCT116, and ZR-75-30, which have been used in our current study, were obtained from the ATCC in 2015, where they were characterized by mycoplasma detection, DNA fingerprinting, isozyme detection, and cell vitality detection. HEK293T, HCT116, and ZR-75-30 cell lines were cultured in DMEM (Invitrogen), McCoy's 5A Medium (Sigma), and RPMI1640 medium (Sigma), respectively, supplemented with 10% FBS (Gibco) in the presence of 100 U/mL penicillin and 100 μg/mL streptomycin at 37°C with 5% CO_2, and cells were immediately stored in liquid nitrogen till use. A new frozen vial of the same batch of cells was restarted every 2 to 3 months.

FASN enzyme activity assay

FASN enzyme activity was determined as described previously (4). Briefly, the reaction mixture consisted of 150 μg particle-free supernatant, 200 mmol/L potassium phosphate buffer (pH 6.6), 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.24 mmol/L NADPH, and 30 μmol/L acetyl-CoA (Sigma) in a total volume of 300 μL. Reactions were initiated by adding 50 μmol/L of malonyl-CoA (Sigma) and analyzed at 25°C. Activities were measured spectrophotometrically by recording oxidation of NADPH at A340 nm (HITACH F-4600 fluorescence spectrophotometer). Rates were corrected for the background rate of NADPH oxidation in the presence of acetyl-CoA. FASN activity was calculated in nmol/L NADPH oxidized min^{-1} mg $protein^{-1}$.

Measurement of cellular lipid content

HCT116 cells were seeded on a microscope cover glass in a 35-mm culture plate at a density of 1×10^6 cells per well. Cells were washed with PBS and then subjected to a hyperspectral SRS microscope to measure biochemical components. Detailed setup of the SRS microscope has been described previously (35), except that the 2.3=MHz acousto-optic was replaced by a 20-MHz electro-optic modulator. Images (512 × 512 pixels) were sequentially acquired via the layer by layer scanning model (step size 1 μm) under frequencies of 2,850 cm^{-1} (lipid) and 2,928 cm^{-1} (protein), and then reconstructed into two 3D images. The intensity of pixels in 3D images was regarded as the quantity of lipids or proteins and added together after the background was eliminated. Lipid contents were normalized to protein contents in each image. All data were processed using Matlab software.

Cell proliferation analysis

HCT116 or ZR-75-30 stable cells were seeded in 6-well plates at a density of 1×10^5 cells per well. Culture medium was refreshed every day. RGFP966 (10 μmol/L) was added into the culture medium at day 1 and refreshed every day. Cell numbers were counted every day over a period of 5 days.

Human hepatocellular carcinoma samples

Following physician-obtained informed consent from patients, hepatocellular carcinoma samples were collected by the Department of Liver Surgery, Liver Cancer Institute, Zhongshan Hospital, Fudan University (Shanghai, China). The procedures related to human subjects were approved by the Ethics Committee of the Institutes of Biomedical Sciences, Fudan University.

Statistical analysis

Statistical analyses were performed with a two-tailed unpaired Student *t* test, except when indicated. All data shown represent the results obtained from triplicated independent experiments (mean ± SD). The values of $P < 0.05$ were considered statistically significant.

Results

Acetylation promotes FASN protein degradation

Multiple acetylated lysine residues were identified in FASN by mass spectrometry (30–32). Western blotting with a pan anti-acetyl lysine antibody demonstrated that Flag-tagged FASN was indeed acetylated, and its acetylation increased by 2.2-fold in HEK293T cells after combined treatment with nicotinamide, an inhibitor of the SIRT family deacetylases (36), and trichostatin A (TSA), an inhibitor of histone deacetylase (HDAC) I and II (Fig. 1A; ref. 37). When FASN-overexpressing cells were separately treated with either nicotinamide or TSA, the acetylation level of FASN-Flag was increased by TSA (2.4-fold), but not by nicotinamide (1.1-fold), suggesting that FASN is deacetylated by an HDAC family deacetylase. In agreement, acetylation of endogenous FASN increased by >2-fold after TSA treatment in three different cell types, HEK293T, HCT116, and ZR-75-30 cells (Fig. 1B and C). Notably, TSA-dependent increase in FASN acetylation was accompanied by decreased FASN protein levels (Fig. 1D and E). *FASN* mRNA expression was, however, either unaffected (HEK293T and HCT116 cells) or even upregulated (ZR-75-30 cells) by TSA treatment (Fig. 1F). Together, these results suggest that acetylation may promote FASN protein degradation.

Acetylation promotes FASN degradation via the ubiquitin–proteasome pathway

Inhibition of protein synthesis with cycloheximide demonstrated that FASN is a rather stable protein in HCT116 cells with an

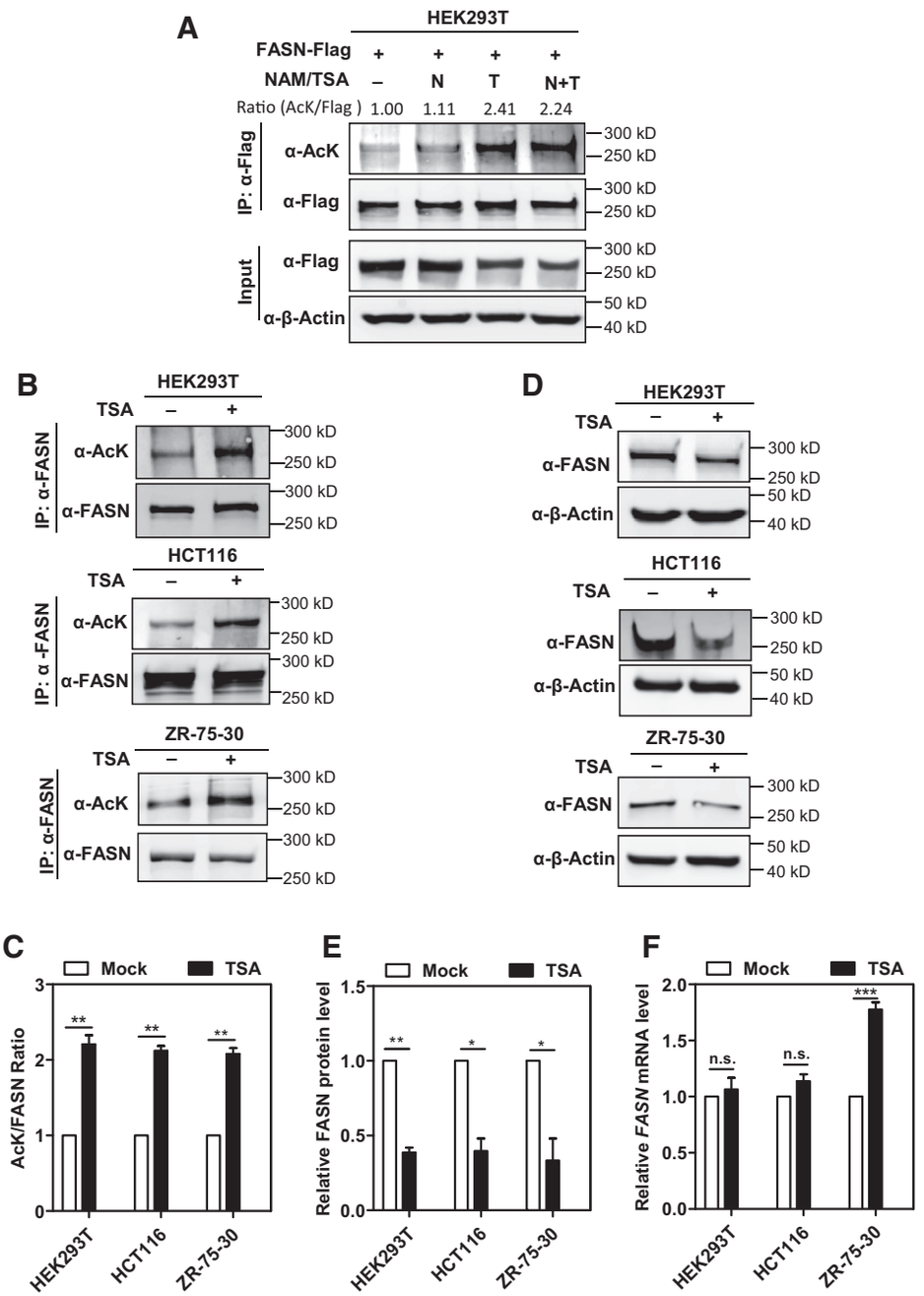

Figure 1.
Acetylation promotes FASN protein degradation. **A,** HEK293T cells overexpressing FASN were treated without or with control solvent, nicotinamide (5 mmol/L, 6 hours), and/or TSA (0.5 mmol/L, 16 hours). Acetylation levels of immunopurified FASN-Flag were determined by Western blotting using a pan-anti-acetyl lysine antibody (α-AcK). IP, immunoprecipitation. Relative FASN acetylation was normalized by Flag protein. **B,** Endogenous FASN protein was immunoprecipitated with an anti-FASN antibody from the indicated cells treated with control solvent alcohol or TSA (0.5 mmol/L, 16 hours). Acetylation levels of endogenous FASN were determined by Western blotting. **C,** Quantification of acetylation levels of endogenous FASN in the indicated cell lines in **B**. Relative FASN acetylation ratios were calculated after normalizing against total FASN protein. **D,** The indicated cells were treated with control solvent alcohol or TSA (0.5 mmol/L, 16 hours), followed by Western blotting to determine endogenous FASN protein levels. **E,** Quantification of endogenous FASN protein levels in the indicated cell lines in **D**. Relative FASN protein levels were normalized by β-actin. **F,** The indicated cells were treated with control solvent alcohol or TSA (0.5 mmol/L, 16 hours), followed by qRT-PCR to determine endogenous *FASN* mRNA expression. Shown are average values with SD of triplicated experiments. *, $P < 0.05$ and **, $P < 0.01$ for the indicated comparison; n.s., not significant.

approximate half-life of 11 hours (Fig. 2A). FASN half-life was reduced to approximately 7 hours by TSA treatment in HCT116 cells (Fig. 2A). Likewise, FASN half-life was also shortened by 4 hours after TSA treatment in HEK293T cells (Supplementary Fig. S1A), suggesting that acetylation destabilizes FASN protein.

In eukaryotes, there are two major pathways by which cellular proteins are degraded, the ubiquitin–proteasome pathway and the autophagy–lysosome pathway. Treatment with leupeptin, an inhibitor of lysosomal degradation, did not increase FASN protein in HEK293T cells (Supplementary Fig. S1B). In contrast, treatment with the proteasome inhibitor MG132 increased FASN protein in HEK293T cells (Fig. 2B). Importantly, although inhibition of HDAC by TSA decreased FASN protein by 40% (Fig. 2C, comparing lane 1 with lane 2), this decrease was restored by the treatment of cells with MG132, an inhibitor of 26S proteasome (comparing lane 2 with lane 3). These results indicate that the acetylation-induced FASN destabilization is mediated by the ubiquitin–proteasome system. Supporting this notion, we found markedly increased levels of polyubiquitylated FASN-Flag in HEK293T cells following TSA treatment, but not nicotinamide treatment (Supplementary Fig. S1C and Fig. 2D). Moreover, FASN polyubiquitylation is further increased by TSA treatment in combination with MG132 in these cells (Fig. 2E). The TSA-dependent increase in endogenous FASN acetylation and

polyubiquitylation and the accompanying reduction in FASN protein level in HEK293T cells (Fig. 2F) further support the idea that acetylation promotes FASN polyubiquitylation and protein destabilization.

In addition, we also compared the acetylation levels of ubiquitylated and nonubiquitylated FASN. HEK293T cells ectopically expressing FASN-Flag and HA-Ub were treated with TSA for 16 hours, and following treatment, cell lysates were subjected to three sequential immunoprecipitations, first with Flag beads to precipitate total FASN proteins, then with HA antibody to enrich ubiquitylated FASN, and finally with FASN antibody to precipitate the nonubiquitylated FASN from the supernatant depleted by the HA antibody (Supplementary Fig. S1D). Compared with nonubiquitylated FASN, we found that ubiquitylated FASN was more heavily acetylated (Supplementary Fig. S1D), further confirming the role of acetylation in promoting FASN polyubiquitylation.

KAT8 is a major acetyltransferase of FASN

The acetylation state of a protein is controlled by lysine acetyltransferases (KAT) and deacetylases (KDAC), enzymes that catalyze the addition and removal of an acetyl group from a lysine residue, respectively. To identify potential KAT(s) that are responsible for FASN acetylation, we ectopically expressed a panel of 12 different human KAT proteins and determined their interaction with endogenous FASN in HEK293T cells. Among the KATs tested, only KAT8 was found to interact with endogenous FASN (Fig. 3A and Supplementary Fig. S2). Reciprocal immunoprecipitation confirmed the protein interaction between endogenous FASN and Myc-KAT8 (Fig. 3B). Overexpression of Myc-KAT8 increased the acetylation level of endogenous FASN by 2.2-fold (Fig. 3C). In contrast, knockdown of *KAT8* decreased endogenous FASN acetylation by as much as 55% (Fig. 3D). Consistent with our earlier observation that acetylation promotes FASN protein degradation (Fig. 1D and E), we found that overexpression of Myc-KAT8 decreased the steady-state level of endogenous FASN protein by 59% without a significant change in *FASN* mRNA expression in HEK293T cells (Fig. 3E). Conversely, *KAT8* knockdown increased endogenous FASN protein level by 1.45-fold, again without significantly affecting *FASN* mRNA level (Fig. 3F). These results collectively indicate that KAT8 is a major acetyltransferase of FASN.

HDAC3 is a major deacetylase of FASN

Our earlier observation that TSA, but not nicotinamide, increases FASN acetylation (Fig. 1A) led us to search for the HDAC family enzyme(s) that is involved in FASN deacetylation. We transiently expressed 9 of 11 human HDAC proteins in combination with FASN-Flag and determined their interaction (Figs. 4A and Supplementary Fig. S3A). Among the tested HDACs, only HA-HDAC3 was found to interact with FASN-Flag. Consistent with this result, the protein interaction between endogenous HDAC3 and FASN was readily detected in HEK293T cells (Fig. 4B). Likewise, overexpression of HA-HDAC3 decreased the acetylation level of endogenous FASN by 35% in HEK293T cells, whereas the expression of a catalytic inactive mutant HDAC3^{Y298H} (38) failed to reduce FASN acetylation (Fig. 4C). Conversely, *HDAC3* knockdown increased the acetylation level of endogenous FASN by >1.5-fold in HEK293T cells (Supplementary Fig. S3B). In HEK293T cells with stable *HDAC3* knockdown, the half-life of endogenous FASN protein was shortened by 4 hours (Supplementary Fig. S3C). Surprisingly, the steady-state level of FASN protein was largely unchanged (Supplementary Fig. S3B). We found that *FASN* mRNA expression was increased by as much as 3-fold in HEK293T cells depleted for *HDAC3* (Supplementary Fig. S3B), which is in line with a previous report that deletion of *HDAC3* leads to upregulated *FASN* mRNA expression in mouse liver cells (39).

To provide direct evidence that the catalytic activity of HDAC3 regulates FASN protein stability, we treated cells with RGFP966, a selective inhibitor for HDAC3 (40). RGFP966 selectively inhibits HDAC3 with IC_{50} of 0.08 μmol/L, but it is much weaker toward other HDACs, such as HDAC1 and HDAC2, whose activity was not significantly affected by RGFP966 concentration of up to 15 μmol/L (40). Our data demonstrated that RGFP966 increased the polyubiquitylation levels of both ectopically expressed and endogenous FASN proteins in HEK293T cells in a dose-dependent manner, with a significant increase in FASN polyubiquitylation in cells treated with 10 μmol/L RGFP966 (Fig. 4D and E). Accordingly, RGFP966 treatment decreased endogenous FASN protein levels in a dose-dependent manner with a notable reduction (49%) at 10 μmol/L RGFP966, but without modulating *FASN* mRNA expression in HEK293T cells (Fig. 4F). The effects of RGFP966 on stimulating endogenous FASN polyubiquitylation and subsequent protein degradation were also detected in HCT116 cells (Supplementary Fig. S3D and S3E). Collectively, these data support a critical role of HDAC3 in deacetylating FASN and thus protecting FASN from degradation.

Acetylation promotes FASN interaction with TRIM21 E3 ubiquitin ligase

To elucidate the mechanism underlying acetylation-dependent FASN polyubiquitylation and protein degradation, we established a HEK293T cell line that stably expressed FASN N-terminally tagged with both Flag and streptavidin-binding peptide. Tandem affinity purification followed by mass spectrometry analysis allowed us to identify potential interacting partners of FASN (Supplementary Table S2). Among these FASN-interacting proteins, TRIM21 (also known as RNF81, Ro52, and SSA1), which is a RING domain E3 ubiquitin ligase (41), DDB1 and SMU1, which are components of the CUL4A and CUL4B-based E3 ubiquitin ligase complexes, FBXO22, which is a substrate recognition component of the SCF (SKP1-CUL1-F-box)-type E3 ubiquitin ligase complex, and CUL5 E3 ubiquitin ligase were identified. Next, we transiently coexpressed FASN-Flag, HA-Ub, and Myc-tagged CUL1, CUL4A, CUL4B, CUL5, and TRIM21 and found that Myc-TRIM21, but not cullin proteins, substantially increased the polyubiquitylation level of FASN-Flag (Supplementary Fig. S4A and S4B). Furthermore, ectopic expression of TRIM21 increased the polyubiquitylation level of endogenous FASN in HCT116 cells (Supplementary Fig. S4C). The association of endogenous FASN with either ectopically expressed or endogenous TRIM21 was confirmed by Western blot analyses, and importantly, the FASN–TRIM21 association is enhanced by treatment with HDAC inhibitors in either 293T or HCT116 cell (Fig. 5A and B and Supplementary Fig. S4D). Ectopic expression of wild-type TRIM21, but not the catalytically inactive RING deletion mutant TRIM21, reduced endogenous FASN protein level by 43% in HEK293T cells (Fig. 5C). Conversely, *TRIM21*

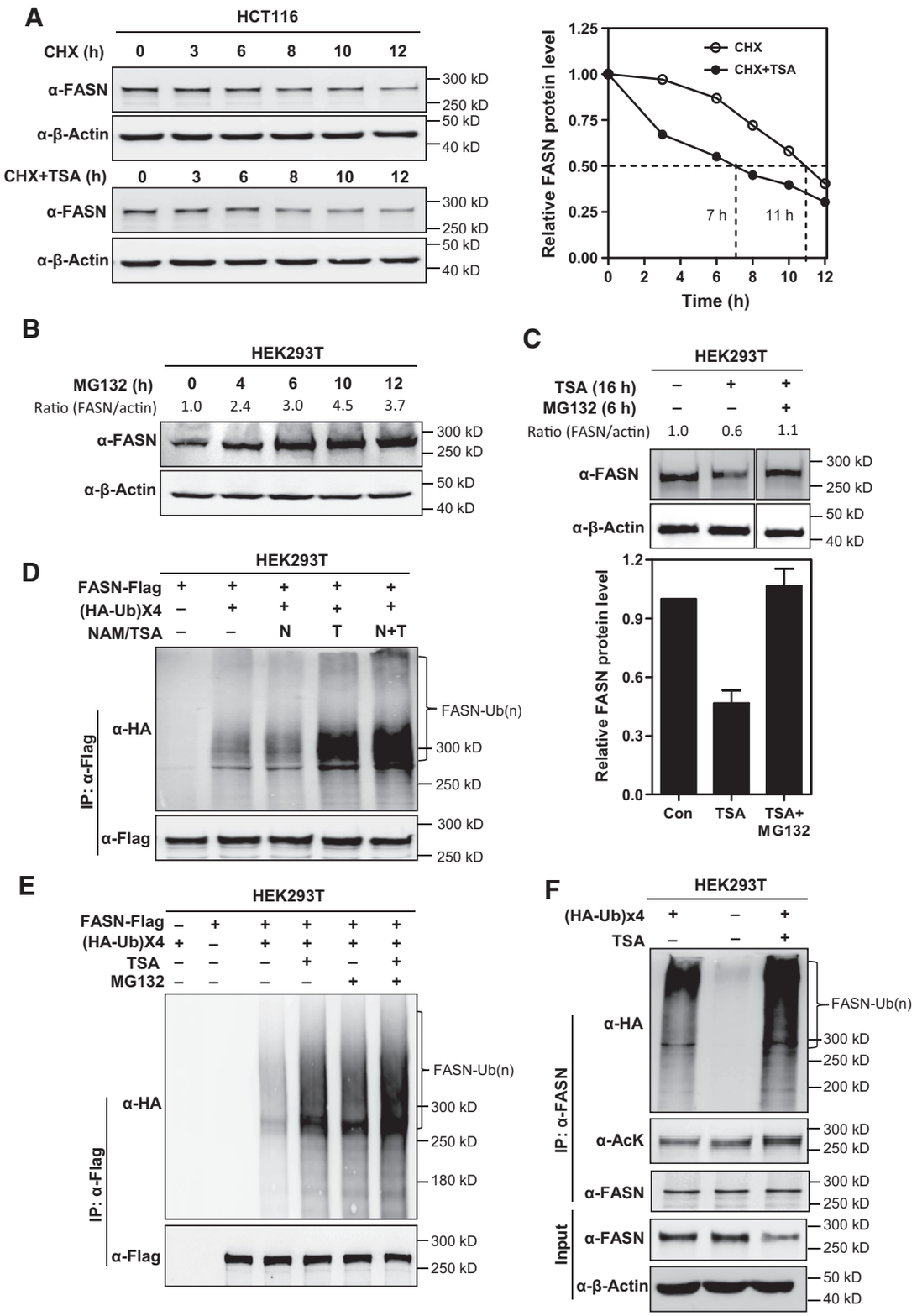

Figure 2.

Acetylation promotes FASN protein polyubiquitylation and destabilization. **A,** HCT116 cells were pretreated with control solvent alcohol or TSA (0.5 mmol/L) and then subjected to cycloheximide (CHX) treatment (10 μg/mL) for the indicated time course. Endogenous FASN protein level was analyzed by Western blotting (left). Quantification of endogenous FASN protein levels is shown (right). (*Continued on the following page.*)

knockout increased endogenous FASN protein levels by >1.5-fold in stable HEK293T cells (Fig. 5D) without altering *FASN* mRNA expression. In addition, we also found that either MG132 or TSA treatment substantially enhanced the increased level of polyubiquitylated FASN-Flag in HEK293T cells ectopically expressing Myc-TRIM21 (Fig. 5E). Notably, the effect of TSA or RGFP966 on enhancing FASN polyubiquitylation was abolished by *TRIM21* deletion in HEK293T cells (Figs. 5F). Together, these results indicate that acetylation enhances FASN interaction with its E3 ubiquitin ligase TRIM21, thereby promoting FASN polyubiquitylation and destabilization.

Acetylation of FASN inhibits *de novo* lipogenesis and tumor cell growth

FASN is essential for *de novo* fatty acid synthesis to support cancer cell growth (15). Inhibition of *de novo* fatty acid synthesis causes cell apoptosis and suppresses tumor cell growth (14, 42). We therefore investigated the role of FASN acetylation in regulating *de novo* lipogenesis and cell proliferation. When HCT116 cells were treated with TSA and RGFP966, we found that the level of endogenous FASN was reduced by 40% and 49%, respectively (Fig. 6A). These reductions in FASN protein led to decreased FASN activity in whole-cell lysate by 50% and 60%, respectively, as measured by spectrophotometrically recorded oxidation of NADPH (Fig. 6A). Stimulated Raman scattering (SRS) microscope has been applied to analyze biological components within cells and tissues by mapping the distribution of diverse biochemical components (35). Here, we employed a hyperspectral femtosecond SRS microscope to detect the lipid content in HCT116 cells upon treatment with TSA or RGFP966. The Raman signals at 2,850 and 2,928 cm^{-1} (S2850 and S2928) reflect the lipid and protein contents, respectively (Supplementary Fig. S5A and S5B). We found in HCT116 cells that the ratio of S2850/S2928 was markedly decreased by 7.93% ($P = 0.02$) and 10.2% ($P = 0.0079$), after TSA and RGFP966 treatment, respectively (Fig. 6B), suggesting that broad inhibition of HDAC enzymes or selective inhibition of HDAC3 reduce intracellular lipid content. Importantly, ectopic expression of FASN rescued the decrease in lipid content caused by either TSA or RGFP966 treatment (Fig. 6B and Supplementary Fig. S5C), supporting the notion that FASN acetylation inhibits *de novo* lipogenesis in cancer cells.

Stable expression of FASN-Flag and treatment with HDAC3 inhibitor RGFP966 resulted in significant increase and decrease of HCT116 cell proliferation (Fig. 6C and Supplementary Fig. S5C). The cell proliferation–stimulatory effect of FASN was blunted by RGFP966 treatment (Fig. 6C). The same effects of FASN overexpression and HDAC3 inhibitor on cell proliferation were also observed in ZR-75-30 breast cancer cells (Supplementary Fig. S5C and Fig. 6E). RGFP966 treatment in both cell lines resulted in decreased levels of FASN protein (Fig. 6D and F). Taken together, these results support the notion that inhibition of HDAC3 promotes FASN protein degradation, thereby decreasing *de novo* lipogenesis and cell proliferation in a cell type–independent manner.

Acetylation of FASN is downregulated in hepatocellular carcinoma tumors

Hepatocellular carcinoma is one of the most common tumors worldwide (43). FASN is highly expressed in many human cancers, including liver cancer (3). Our findings that acetylation promotes FASN degradation and inhibits the ability of FASN to support cancer cell proliferation prompted us to examine both acetylation and total protein levels of FASN in human liver cancers. We carried out direct immunoblot analysis of a panel of 17 pairs of primary hepatocellular carcinoma tumors and their adjacent normal tissues. This analysis revealed that the acetylation level of FASN was invariably reduced in all hepatocellular carcinoma tumor tissues tested when compared with adjacent normal tissues (Fig. 7A and C and Supplementary Fig. S6A). Of these 17 pairs of samples, 14 tumor samples exhibited a significant increase in the steady-state level of FASN protein, whereas three tumor samples had reduced FASN protein levels compared with their corresponding normal tissues (Fig. 7A and B and Supplementary Fig. S6B). Interestingly, most of the tested hepatocellular carcinoma tumors (15/17 pairs) exhibited increased HDAC3 protein expression compared with adjacent normal tissues (Fig. 7A and D and Supplementary Fig. S6B). Finally, our data demonstrate that FASN protein levels had a negative correlation with FASN acetylation levels in hepatocellular carcinoma tumors and adjacent normal tissues ($R^2 = 0.1510$, $P = 0.0231$; Fig. 7E), and HDAC3 protein levels had a positive correlation with FASN protein levels in hepatocellular carcinoma tumors and adjacent normal tissues ($R^2 = 0.1596$, $P = 0.0192$; Fig. 7F). Furthermore, FASN protein levels had no significant association with either KAT8 (Fig. 7A and Supplementary Fig. S6B) or TRIM21 (Supplementary Fig. S6C–S6F) in hepatocellular carcinoma tumors and adjacent normal tissues. Collectively, these data indicate that acetylation of FASN is frequently downregulated in hepatocellular carcinoma tumors, which is at least in part associated with increased HDAC3. Thus, FASN acetylation and HDAC3 may be potential biomarkers for hepatocellular carcinoma tumors.

Discussion

Reprogramming of metabolic pathways is a hallmark of cancer (15, 44). Proliferating cancer cells require continuous *de novo* fatty acid synthesis to provide precursors for membrane biogenesis. Deregulated *de novo* lipogenesis causes abnormal accumulation of fatty acids, affects signal transduction and

(*Continued.*) **B,** HEK293T cells were treated with MG132 (10 μmol/L) for the indicated time course. Endogenous FASN protein level was analyzed by Western blotting. Relative FASN protein levels were normalized by β-actin. **C,** HEK293T cells were treated with control solvent alcohol or TSA (0.5 μmol/L) in the absence or presence of MG132 (10 μmol/L). Endogenous FASN protein level was analyzed by Western blotting, normalized by β-actin protein (top). Please note that the protein levels of endogenous FASN or β-actin in lane 1/2 and lane 3 were determined on the same blot. Quantification of endogenous FASN protein levels are shown (bottom). Shown are average values with SD of triplicated experiments. **D** and **E,** Polyubiquitylation levels of affinity purified FASN-Flag proteins were detected by Western blotting in transfected HEK293T cells under treatment with control solvent, nicotinamide, and/or TSA in the absence (**D**) or presence (**E**) of MG132 (10 μmol/L, 6 hours). N, normal; T, tumor; IP, immunoprecipitation. **F,** Polyubiquitylation, acetylation, and the protein levels of endogenous FASN in HEK293T cells under treatment with control solvent alcohol or TSA (0.5 mmol/L, 16 hours) were detected by Western blotting.

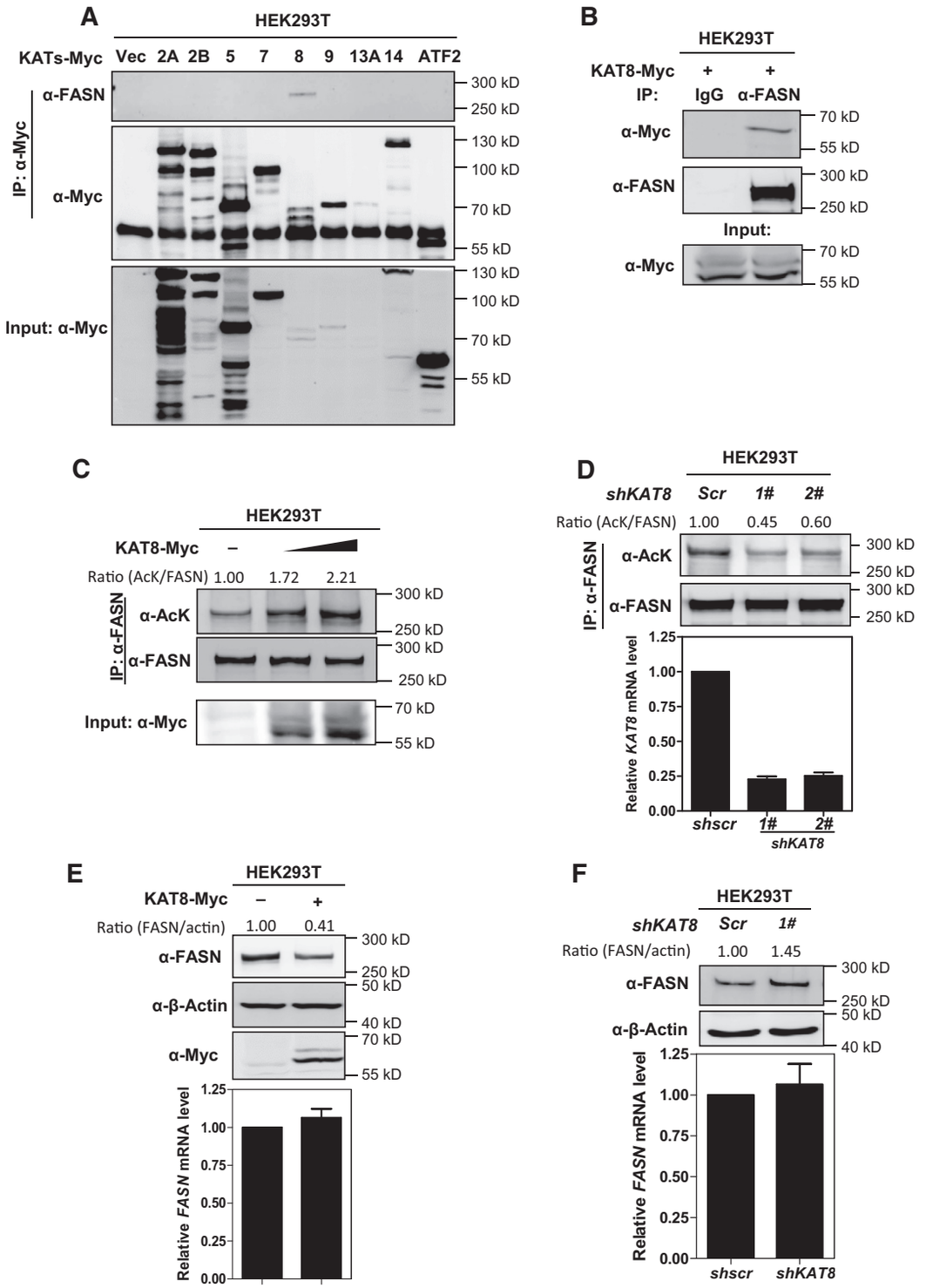

Figure 3.

KAT8 is a major acetyltransferase of FASN. **A** and **B,** Interaction between the indicated Myc-tagged KAT proteins and endogenous FASN was detected by Western blotting. IP, immunoprecipitation. **C,** Increased amount of Myc-KAT8 was transiently overexpressed in HEK293T cells, and then acetylation levels of endogenous FASN proteins were determined. **D,** Stable HEK293T cells with *KAT8* knockdown were generated by retroviral infection, and the knockdown efficiency was determined by qRT-PCR (bottom). Acetylation levels of endogenous FASN were detected by Western blotting (top). **E,** Myc-KAT8 was transiently overexpressed in HEK293T cells, and then mRNA and protein levels of endogenous FASN were determined by qRT-PCR and Western blotting, respectively. Relative FASN protein levels were normalized by β-actin. **F,** In stable HEK293T cells with *KAT8* knockdown, mRNA and protein levels of endogenous FASN were determined by qRT-PCR and Western blotting, respectively. Shown are average values with SD of triplicated experiments.

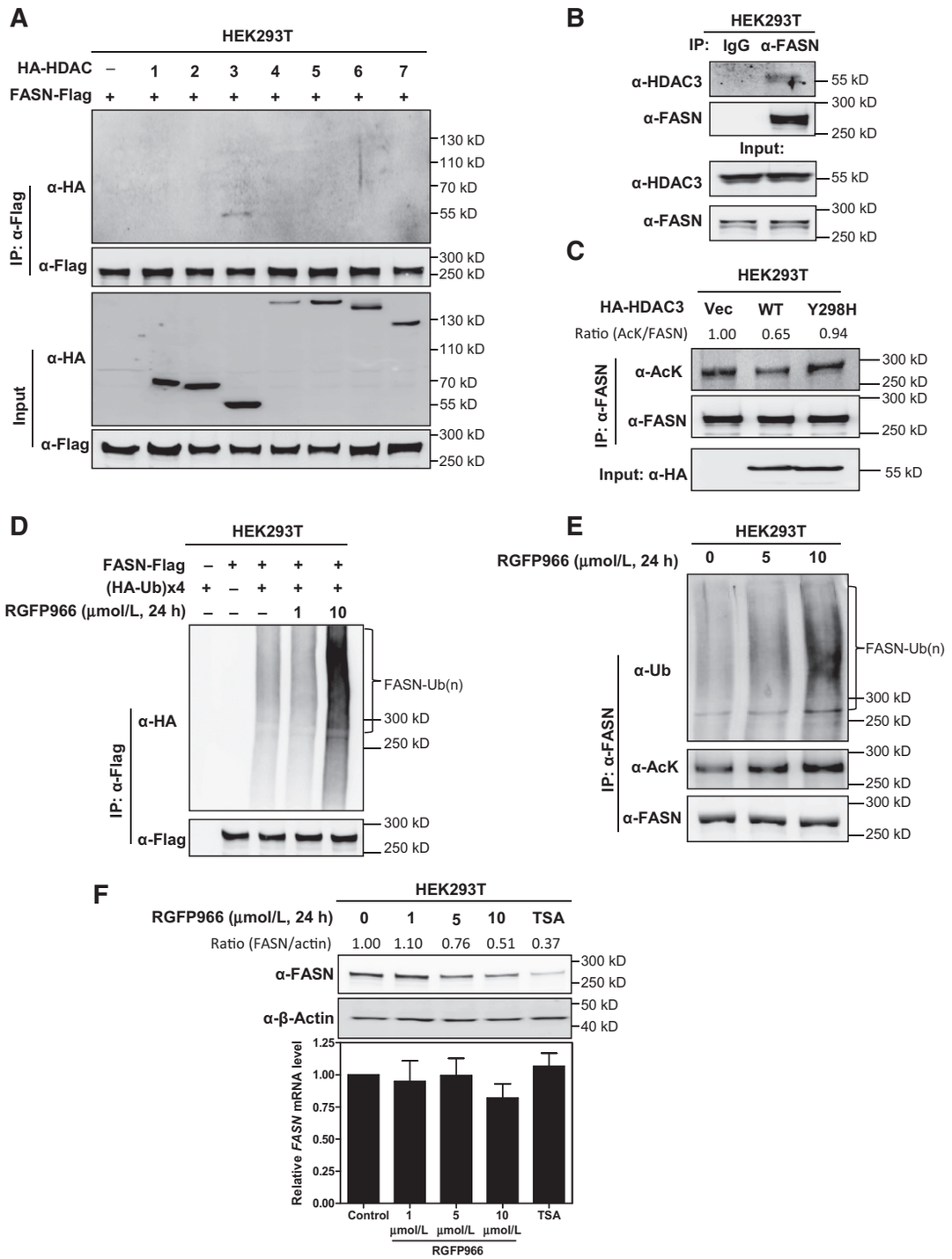

Figure 4.

HDAC3 is a major acetyltransferase of FASN. **A,** Flag-tagged FASN was overexpressed in HEK293T cells in combination with different HA-tagged HDACs as indicated. Flag-FASN protein was immunopurified, followed by Western blotting to detect its interaction with HA-tagged HDACs. IP, immunoprecipitation. **B,** In HEK293T cells, FASN protein was immunopurified with FASN antibody, followed by Western blotting to detect HDAC3 interaction. **C,** HA-tagged wild-type HDAC3 or its catalytically inactive mutant HDAC3^{Y298H} was expressed in HEK293T cells. FASN protein was immunopurified with FASN antibody, followed by Western blotting to detect its acetylation. **D,** Flag-tagged FASN and HA-tagged Ub plasmids were co-overexpressed in HEK293T cells, which were then subjected to increased concentrations of RGFP966 as indicated. FASN protein was immunopurified with Flag beads, followed by Western blotting to detect FASN polyubiquitylation with a HA antibody. **E,** HEK293T cells were subjected to increased concentrations of RGFP966 as indicated. Endogenous FASN was immunopurified, followed by Western blotting to detect its acetylation and ubiquitylation levels. **F,** HEK293T cells were subjected to increasing concentrations of RGFP966 or TSA as indicated. Endogenous FASN mRNA and protein levels were determined by qRT-PCR and Western blotting, respectively. Shown are average values with SD of triplicated experiments.

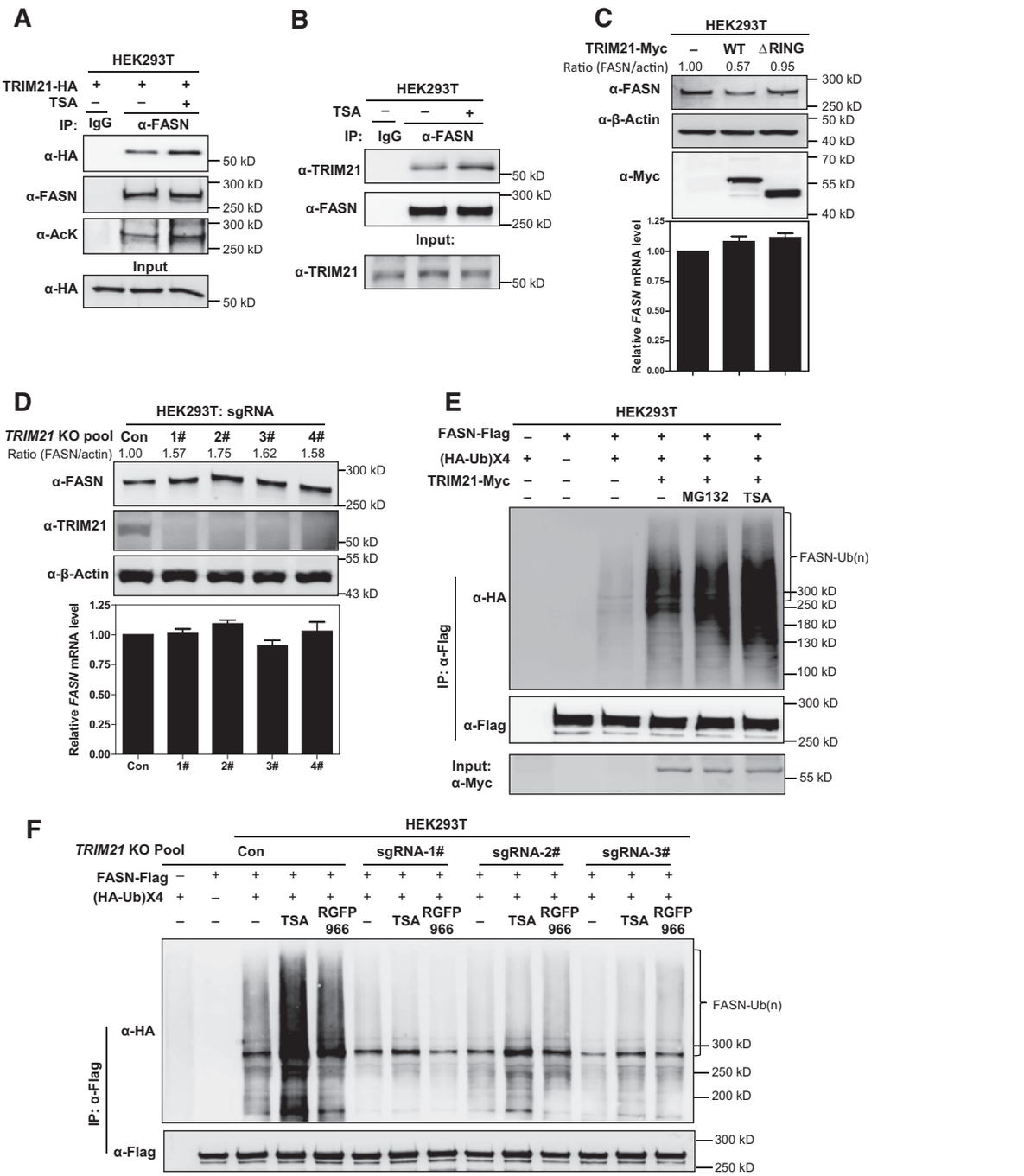

Figure 5.

Acetylation promotes FASN interaction with ubiquitin E3 ligase TRIM21. **A,** HA-TRIM21 was overexpressed in HEK293T cells, and the transfected cells were treated with control solvent alcohol or TSA (0.5 mmol/L, 16 hours). FASN protein was immunopurified, followed by Western blotting to detect its interaction with HA-TRIM21. IP, immunoprecipitation. **B,** In HEK293T cells treated with control solvent alcohol or TSA (0.5 mmol/L, 16 hours), FASN protein was immunopurified with FASN antibody, followed by Western blotting to detect its interaction with endogenous TRIM21. **C,** Myc-tagged wild-type or E3 ligase catalytic inactive mutant TRIM21$^{\Delta RING}$ was overexpressed in HEK293T cells. Endogenous FASN mRNA and protein levels were determined by qRT-PCR and Western blotting, respectively. Shown are average values with SD of triplicated experiments. **D,** In HEK293T cells, *TRIM21* gene was deleted by the CRISPR-Cas9 system. TRIM21 knockout (KO) efficiency and endogenous FASN protein level were determined by Western blotting with the indicated antibodies. Relative FASN protein levels were normalized by β-actin. **E,** TRIM21 promotes FASN polyubiquitylation. FASN-Flag, Myc-TRIM21, and HA-Ub were co-overexpressed in HEK293T cells as indicated. The transfected cells were treated with control (Con) solvents or TSA (0.5 mmol/L, 16 hours) or MG132 (10 μmol/L, 6 hours). FASN-Flag was immunopurified with Flag beads, followed by Western blotting to detect its polyubiquitylation with a HA antibody. **F,** FASN-Flag and HA-Ub were co-overexpressed in *TRIM21* knockout HEK293T cell pools and control cells. The transfected cells were treated with control solvents, TSA (0.5 mmol/L, 16 hours), or RGFP966 (10 μmol/L, 24 hours). FASN-Flag protein was immunopurified, followed by Western blotting to detect FASN polyubiquitylation with an HA antibody.

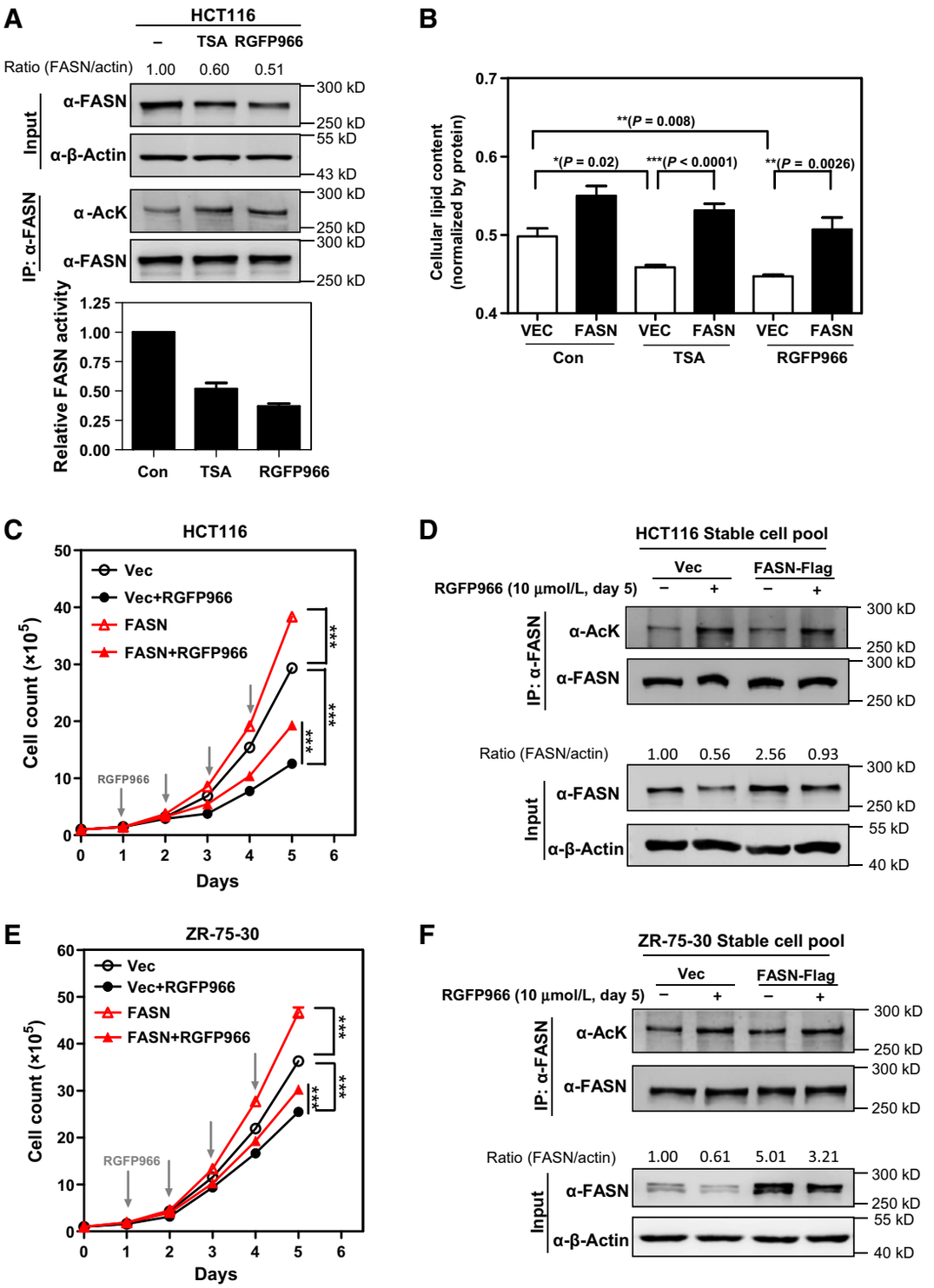

Figure 6.

Acetylation of FASN inhibits *de novo* lipogenesis and suppresses tumor cell growth. **A,** HCT116 cells were treated with control (Con) solvents, TSA (0.5 mmol/L, 16 hours), or RGFP966 (10 μmol/L, 24 hours). IP, immunoprecipitation. FASN activity in the whole-cell lysates was measured (right). FASN protein was immunopurified, followed by Western blotting to detect its acetylation. Relative FASN protein levels were normalized by β-actin. **B,** HCT116 cells stably expressing empty vector or FASN were treated as mentioned in **A**. Lipid content was measured and quantified by employing a SRS microscope as described in Materials and Methods. **C–F,** HCT116 or ZR-75-30 cells stably expressing empty vector or FASN were seeded in 6-well plates and were treated with control solvent DMSO or RGFP966 (10 μmol/L) as indicated. Cell numbers were counted every day over a period of 5 days (**C** and **E**). At day 5, cells were harvested, and the acetylation and protein levels of FASN were determined by Western blotting (**D** and **F**). Relative FASN protein levels were normalized by β-actin.

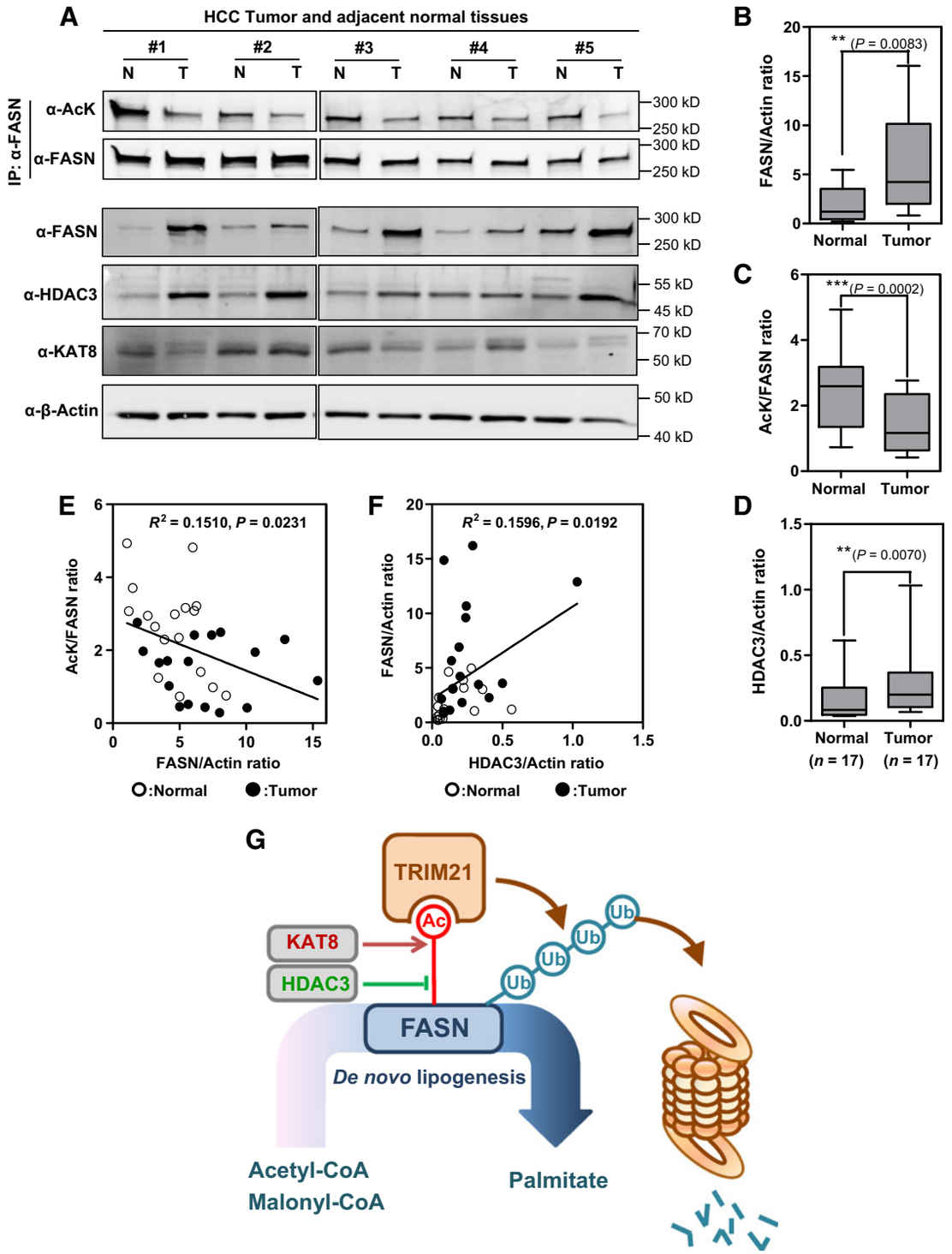

Figure 7.

Acetylation of FASN is downregulated in human hepatocellular cancers (HCC). **A,** In total, 17 pairs of tumor tissues (T) and adjacent normal tissues (N) were lysed. FASN protein was immunopurified with FASN antibody, followed by Western blotting to detect its acetylation. IP, immunoprecipitation. Protein levels of FASN, HDAC3, and KAT8 were determined by direct Western blotting. Relative FASN and HDAC3 protein levels were normalized by β-actin. Shown are five pairs of samples. See Supplementary Fig. S6 for the other 12 pairs of samples. **B–D,** Quantification of relative FASN protein, FASN acetylation, and relative HDAC3 protein levels in the 17 pairs of samples tested. **E** and **F,** Correlation between FASN protein levels and its acetylation levels (**E**) or HDAC3 protein levels (**F**) in the tested 17 pairs of samples. Shown are average values with SD. Statistical analyses were performed with a two-tailed paired *t* test. **, $P < 0.01$ and ***, $P < 0.001$ for the indicated comparison. **G,** Shown is a working model depicting how acetylation promotes FASN protein degradation through the ubiquitin–proteasome pathway, thereby inhibiting *de novo* lipogenesis.

gene expression, and eventually influence tumor cell survival. FASN, as one of the rate-limiting enzymes in the *de novo* lipogenesis pathway, has been found to be overexpressed in a wide variety of human cancers by multiple mechanisms (2, 7, 9, 45). For instance, *FASN* gene expression is controlled by SREBP-1, which is the major transcriptional regulator of *FASN* and is upregulated by hypoxia in solid tumors (19); Isopeptidase USP2a interacts with and stabilizes FASN by deubiquitinating and preventing its proteasomal degradation (45). In this study, we uncovered a novel biochemical mechanism of FASN regulation at the posttranscriptional level. Our data demonstrate that KAT8 catalyzes FASN acetylation, which then promotes FASN interaction with TRIM21 E3 ubiquitin ligase, leading to subsequent polyubiquitylation and proteasomal degradation. Conversely, HDAC3-mediated deacetylation of FASN contributes to increased FASN protein and elevated *de novo* fatty acid synthesis in tumor cells (Fig. 7G).

TRIM21 belongs to the tripartite motif (TRIM) family and acts as a RING finger domain–containing E3 ligase (41). Several reported substrates of TRIM21 are all involved in innate and adaptive immunity, including IRF3, IRF5, IRF7, IRF8, and SQSTM1/p62 (41, 46–50). Results from our study demonstrate that FASN is a novel substrate of TRIM21. We show that TRIM21 physically interacts with and ubiquitylates FASN to regulate its protein stability. In addition, our data revealed that FASN acetylation enhanced its interaction with TRIM21. It will be interesting to determine how TRIM21-mediated FASN ubiquitylation affects cellular immunity and other nonproliferative property of the cell.

Our study also shows that HDAC3 inhibitor RGFP966 increases FASN acetylation and destabilizes FASN protein, supporting a critical role for HDAC3-regulated FASN acetylation in the control of *de novo* lipid synthesis and the regulation of tumor cell growth/survival. We found that in primary hepatocellular carcinoma cancer samples, the acetylation level of FASN is frequently reduced, and both FASN and HDAC3 protein levels are upregulated when compared with adjacent normal tissues. These findings indicate that HDAC3 may function as an oncogenic factor with a potential role in deacetylating and stabilizing FASN, thereby promoting *de novo* lipid synthesis to support rapid cancer cell growth. These findings suggest that HDAC3 inhibitors may merit exploration as a therapeutic agent for cancers such as hepatocellular carcinoma.

Disclosure of Potential Conflicts of Interest

K.-L. Guan has ownership interest (including patents) in Vivace Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.-P. Lin, Z.-L. Cheng, R.-Y. He, K.-L. Guan, Y. Xiong
Writing, review, and/or revision of the manuscript: H.-P. Lin, R.-Y. He, B.S. Groh, K.-L. Guan, D. Ye, Y. Xiong
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-P. Lin, R.-Y. He, L. Song, L.-S. Zhou, B.S. Groh, Y. Xiong
Study supervision: D. Ye, Y. Xiong

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