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Ubiquitin-specific peptidase 7 (USP7)-mediated deubiquitination of the histone deacetylase SIRT7 regulates gluconeogenesis

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Sirtuin 7 (SIRT7), a member of the NAD$^+$-dependent class III histone deacetylases, is involved in the regulation of various cellular processes and in resisting various stresses, such as hypoxia, low glucose levels, and DNA damage. Interestingly, SIRT7 is linked to the control of glycolysis, suggesting a role in glucose metabolism. Given the important roles of SIRT7, it is critical to clarify how SIRT7 activity is potentially regulated. It has been reported that some transcriptional and post-transcriptional regulatory mechanisms are involved. However, little is known how SIRT7 is regulated by the post-translational modifications. Here, we identified ubiquitin-specific peptidase 7 (USP7), a deubiquitinase, as a negative regulator of SIRT7. We showed that USP7 interacts with SIRT7 both *in vitro* and *in vivo,* and we further demonstrated that SIRT7 undergoes endogenous Lys-63-linked polyubiquitination, which is removed by USP7. Although the USP7-mediated deubiquitination of SIRT7 had no effect on its stability, the deubiquitination repressed its enzymatic activity. We also showed that USP7 coordinates with SIRT7 to regulate the expression of glucose-6-phosphatase catalytic subunit (*G6PC*), a gluconeogenic gene. USP7 depletion by RNA interference increased both *G6PC* expression and SIRT7 enzymatic activity. Moreover, SIRT7 targeted the *G6PC* promoter through the transcription factor ELK4 but not through forkhead box O1 (FoxO1). In summary, SIRT7 is a USP7 substrate and has a novel role as a regulator of gluconeogenesis. Our study may provide the basis for new clinical approaches to treat metabolic disorders related to glucose metabolism.

SIRT7, a member of the mammalian sirtuins, belongs to a family of NAD$^+$-dependent class III histone deacetylases (HDACs)[3] (1–3). Thus far, only a few of its molecular substrates, including H3K18 (4), PAF53 (5), NPM1 (6), GABPβ1 (7), U3-55K (8), PGK1 (9), CDK9 (10), and FKBP51 (11) have been reported. Recently, we systematically identified a comprehensive list of SIRT7 candidate substrates via SILAC-coupled bioinformatics-based prediction analysis, which will contribute to further understanding of the biological role of SIRT7 in the future (12). SIRT7 has important roles in the control of diverse cellular processes, ranging from cellular homeostasis, aging, and DNA repair to cancer (13). SIRT7-deficient mice are associated with a reduced life span, the accumulation of lactate, reduced exercise performance, cardiac dysfunction and hypertrophy, hepatic microvesicular steatosis, and hearing defects (7, 14). SIRT7 has oncogenic potential because its expression is elevated in several cancers and is associated with tumor progression and aggressiveness (4, 15–20). SIRT7 mainly concentrates in the nucleolus, where it associates with the rDNA transcription machinery and positively regulates rDNA transcription and ribosome biogenesis (2, 5, 21, 22). As a chromatin-enriched protein, SIRT7 selectively targets acetylated histone H3K18, through which it modulates chromatin structure and transcriptional competency to maintain the transformation of cancer cells (4). SIRT7 also has a role in resisting various types of stresses, such as hypoxia, endoplasmic reticulum stresses, low glucose levels, and genomic insults (5, 23–25). Moreover, SIRT7 is involved in DNA repair and the maintenance of genomic integrity (26–29). Similar to the role of the sirtuins in metabolic regulation (30–35), SIRT7 plays a significant role in hepatic lipid metabolism (24, 36) and mitochondrial homeostasis (7, 37). Interestingly, SIRT7 is linked to the control of glycolysis through the deacetylation of PGK1 in liver cancer cells (9). Whether SIRT7 contributes to other glucose metabolic pathways has not been specifically addressed.

Given its important physiological functions, it is critical to understand the precise molecular mechanisms by which

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[3] The abbreviations used are: HDAC, histone deacetylase; G6PC, glucose-6-phosphatase catalytic subunit; qPCR, quantitative PCR; Ub, ubiquitin; IP, immunoprecipitation.

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SIRT7 is regulated. Transcriptional and post-transcriptional regulatory mechanisms are involved in the regulation of SIRT7 activity. C/EBPα negatively regulates SIRT7 expression by recruiting HDAC3 to the upstream promoter of *SIRT7* in hepatocellular carcinoma cells (38). Several microRNAs are involved in negatively regulating SIRT7 (16, 39–41). By contrast, little is known about the post-translational mechanisms that regulate SIRT7 activity.

Deubiquitination, the reverse of ubiquitination, removes ubiquitin from modified proteins via deubiquitinating enzymes and is essential for the regulation of transcription, DNA repair, cell cycle progression, protein stability, and endocytosis (42, 43). Based on Ub–protease domains, the cysteine protease-deubiquitinating enzymes are divided into four classes. USP7, also known as herpesvirus-associated ubiquitin-specific protease, belongs to the largest ubiquitin-specific protease subfamily (42, 44). USP7 was originally identified through its interaction with a herpes viral protein, ICP0 (45). USP7 knock-out (KO) mice died during embryonic development (46). A wide range of substrates of USP7 have been identified, including p53 (47), MDM2 (48), FOXO4 (49), PTEN (50), UHRF1 (51, 52), TIP60 (53), DNMT1 (54), and histone H2B (55). Through deubiquitinating a broad range of targets, USP7 controls a variety of biological processes, such as tumor suppression, DNA repair, and epigenetic regulation. Consistent with this broad range of effects, USP7 is abnormally expressed in many solid and non-solid tumors (50, 56, 57), and thus it is considered as a potential drug target (58, 59).

In this study, we identified SIRT7 as a novel substrate of USP7. We demonstrate that USP7 interacts with SIRT7 *in vivo* and *in vitro*. USP7 specifically removes the Lys-63-linked polyubiquitin on SIRT7, which increases the enzymatic activity of SIRT7. The functional association between USP7 and SIRT7 may include their involvement in gluconeogenesis through their influence on the expression of *G6PC*.

Results

USP7 is a SIRT7-interacting protein

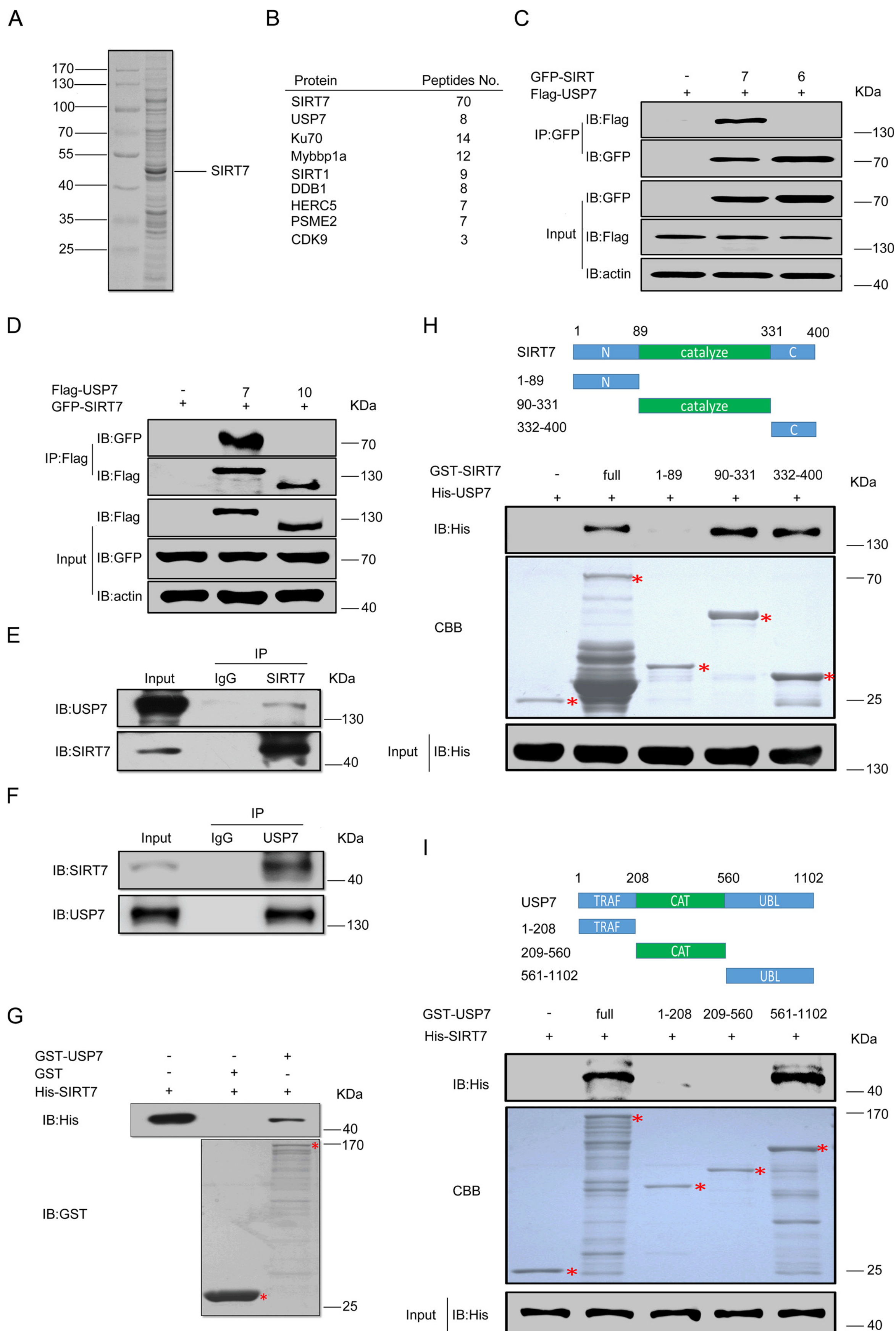
To elucidate the potential post-translational modifications that regulate SIRT7, we used large-scale affinity purification and mass spectrometry to screen for novel SIRT7-interacting proteins in HCT116 cells (Fig. 1*A*). We found previously identified SIRT7-associated proteins, such as SIRT1, Mybbp1a, Ku70, and DDB1 in this purification (Fig. 1*B*) (36, 60, 61), indicating that our approach yielded a reliable identification of SIRT7-binding proteins from HCT116 cells. In addition, we found that USP7 was a major SIRT7-associated protein (Fig. 1*B*). Considering previous reports that other ubiquitin-specific peptidase members, such as USP22 and USP10, regulate the ubiquitination of SIRT1 and SIRT6 (62, 63), we hypothesized that USP7 may regulate the ubiquitination of SIRT7. Therefore, we first investigated whether SIRT7 interacts with USP7. As indicated in Fig. 1*C*, USP7 interacts with SIRT7 but not with SIRT6, another sirtuin family member. Conversely, SIRT7 specifically interacts with USP7 but not with USP10 (Fig. 1*D*). Moreover, an endogenous, reciprocal co-IP assay using antibodies directed against SIRT7 or USP7 confirmed the interaction between SIRT7 and USP7 in physiological conditions (Fig. 1, *E* and *F*). Therefore, the results clearly show USP7 as a specific interacting protein of SIRT7. To investigate whether SIRT7 interacts directly with USP7, a His-tagged SIRT7 expressed in bacteria was incubated with GST or GST-USP7. As shown in Fig. 1*G*, His-SIRT7 showed a direct interaction with GST-USP7 but not with GST alone. To map the region of SIRT7 that mediates its interaction with USP7, truncated mutants of SIRT7 were generated, and as shown in Fig. 1*H*, the catalytic domain and the C terminus of SIRT7 bound USP7. Similarly, using various deletion plasmids of USP7, we showed that the UBL domain in the C terminus of USP7 interacts with SIRT7 (Fig. 1*I*). Overall, our data clearly demonstrate that SIRT7 and USP7 physically interact with each other *in vivo* and *in vitro*.

USP7 deubiquitinates SIRT7 in vivo and in vitro

To determine whether USP7 regulates the function of SIRT7 through its deubiquitinase activity, we first investigated whether SIRT7 is ubiquitinated. We performed an *in vivo* ubiquitination assay for SIRT7 and found that endogenous SIRT7 undergoes polyubiquitination *in vivo* (Fig. 2*A*). We also demonstrated that the co-expression of HA-ubiquitin and FLAG-SIRT7 caused a dramatic increase in ubiquitinated SIRT7 in HCT116 cells, which was not observed in the absence of HA-ubiquitin (Fig. 2*B*). The results indicate that SIRT7 undergoes polyubiquitination *in vivo*. Next, to investigate the deubiquitinating capability of USP7 toward SIRT7, we overexpressed USP7 and checked the ubiquitination level of overexpressed SIRT7 in HCT116 cells. We found that the overexpression of USP7 significantly reduced the level of polyubiquitination on overexpressed SIRT7 (Fig. 2*C*); by contrast, knockdown of USP7 by siRNA increased the ubiquitination of endogenous SIRT7 (Fig. 2*D*), indicating that USP7 can deubiquitinate SIRT7 *in vivo*. In addition, we found that a catalytic mutant USP7 (C223S) failed to reduce the ubiquitination level of SIRT7 (Fig. 2*E*). Thus, our results suggest that USP7 is a potential deubiquitinase for SIRT7. To further confirm whether USP7 directly deubiquitinates SIRT7, we performed an *in vitro* deubiquitination assay. Ubiquitinated SIRT7 from HCT116 cells was incubated with purified GST, and the GST-USP7 fusion protein, as shown in Fig. 2*F*, GST-USP7, but not GST alone, inhibited SIRT7 ubiquitination. Taken together, these results demonstrate that SIRT7 is a direct substrate of USP7.

USP7 does not control SIRT7 stability

Deubiquitinase usually protects proteins from proteasome degradation via its deubiquitinating capacity. To further investigate whether USP7 stabilizes the endogenous protein levels of SIRT7, we overexpressed USP7 in a dose-dependent manner and measured endogenous SIRT7 expression levels in HCT116 cells. Interestingly, endogenous SIRT7 protein levels were not increased by the dose-dependent overexpression of USP7 (Fig. 3*A*). Similarly, USP7 overexpression had no obvious effect on the exogenous SIRT7 (Fig. 3*B*); in addition, suppression of USP7 by siRNA did not affect the expression level of SIRT7 in HCT116 cells (Fig. 3*C*). To study the effect of USP7 on SIRT7 protein stability in more detail, we measured the half-life of



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SIRT7 after treatment with cycloheximide. As shown in Fig. 3*D*, transient transfection of USP7 did not affect the half-life of SIRT7. Moreover, there were no significant changes in SIRT7 transcriptional levels upon USP7 knockdown or overexpression (Fig. 3*E*). Collectively, these findings suggest that the polyubiquitination of SIRT7 may not be responsible for its degradation in the cell. Importantly, the cellular function of ubiquitin varies depending on the position of the Lys residues involved in the polyubiquitin chain (64). Therefore, we further investigated the type of ubiquitin chains conjugated to SIRT7. Using mutant ubiquitin constructs, we showed that SIRT7 could be associated with Lys-63-branched but not Lys-48-branched, ubiquitin (Fig. 3*F*). Furthermore, data from the immunoprecipitation assay showed that USP7 removed Lys-63-linked polyubiquitin from SIRT7 (Fig. 3*G*). We further confirmed the deubiquitinating activity of USP7 on Lys-63-linked polyubiquitination using a Lys-63-linked specific antibody (Fig. 3*H*). Finally, we found that SIRT7 ubiquitination dramatically decreased with a mutant in which only Lys-48 was replaced by arginine residues, further excluding the possibility of removing Lys-48 ubiquitination (Fig. 3*I*). Taken together, our data indicate that USP7 removes Lys-63-linked polyubiquitination on SIRT7 and had no effect on SIRT7 protein stabilization.

USP7 negatively regulates SIRT7 deacetylase activity

Lys-63-linked polyubiquitin chains regulate protein activation (65). Therefore, we investigated whether USP7 regulates the enzymatic activity of SIRT7. Because H3K18Ac is the typical substrate of SIRT7, we hypothesized that USP7 would affect H3K18 acetylation levels by deubiquitinating SIRT7. As shown in Fig. 4*A*, USP7 knockdown with two different siRNAs decreased the acetylation of H3K18 in HCT116 cells, whereas total histone levels remained unchanged. Similar results were obtained in the LoVo colon cancer cell line (Fig. 4*B*). Moreover, this decrease in H3K18 acetylation recovered when both SIRT7 and USP7 were knocked down by siRNA (Fig. 4*C*), which confirmed the inhibition of SIRT7 deacetylase activity by USP7. To further determine whether the catalytic activity of USP7 is required for SIRT7-mediated H3K18 acetylation, we performed a rescue assay. As shown in Fig. 4*D*, H3K18 acetylation levels were significantly decreased in USP7 knockdown cells, and the decreased acetylation was rescued upon the co-expression of wild-type USP7 but not of the catalytic mutant USP7 (C223S). These results show that the deubiquitinase activity of USP7 suppresses SIRT7-dependent H3K18 acetylation.

Glucose starvation attenuates the interaction between USP7 and SIRT7

We further investigated the function of the interaction between USP7 and SIRT7 in glucose starvation because the binding affinity of USP7 to SIRT7 decreased upon glucose starvation (Fig. 4*E*). Consistent with this result, we found that the ubiquitination levels of SIRT7 decreased in a glucose dose-dependent manner when cells were cultured in different concentrations of glucose (0–30 mM) (Fig. 4*F*). Moreover, glucose starvation significantly enhanced the ubiquitination level of SIRT7 in HCT116 cells in a time-dependent manner (Fig. 4*G*). The fact that SIRT7 ubiquitination was elevated in USP7 depletion and glucose starvation conditions prompted us to test the possible role of USP7 in the regulation of SIRT7 activity under glucose starvation. We first examined the activity of SIRT7 in glucose-starved HCT116 cells. As shown in Fig. 4*H*, H3K18 acetylation gradually decreased following glucose starvation, and the decrease in H3K18Ac was blocked by SIRT7-siRNA. By contrast, the already low H3K18 acetylation triggered by glucose starvation decreased further with the overexpression of SIRT7 (Fig. 4*I*), which indicated that the low H3K18 acetylation observed under glucose deprivation could be mediated by SIRT7. Next, we validated that USP7 was associated with the activation of SIRT7 upon glucose starvation because the cells transfected with USP7-siRNA had significantly decreased levels of H3K18Ac under glucose starvation (Fig. 4*J*), demonstrating that changes in SIRT7 activity resulted from USP7 siRNA treatment. Moreover, we found that compared with the expression of SIRT7 alone, the co-expression of USP7 with SIRT7 upon glucose starvation blocked the deacetylation of H3K18 (Fig. 4*K*), whereas the catalytically inactive mutant USP7 (C223S) failed to block the decrease in H3K18Ac (Fig. 4*K*). In summary, these data suggested that the interaction between SIRT7 and USP7 decreased in response to glucose starvation, accompanied by reduced H3K18 acetylation. Therefore, we propose that USP7 is a regulator of SIRT7 activity during glucose starvation.

USP7 and SIRT7 regulate common target gene of gluconeogenesis

As NAD^+-dependent histone deacetylases, sirtuins are linked to cellular energy status and gene transcription (66, 67). Therefore, we investigated whether a combination of SIRT7 and USP7 was functionally linked to glucose metabolism. We first compared the expression levels of *G6PC*, a key regulatory enzyme of gluconeogenesis in HCT116 cells, upon SIRT7

Figure 1. USP7 is a SIRT7-interacting protein. *A,* proteins pulled down with FLAG-SIRT7 from HCT116 cells were separated by SDS-PAGE and visualized by Coomassie Brilliant Blue (*CBB*) staining. *B,* some of the SIRT7-interacting proteins identified by mass spectrometry are listed in the *table*, and the total peptide numbers for each protein are indicated. *C,* FLAG-tagged USP7 with GFP-tagged SIRT7 or SIRT6 plasmids were transfected into HCT116 cells; proteins were extracted for co-IP with anti-GFP. Western blotting was performed with anti-GFP or anti-FLAG. Actin was probed as a loading control. *D,* FLAG-tagged USP7 or USP10 with GFP-tagged SIRT7 plasmids were transfected into HCT116 cells; proteins were extracted for co-IP with anti-FLAG. Western blotting was performed with anti-GFP or anti-FLAG. Actin was probed as a loading control. *E* and *F,* HCT116 cell lysates were subjected to co-IP with anti-SIRT7 or anti-USP7 antibody, respectively, and normal rabbit IgG was the control, followed by Western blotting using the indicated antibodies. *G,* fusion protein His-SIRT7 was incubated with GST or GST-USP7 *in vitro* for GST pulldown assays. The interaction of SIRT7 and USP7 was detected by Western blotting using an anti-His antibody. GST or GST-USP7 was detected by Western blotting using an anti-GST antibody. *H,* structures of SIRT7 and its truncated mutants are shown in the *top panels*. His-tagged USP7 was incubated with GST-tagged SIRT7 or each of its mutants, and Western blotting was performed with an anti-His antibody. *I,* structures of USP7 and its truncated mutants are shown in the *top panel*. His-tagged SIRT7 was incubated with GST-tagged USP7 or each of its mutants, and Western blotting was performed with an anti-His antibody. * represents specific bands corresponding to the induced GST-tagged proteins stained by Coomassie Brilliant Blue. *IB*, immunoblotting.

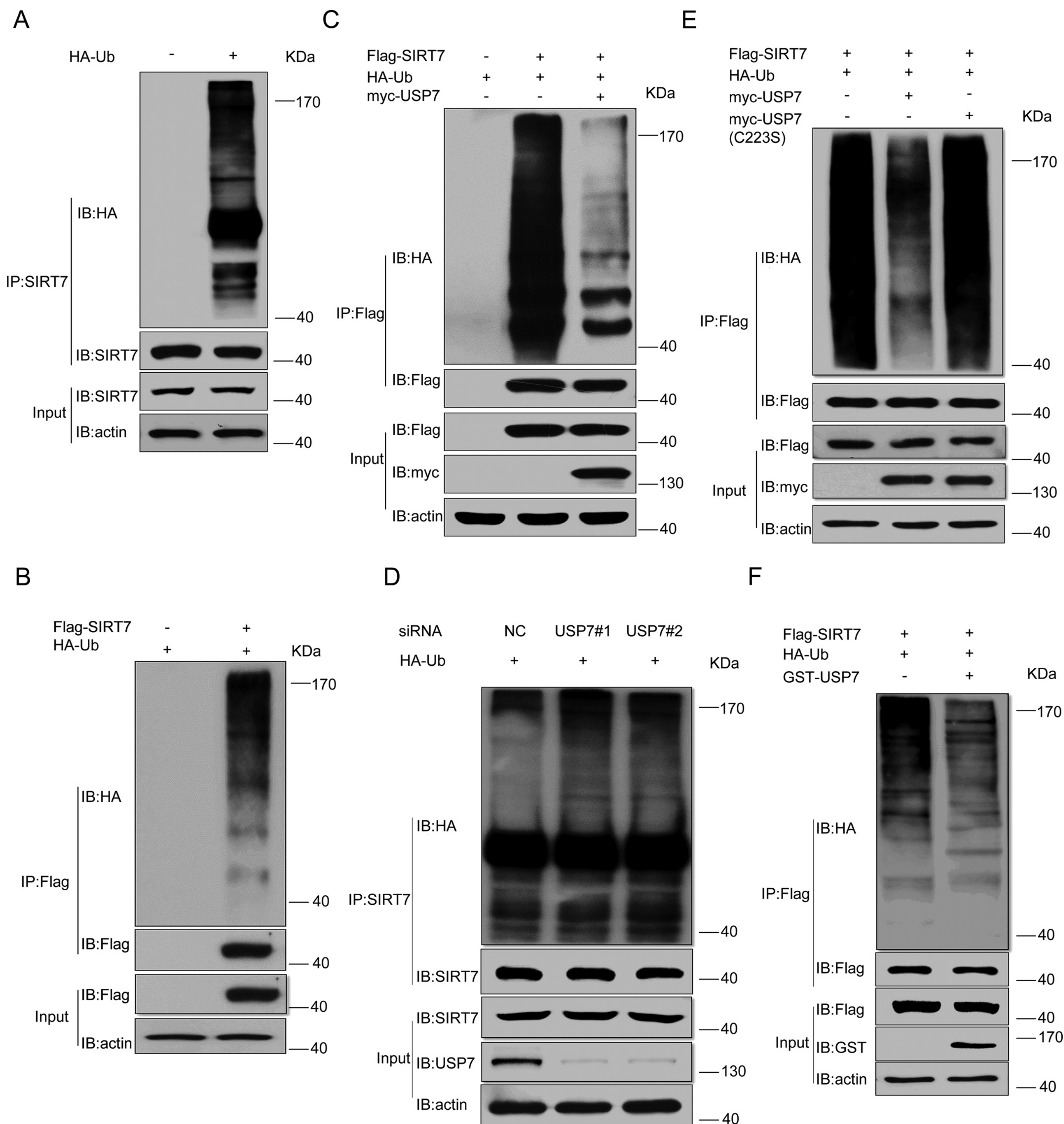

Figure 2. USP7 deubiquitinates SIRT7 *in vivo* and *in vitro*. *A*, HA-Ub plasmid was transfected into HCT116 cells. The ubiquitination of endogenous SIRT7 was analyzed by co-IP with a SIRT7 antibody and Western blotting with an anti-HA antibody. SIRT7 protein levels were confirmed by Western blotting. *B*, FLAG-SIRT7 and HA-Ub plasmids were transfected into HCT116 cells. The ubiquitination of SIRT7 was analyzed by co-IP with anti-FLAG antibody and Western blotting with anti-HA antibody. *C*, FLAG-SIRT7 and HA-Ub were transfected into HCT116 cells with or without Myc-USP7. The ubiquitination of SIRT7 was detected by co-IP with an anti-FLAG antibody and Western blotting with an anti-HA antibody (*top panel*). *D*, HA-Ub was transfected into HCT116 cells with two different siRNAs of USP7 or the negative control (*NC*). The ubiquitination of endogenous SIRT7 was detected. *E*, FLAG-SIRT7 and HA-Ub were transfected into HCT116 cells with Myc-USP7 or a Myc-USP7(C223S) mutant. The ubiquitination of SIRT7 was detected. *F*, FLAG-SIRT7 and HA-Ub were transfected into HCT116 cells. Ubiquitinated FLAG-SIRT7 proteins were pulled down and incubated with purified GST or GST-USP7 fusion proteins. The ubiquitination of SIRT7 was detected. *IB*, immunoblotting.

knockdown. As shown in Fig. 5*A*, transfection with two different siRNAs of SIRT7 resulted in the reduced expression of *G6PC*, consistent with results in KO mice (36). In addition, SIRT7 WT, but not SIRT7 (H187Y) overexpression, increased the expression of *G6PC* (Fig. 5*B*), indicating that the deacetylase activity of SIRT7 is required for *G6PC* expression. Moreover,

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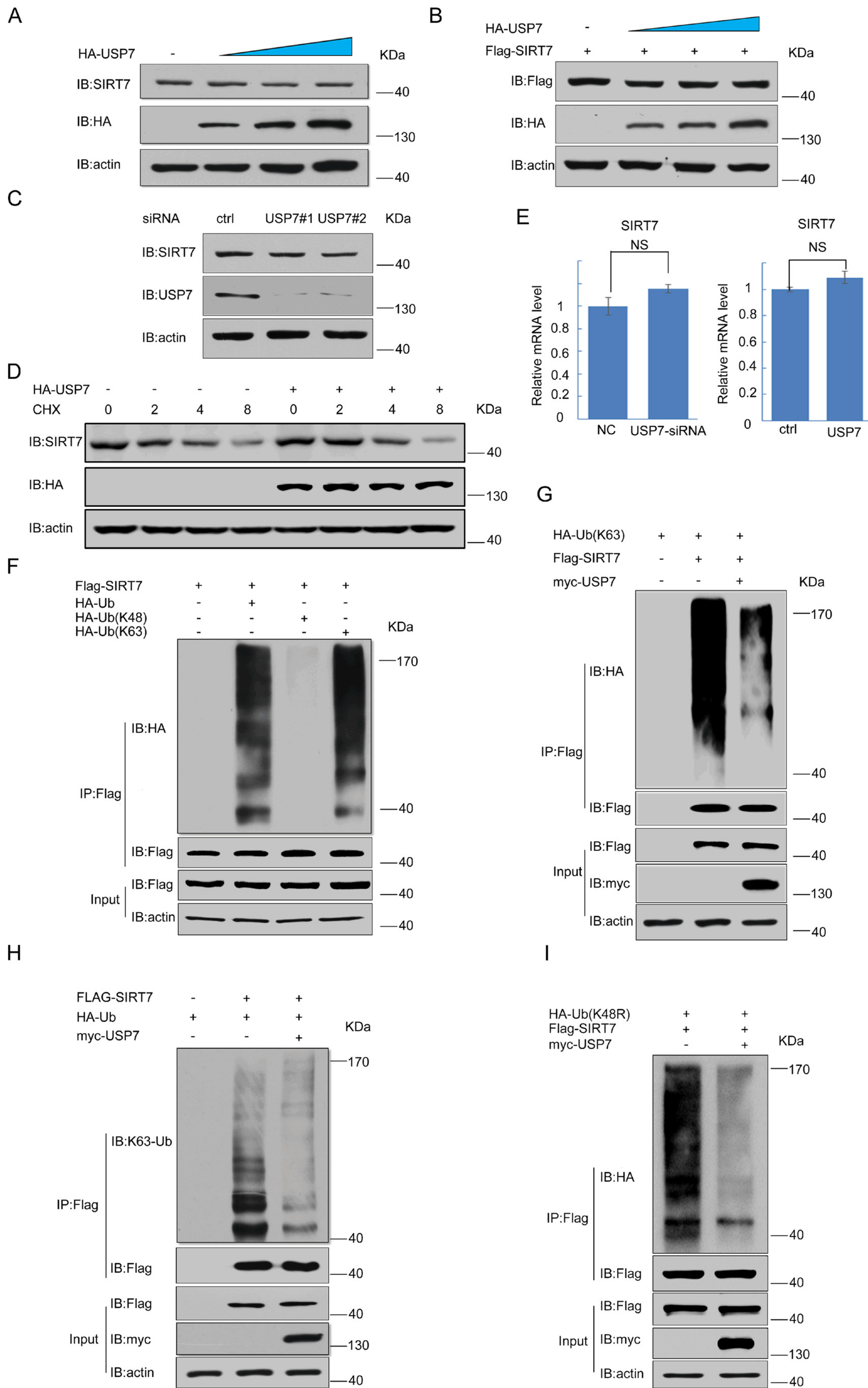

A
HA-USP7
KDa
IB:SIRT7
IB:HA
IB:actin
40
130
40
B
HA-USP7
Flag-SIRT7
IB:Flag
IB:HA
IB:actin
C
siRNA
ctrl
USP7#1
USP7#2
IB:SIRT7
IB:USP7
IB:actin
D
HA-USP7
CHX
0
2
4
8
E
SIRT7
NS
Relative mRNA level
NC
USP7-siRNA
ctrl
USP7
F
Flag-SIRT7
HA-Ub
HA-Ub(K48)
HA-Ub(K63)
IP:Flag
Input
170
G
HA-Ub(K63)
myc-USP7
IB:myc
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FLAG-SIRT7
IB:K63-Ub
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HA-Ub(K48R)

transient transfection of SIRT7 resulted in elevated G6PC protein expression (Fig. 5*C*). In contrast, SIRT7 knockdown did not affect the expression of *PCK1*, another key enzyme gene of gluconeogenesis (Fig. 5*D*), indicating the specific regulation of *G6PC* expression by SIRT7. Furthermore, we showed that the co-transfection of SIRT7 and USP7 WT, but not the USP7 (C223S) mutant, blocked the increase in *G6PC* expression (Fig. 5*E*) and that in the absence of USP7, the overexpression of SIRT7 compared with SIRT7 alone led to a dramatic increase in the levels of *G6PC* (Fig. 5*E*), which indicates that USP7 negatively regulates the effect of SIRT7 on the *G6PC* expression. Next, we analyzed whether upon glucose starvation USP7 was required for the regulation of *G6PC*. Again, we observed that compared with glucose starvation only, the combination of glucose starvation and the expression of SIRT7 WT, but not SIRT7 (H187Y), increased *G6PC* mRNA levels in HCT116 cells (Fig. 5*F*). Furthermore, the expression level of *G6PC* was reduced in the presence of USP7 WT, but not USP7(C223S), in the condition of glucose starvation (Fig. 5*F*), which suggests that USP7 regulates *G6PC* expression by deubiquitinating SIRT7. These results were not unique to the HCT116 cell line, and similar results were observed in HepG2 cells (Fig. 5*G*). Together, these data suggest that the deubiquitination of SIRT7 by USP7 suppresses SIRT7-mediated *G6PC* expression.

SIRT7 binds to the G6PC promoter through ELK4

From our preceding data, glucose starvation induction of *G6PC* transcription was SIRT7-dependent. Because SIRT7 lacks sequence-specific DNA-binding domains, we wanted to know how SIRT7 is recruited to the *G6PC* promoter. The transcription factor FoxO1 activates gluconeogenesis through direct binding to the *G6PC* promoter (68), and USP7 attenuates hepatic gluconeogenesis by modulating FoxO1 occupancy on the *G6PC* promoter (69). To investigate whether SIRT7 recruitment to the *G6PC* promoter was mediated by FoxO1, we first performed a co-immunoprecipitation experiment to confirm the interaction between SIRT7 and FoxO1. We found that SIRT7 physically interacted with FoxO1 (Fig. 6, *A* and *B*). Accordingly, using ChIP analysis, we found a specific occupancy of SIRT7 on the *G6PC* promoter region (−237 to −70 bp) (Fig. 6*C*). This region has been shown to contain the FoxO1-binding site (70, 71). To further confirm FoxO1's requirement for SIRT7 recruitment, we examined the effects of FoxO1 knockdown on SIRT7 occupancy at the *G6PC* promoter. Unexpectedly, FoxO1 depletion had no effect on SIRT7 occupancy at the *G6PC* promoter (Fig. 6*D*). It is worth noting that SIRT7 has been reported to be recruited to specific target promoters through ELK4 (4). Therefore, we examined the effects of ELK4 knockdown on SIRT7 ChIP occupancy at the *G6PC* promoter. As shown in Fig. 6*E*, ELK4 depletion led to a significant decrease in SIRT7 occupancy at the *G6PC* promoter. Consistent with this result, we predicted ELK4 can bind to this region of the *G6PC* promoter in the JASPAR CORE database (72). Taken together, these data suggest that SIRT7 binds to the *G6PC* promoter via ELK4 rather than via FoxO1.

Because SIRT7 is a histone H3K18 deacetylase, we tested whether SIRT7-deficient cells exhibited increased H3K18 acetylation in the *G6PC* promoter. ChIP analysis with an anti-H3K18Ac antibody clearly showed increased H3K18 acetylation in the *G6PC* promoter (Fig. 7*A*), suggesting that SIRT7 is one of the main H3K18 deacetylases of the *G6PC* promoter.

To further understand the role of USP7 in the recruitment of SIRT7 to the *G6PC* promoter, we analyzed the occupancy of SIRT7 and H3K18AC in the *G6PC* promoter in WT cells *versus* siRNA-mediated USP7 knockdown cells. Consistent with the ChIP results (Figs. 6*C* and 7*A*), we observed the presence of SIRT7 and H3K18 acetylation in the *G6PC* promoter, whereas RNAi-mediated knockdown of USP7 induced a higher occupancy of SIRT7 in the *G6PC* promoter (Fig. 7*B*). Accordingly, H3K18 acetylation occupancy decreased in response to USP7 depletion (Fig. 7*C*). These data suggest that USP7 plays an important role in regulating SIRT7 binding with the *G6PC* promoter. To further confirm the role of USP7 in regulation of SIRT7-mediated *G6PC* expression under glucose deprivation, we first cultured HepG2 cells in a medium without glucose, the cells were then released from glucose deprivation, and ChIP assays were used to measure the occupancy of USP7 and SIRT7 on the *G6PC* gene promoter. We found that glucose deprivation stimulates SIRT7 binding to the endogenous *G6PC* promoter (Fig. 7*D*), whereas the chromatin combination of USP7 on the *G6PC* promoter was decreased (Fig. 7*D*). When the cells were released from absence of glucose, the binding capacity of SIRT7 and USP7 on the *G6PC* promoter was rescued (Fig. 7*D*). Consistently, the increased *G6PC* transcription upon glucose starvation was also restored when the cells were released from glucose deprivation (Fig. 7*E*). Taken together, all results support the role of USP7 in suppression of the *G6PC* gene through SIRT7 under glucose deprivation stress.

Discussion

In this study, we demonstrate for the first time that SIRT7 undergoes Lys-63-linked polyubiquitination. USP7 is a deubiquitinase for SIRT7; it removes the polyubiquitination of SIRT7 to negatively control its enzymatic activity and the SIRT7-me-

Figure 3. USP7 does not alter SIRT7 protein stability. *A,* HA-USP7 was transfected into HCT116 cells in a dose-dependent manner. The protein level of endogenous SIRT7 was detected with anti-SIRT7 antibody. *B,* HA-USP7 was transfected into HCT116 cells in a dose-dependent manner with FLAG-SIRT7. The protein level of SIRT7 was detected by anti-FLAG antibody. *C,* siRNAs directed against USP7 or the control (negative control, *NC*) were transfected into HCT116 cells. The endogenous SIRT7 protein level was detected using an anti-SIRT7 antibody. *D,* USP7 expression plasmids or empty vectors were transfected into HCT116 cells. The transfected cells were treated with cycloheximide (*CHX*) for different times. The protein levels were determined by Western blotting using indicated antibodies. *E,* total RNA was extracted from HCT116 cells that either overexpressed USP7 or had USP7 expression knocked down. The mRNA levels of SIRT7 were measured using qRT-PCR. Three independent samples were averaged, and the values were normalized to GAPDH. Statistically not significant differences are denotes as *NS*. *F,* FLAG-SIRT7 and HA-Ub, HA-Ub (Lys-48), or HA-Ub (Lys-63) were transfected into HCT116 cells. The ubiquitination of SIRT7 was detected by co-IP with an anti-FLAG antibody and Western blotting with an anti-HA antibody. *G,* FLAG-SIRT7 and HA-Ub (Lys-63) were transfected into HCT116 cells with or without Myc-USP7. The ubiquitination of SIRT7 was detected. *H,* FLAG-SIRT7 and HA-Ub were transfected into HCT116 cells with or without Myc-USP7. The ubiquitination of SIRT7 was detected by co-IP with an anti-FLAG antibody and Western blotting with an anti-Lys-63–Ub antibody. *I,* FLAG-SIRT7 and HA-Ub (K48R) were transfected into HCT116 cells with or without Myc-USP7. The ubiquitination of SIRT7 was analyzed by co-IP with anti-FLAG antibody and Western blotting with anti-HA antibody. *IB,* immunoblotting.

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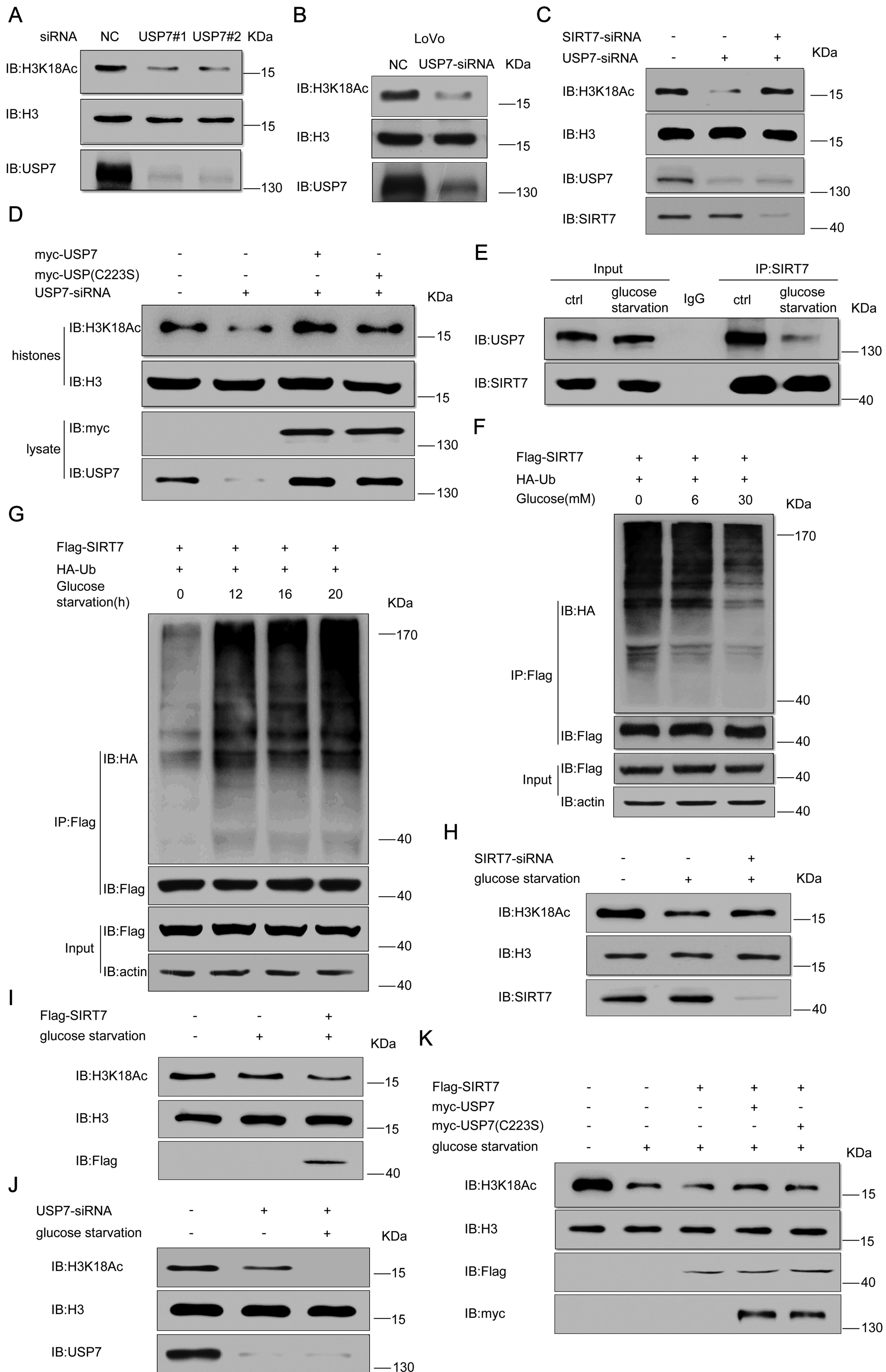
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siRNA NC USP7#1 USP7#2 KDa
IB:H3K18Ac
IB:H3
IB:USP7
B
LoVo
NC USP7-siRNA KDa
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IB:H3
IB:USP7
C
SIRT7-siRNA
USP7-siRNA
IB:H3K18Ac
IB:H3
IB:USP7
IB:SIRT7
D
myc-USP7
myc-USP(C223S)
USP7-siRNA
histones
IB:H3K18Ac
IB:H3
lysate
IB:myc
IB:USP7
E
Input
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IB:USP7
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F
Flag-SIRT7
HA-Ub
Glucose(mM) 0 6 30
IB:HA
IP:Flag
IB:Flag
Input
IB:actin
G
Flag-SIRT7
HA-Ub
Glucose starvation(h) 0 12 16 20
IB:HA
IP:Flag
IB:Flag
Input
IB:actin
H
SIRT7-siRNA
glucose starvation
IB:H3K18Ac
IB:H3
IB:SIRT7
I
Flag-SIRT7
glucose starvation
IB:H3K18Ac
IB:H3
IB:Flag
J
USP7-siRNA
glucose starvation
IB:H3K18Ac
IB:H3
IB:USP7
K
Flag-SIRT7
myc-USP7
myc-USP7(C223S)
glucose starvation
IB:H3K18Ac
IB:H3
IB:Flag
IB:myc

diated gluconeogenic expression of *G6PC*. Our data reveal a novel molecular mechanism by which USP7 antagonizes the activity of SIRT7 in the regulation of gluconeogenesis (Fig. 7*F*).

The diverse roles of SIRT7 suggest that it should be tightly regulated by multiple mechanisms, as is SIRT1 (73, 74). However, aside from transcriptional and post-transcriptional regulatory mechanisms, there are few reports on whether and how SIRT7 is regulated by post-translational modifications. SIRT7 has been reported to be phosphorylated during mitosis by the cyclin-dependent kinase 1 (CDK1)–cyclin B pathway, although the sites and the function have not been defined (22). SIRT7 is phosphorylated by AMPK under energy stress, which plays a crucial role in determining the subcellular distribution and degradation of SIRT7, and SIRT7 is also modified by ubiquitination (75). Consistent with these data, our study showed that SIRT7 was modified by Lys-63-linked polyubiquitination. In addition, we identified USP7 as a deubiquitinase of SIRT7. Through its action on SIRT7, USP7 counteracts its ubiquitination, which is essential for the regulation of gluconeogenesis. Different ubiquitin linkages can result in different cellular fates. For example, a Lys-48 linkage mainly targets proteins for proteasome degradation, whereas a Lys-63 linkage seems to play important roles beyond degradation, including protein activation, signal transduction, cellular localization and responses to stress (64, 76–81). USP7 has the ability to cleave both Lys-48- and Lys-63-linked ubiquitin chains (82). USP7 interacts with different substrates for deubiquitination and subsequent protein stabilization (47, 53, 83) or control of subcellular trafficking (50). Our study showed that USP7 controlled the enzymatic activity of SIRT7 by interacting with and deubiquitinating it.

Among the ubiquitin-specific protease family, USP22-mediated deubiquitination and stabilization of SIRT1 suppress the transcriptional and proapoptotic functions of p53 (62). USP10 suppresses SIRT6 ubiquitination to protect SIRT6 from proteasome degradation, which inhibits c-Myc transcriptional activity and tumor growth (63). Unlike USP22 and USP10, the USP7-mediated deubiquitination of SIRT7 had no effect on SIRT7 protein stability, suggesting that although SIRT7 is polyubiquitinated, ubiquitin-mediated degradation may not be the key process responsible for its turnover in the cell. Our present observation may be justified in light of a recent report that SIRT7 is degraded by a ubiquitin-independent proteasome (29, 75).

To date, the E3 ubiquitin ligases that catalyze SIRT7 ubiquitination have not been identified. Although the E3 ligase HERC5 was identified as a SIRT7-interacting protein in our proteomic assay, we could not detect any change to SIRT7 ubiquitination upon its overexpression, thereby excluding the possibility that it is a ubiquitin ligase of SIRT7. Additional studies are needed to further identify the E3 ligase(s) that catalyze(s) SIRT7 ubiquitination.

Accumulating evidence suggests that sirtuins play important roles in controlling gluconeogenesis. With respect to the nuclear sirtuins, SIRT1 interacts with and deacetylates the transcriptional co-activator PGC-1α to induce gluconeogenesis in response to fasting (84). SIRT1 also binds and deacetylates transcription factor FoxO1, resulting in FoxO1-mediated glucogenic gene transcription (85). Moreover, SIRT1 attenuates gluconeogenesis during late fasting through the deacetylation of the cAMP-response element-binding protein-regulated transcription co-activator 2 (CRTC2) and the promotion of its ubiquitin-dependent degradation (86). By contrast, SIRT6 indirectly down-regulates PGC-1α through activation of the acetyltransferase GCN5 and suppresses hepatic glucose production (87). We previously reported that SIRT6, whose interaction with FoxO1 led to FoxO1 deacetylation and export to the cytoplasm, down-regulates the key gluconeogenic enzymes G6PC and PCK1 to suppress hepatic gluconeogenesis (88). In contrast to how SIRT1 and SIRT6 regulate gluconeogenesis indirectly, we show here that SIRT7 directly promotes gluconeogenesis by binding to the promoter of *G6PC* and deacetylation of histone H3 lysine 18 (H3K18) in the *G6PC* promoter. Furthermore, our results on the effect of SIRT7 on *G6PC* agree with a recent report showing that the hepatic expression of *G6PC* was reduced in SIRT7 KO mice and displayed resistance to glucose intolerance, although no mechanism was proposed (36). Beyond recent reports of the effects of SIRT7 on energy and lipid metabolism, our findings add an additional layer for SIRT7 in the regulation of glucose homeostasis. We also uncovered a new sirtuin-mediated gluconeogenesis regulatory pathway for the control of *G6PC* expression. As the last rate-limiting enzyme catalyzing gluconeogenesis, the expression of *G6PC* is controlled mainly at the transcription level. FoxO1 is an important transcription factor for the activation of gluconeogenesis through direct binding to the *G6PC* promoters (68, 89, 90). USP7 attenuates *G6PC* expression in primary hepatocytes by suppressing FoxO1 occupancy on the *G6PC* promoter (69). Interestingly, our results indicate the different mechanisms by which USP7 regulates *G6PC* expression. Thus, we revealed a novel pathway through which USP7 regulates *G6PC* expression under glucose deprivation conditions.

In summary, our data show that USP7 negatively regulates the enzymatic activity of SIRT7 through deubiquitination, which is involved in the control of gluconeogenesis by regulat-

Figure 4. USP7 regulates SIRT7 deacetylase activity. *A,* siRNAs directed against USP7 or the control (negative control, *NC*) were transfected into HCT116 cells, and the acid-extracted histone was subjected to immunoblotting (*IB*) with an anti-H3K18Ac antibody. *B,* control (*NC*) or USP7 siRNA was transfected into LoVo cells. The level of H3K18Ac was confirmed as described in *A*. *C,* USP7 siRNA was transfected into HCT116 cells with or without SIRT7 siRNA. The level of H3K18Ac was detected as described in *A*. *D,* USP7 siRNA was transfected into HCT116 cells with Myc-USP7 or Myc-USP7 (C223S). The level of H3K18Ac was confirmed as indicated in *A*. *E,* HCT116 cells were treated with or without glucose starvation for 12 h. The cell lysates were precipitated with an anti-SIRT7 antibody and normal rabbit IgG to examine the change in the interaction of endogenous USP7 and SIRT7. *F,* ubiquitination of SIRT7 was analyzed in HCT116 cells transfected with FLAG-SIRT7 and HA-Ub and treated with glucose starvation in a dose-dependent manner. *G,* FLAG-SIRT7 and HA-Ub were transfected into HCT116 cells and treated with glucose starvation in a time-dependent manner. The ubiquitination of SIRT7 was analyzed by co-IP with anti-FLAG antibody and Western blotting with anti-HA antibody. *H,* HCT116 cells were glucose-starved for 12 h with or without SIRT7 siRNA. The level of H3K18Ac was analyzed. *I,* USP7 siRNA was transfected into HCT116 cells with or without glucose starvation for 12 h. The level of H3K18Ac was analyzed. *J,* HCT116 cells with or without overexpressed FLAG-SIRT7 were treated with glucose starvation for 12 h. The level of H3K18Ac was tested. *K,* HCT116 cells transfected with FLAG-SIRT7, Myc-USP7, and Myc-USP7(C223S) were glucose-starved for 12 h. The level of H3K18Ac was confirmed as described in *A*.

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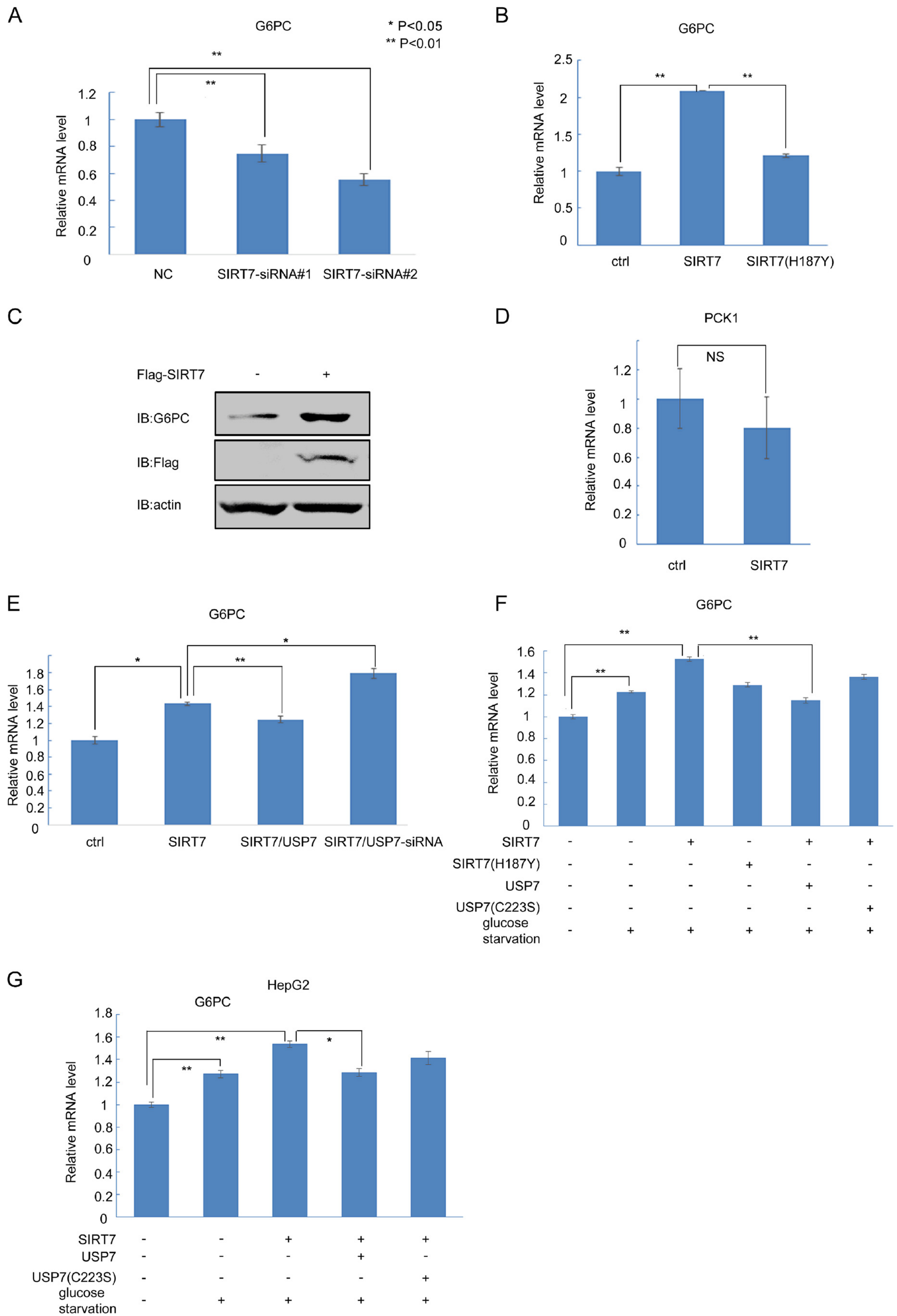
A
G6PC
* P<0.05
** P<0.01
Relative mRNA level
NC
SIRT7-siRNA#1
SIRT7-siRNA#2
B
G6PC
Relative mRNA level
ctrl
SIRT7
SIRT7(H187Y)
C
Flag-SIRT7
IB:G6PC
IB:Flag
IB:actin
D
PCK1
NS
Relative mRNA level
ctrl
SIRT7
E
G6PC
Relative mRNA level
ctrl
SIRT7
SIRT7/USP7
SIRT7/USP7-siRNA
F
G6PC
Relative mRNA level
SIRT7
SIRT7(H187Y)
USP7
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glucose starvation

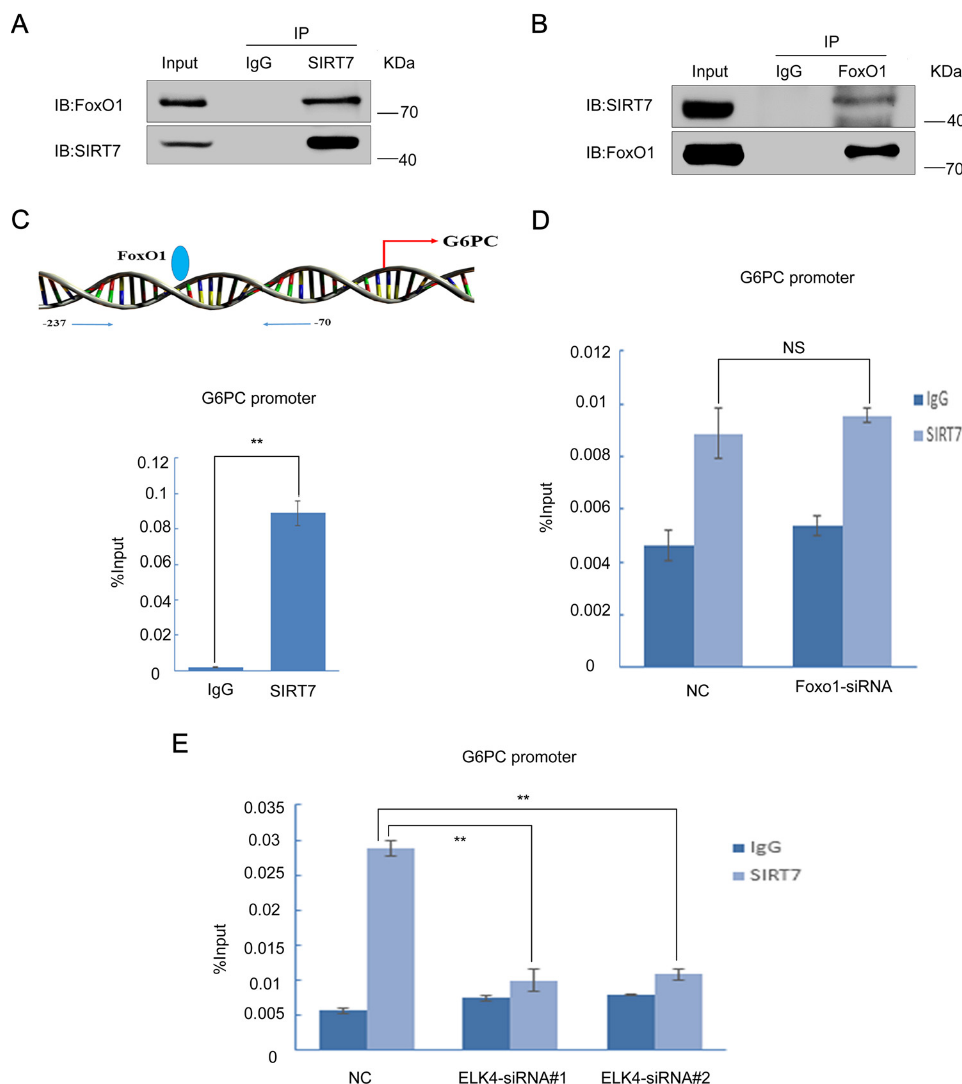

Figure 6. USP7 regulates SIRT7-dependent *G6PC* expression through ELK4. *A* and *B*, interaction of endogenous SIRT7 and FoxO1. HCT116 Cell lysates were subjected to co-IP with anti-SIRT7 or anti-USP7 antibody, respectively, and normal rabbit IgG was control, followed by Western blotting analysis using indicated antibodies. *C*, occupancy of SIRT7 at the *G6PC* promoter in HCT116 cells was determined by ChIP-qPCR, and the data were compared with data from IgG negative control samples. *D*, occupancy of SIRT7 at the *G6PC* promoter was shown in control or FoxO1 siRNA expressed HCT116 cells by ChIP, compared with IgG negative control samples. *E*, occupancy of SIRT7 at the *G6PC* promoter was determined in control or two different ELK4 siRNA knockdown HCT116 cell groups by ChIP and compared with IgG negative control samples. *IB*, immunoblot. Statistically significant differences are denoted as **, $p < 0.01$ and are denoted as *NS*.

ing *G6PC* expression. Overall, these findings demonstrate that USP7 is a novel regulator of SIRT7 activity and that SIRT7 is a new substrate of USP7. Our study may provide the basis for novel clinical approaches to treat metabolic disorders related to glucose metabolism.

Experimental procedures

Cell culture and RNA interference

The human colon cancer cell lines HCT116 and LoVo, the human embryonic kidney cell line HEK293T, the human cervical cancer cell line HeLa, and the human liver cancer cell line HepG2 were grown in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified incubator containing 5% CO_2. Protein half-life studies were performed by incubating cells with cycloheximide (Amresco, 50 μg/ml) and proteasome inhibitor MG132 (Sigma, 20 μM) for the indicated time points. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. The RNAi oligonucleotides (Gene Pharma Co.) were as follows: USP7, 5′-GUGUAAAGA-

Figure 5. USP7 and SIRT7 regulate *G6PC* expression. *A*, total RNA was extracted from control and siRNA-mediated SIRT7-depleted HCT116 cells. The mRNA level of *G6PC* was analyzed by real-time PCR. Statistically significant differences are denoted as follows: *, $p < 0.05$, or **, $p < 0.01$. *B*, total RNA was extracted from HCT116 cells overexpressing the SIRT7 or SIRT7 (H187Y) plasmids. The mRNA level of *G6PC* was analyzed using real-time PCR. *C*, FLAG-SIRT7 or control was transfected into HCT116 cells. The protein levels of G6PC were detected using anti-G6PC. *D*, total RNA was extracted from HCT116 cells overexpressed SIRT7 plasmid, and the mRNA level of PCK1 was analyzed by real-time PCR. Statistically not significant differences are denotes as *NS*. *E*, total RNA was isolated from HCT116 cells expressing the SIRT7 plasmid with a USP7 plasmid or a USP7-specific siRNA. The mRNA level of *G6PC* was determined by real-time PCR. *F*, total RNA was extracted from HCT116 cells expressing SIRT7, SIRT7 (H17Y), USP7 and/or USP7(C223S) after glucose starvation for 12 h. The mRNA level of *G6PC* was determined by real-time PCR. *G*, HepG2 cells expressing SIRT7, SIRT7 (H187Y), USP7, and USP7(C223S) treat with glucose starvation for 12 h. Total RNA was extracted from analyzing the mRNA level of *G6PC* by real-time PCR. *NC*, negative control; *IB*, immunoblot.

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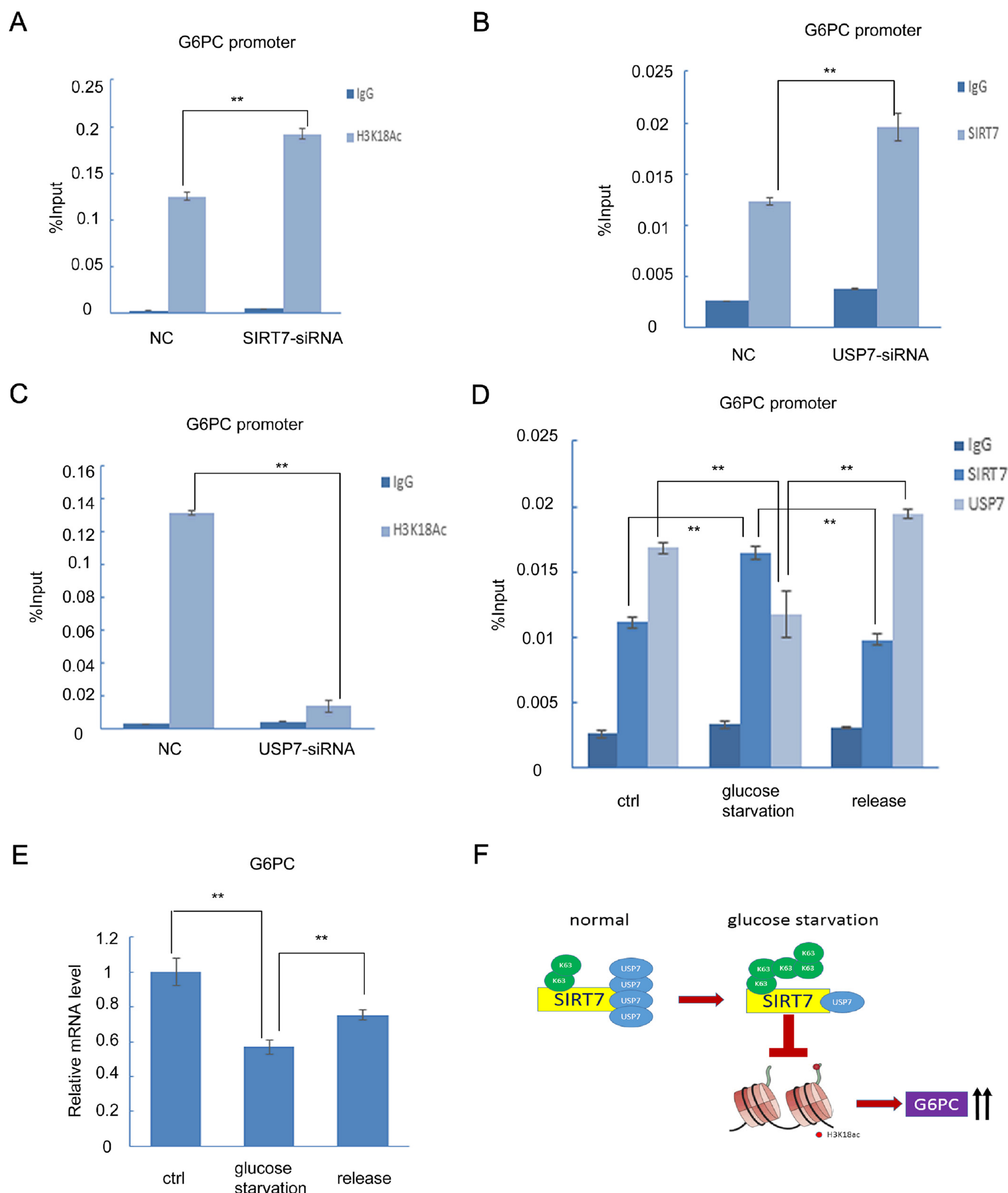

Figure 7. USP7 regulates SIRT7-mediated *G6PC* expression upon glucose starvation. *A,* hyperacetylation of H3K18 at the *G6PC* promoter was determined in control or siRNA-mediated SIRT7 knockdown HCT116 cells by ChIP and compared with IgG negative control (*NC*) samples. *B,* occupancy of SIRT7 at the *G6PC* promoter was determined in control or siRNA-mediated USP7 knockdown HCT116 cells by ChIP-qPCR and compared with IgG negative control samples. *C,* hypoacetylation of H3K18 at the *G6PC* promoter was determined in control or siRNA-mediated USP7 knockdown HCT116 cells by ChIP and compared with IgG negative control samples. *D,* HepG2 cells were cultured with a medium without glucose for 12 h, and the cells were then changed to normal glucose medium for other 12 h, and ChIP assays were performed with IgG, SIRT7, and USP7 antibodies, respectively. *E,* cells were treated as *D,* and total RNA was extracted, and the expression of G6PC was analyzed by real-time PCR. *F,* model of USP7 regulates SIRT7-mediated *G6PC* expression upon glucose starvation. Statistically significant differences are denoted as **, $p < 0.01$.

AGUAGACUAU-3′ and 5′-GGUUCAUAGUGGAGAUAAU-3′; SIRT7, 5′-TAGCCATTTGTCCTTGAGGAA-3′ and 5′-GAACGGAACTCGGGTTATT-3′; ELK4, 5′-CGACACAGACATTGATTCA-3′ and 5′-GAGAATGGAGGGAAAGATA-3′; FoxO1, 5′-GAGCGTGCCCTACTTCAAG-3′; control (NC), 5′-UUCUCCGAACGUGUCACGU-3′.

Western blotting

Western blotting was carried out as described previously (91). The antibodies used were as follows: anti-FLAG (Sigma, F1804); anti-HA (MBL, M180-3); anti-Myc (MBL, M407-); anti-His (MBL, PM032); anti-SIRT7 (Santa Cruz Biotechnology, sc-135055); anti-USP7 (Bethyl Laboratories, A300-033A); anti-Ub (Abcam, ab19247); anti-Lys-63–Ub (Cell Signaling Technology, D7A11); anti-G6PC (Proteintech, 22169-1-AP); and anti-H3K18Ac (Abcam, ab61233).

Co-immunoprecipitation

Cells were harvested and then lysed in lysis buffer (1% Nonidet P-40, 150 mM NaCl, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.05% SDS, and 1% EDTA-free protease and phosphatase inhibitor mixtures (Roche Applied Science)) on ice for 30 min. After centrifugation at 4 °C, 12,000 rpm for 15 min, the indicated antibody was added to the supernatant and incubated overnight at 4 °C. Then, protein G- or A-Sepharose beads (GE Healthcare) were incubated with the mixture for 2 h at 4 °C. The beads were washed with Nonidet P-40 buffer three times. The pellets were dissolved in 2× SDS loading buffer and analyzed by Western blotting.

GST pulldown assay

GST fusion proteins were expressed in *Escherichia coli* and purified using glutathione-Sepharose 4B beads (GE Healthcare). Recombinant His-tagged proteins purified from *E. coli* by Ni(II)-Sepharose affinity (GE Healthcare) were incubated with GST fusion proteins in TEN buffers (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl) for 4 h at 4 °C. The beads were washed three times with TEN buffers and boiled with 2× SDS loading buffer. Proteins were analyzed by Western blotting using anti-GST or anti-His antibodies.

In vitro deubiquitination assay

HCT116 cells were transfected with FLAG-SIRT7 and HA-Ub. After 48 h, the cells were harvested and precipitated using anti-FLAG-agarose beads (immunoprecipitation). Ubiquitinated SIRT7 proteins were eluted using a 3× FLAG peptide and incubated with GST or GST-USP7 in the deubiquitination buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 10 mM DTT, 5% glycerol) at 37 °C for 2 h. The ubiquitination of SIRT7 was detected by Western blotting.

Acid extraction of histones

Cells were harvested and lysed in hypotonic lysis buffer (10 mM Tris-HCl, pH 8.0, 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 1% protease inhibitor mixture) and incubated on ice for 30 min. After centrifugation at 11,000 × *g* for 10 min at 4 °C, the cell supernatant fraction that contained acid-soluble proteins was retained. The supernatant was dialyzed against 200 ml of 0.1 N acetic acid twice for 1–2 h each time and then dialyzed against 200 ml of H_2O for 1 and 3 h and overnight. Proteins (20 μg for each lane) were quantified and fractionated by size using SDS-PAGE (15%) for Western blotting with the indicated antibodies.

RNA extraction and RT-qPCR

Total RNA was extracted by TRIzol reagent (Invitrogen), precipitated using ethanol, and then converted to cDNA using a Quantscript RT kit (Promega, WI) according to the manufacturer's instructions. Quantitative real-time RT-PCR was performed with SYBR Green dye to analyze the target genes. The primers used for RT-PCR were as follows: *SIRT7* forward, 5′-ACGCCAAATACTTGGTCGTCT-3′, and reverse 5′-AGCACTAACGCTTCTCCCTTT-3′; USP7 forward, 5′-CCCTCCGTGTTTTGTGCGA-3′, and reverse 5′-AGACCATGACGTGGAATCAGA-3′; *G6PC* forward, 5′-GGCTCAACCTCGTCTTTAAGTG-3′, and reverse, 5′-CTCCCTGGTCCAGTCTCACA-3′; and *PCK1* forward, 5′-ACGGATTCACCCTACGTGGT-3′, and reverse, 5′-CCCCACAGAATGGAGGCATTT-3′.

Chromatin immunoprecipitation

HCT116 cells were fixed with 1% formaldehyde for 10 min at room temperature and then lysed with lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1% SDS, 1% PMSF, and 1% protease inhibitor mixture) on ice for 15 min. Cell lysates were sonicated and centrifuged at 12,000 rpm for 10 min at 4 °C. Ten percent of the supernatant was removed for input, and the remaining supernatant was diluted (1:5) in dilution buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, and 1% Triton X-100). Immunoprecipitation was performed with the indicated antibodies or normal mouse/rabbit IgG and then rotated at 4 °C overnight. Protein A/G-Sepharose beads and salmon DNA were added to each sample for 2 h at 4 °C. The supernatant was discarded after centrifugation (1000 rpm, 2 min, 4 °C), and the beads were sequentially washed with TSE I buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 150 mM NaCl, 0.1% SDS, and 1% Triton X-100); TSE II buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 500 mM NaCl, 0.1% SDS, 1% Triton X-100); buffer III (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 250 mM LiCl, 1% Nonidet P-40, 1% deoxycholate (Sigma, D6750)); and TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). DNA was purified with a DNA extraction kit (Qiagen) after incubation for at least 6 h at 65 °C. Real-time PCR was performed with primers designed using the *G6PC* promoter sequence (−237 to −70) (forward, 5′-CATTGGCCCTGCTGAGTACA-3′, and reverse, 5′-AACCCAGCCCTGATCTTTGG-3′).

Author contributions—L. J. performed most of the experimental work. M. T., C. Z., Z. C., Y. C., X. L., Y. L., and H. W. assisted in the experimental process. J. Z., J. X., and L. W. analyzed the data and performed material preparation. J. W. discussed the results and commented on the manuscript. W. G. Z. supervised the project. H. Y. W. designed and supervised the experiments and wrote the manuscript. All authors critically read the paper.

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