PNAS RESEARCH ARTICLE | MEDICAL SCIENCES

Proline hydroxylation of CREB-regulated transcriptional coactivator 2 controls hepatic glucose metabolism

Yaqian Xue[a,1], Aoyuan Cui[a,1], Shuang Wei[a], Fengguang Ma[a], Zhengshuai Liu[a], Xia Fang[a,b], Shaofeng Huo[c], Xiaoyang Sun[d], Wenjing Li[a], Zhimin Hu[a], Yuxiao Liu[a], Genxiang Cai[a], Weitong Su[a], Jiuxiang Zhao[e], Xi Yan[f], Chenlin Gao[b], Jian Wen[a,g], Haibing Zhang[a], Hong Li[f], Yi Liu[h], Xu Lin[a], Yong Xu[b], Wenguang Fu[g,2], Jing Fang[i,2], and Yu Li[a,2]

Edited by Marc Montminy, Salk Institute for Biological Studies, La Jolla, CA; received November 17, 2022; accepted April 25, 2023

Prolyl hydroxylase domain (PHD) enzymes change HIF activity according to oxygen signal; whether it is regulated by other physiological conditions remains largely unknown. Here, we report that PHD3 is induced by fasting and regulates hepatic gluconeogenesis through interaction and hydroxylation of CRTC2. Pro129 and Pro615 hydroxylation of CRTC2 following PHD3 activation is necessary for its association with cAMP-response element binding protein (CREB) and nuclear translocation, and enhanced binding to promoters of gluconeogenic genes by fasting or forskolin. CRTC2 hydroxylation–stimulated gluconeogenic gene expression is independent of SIK-mediated phosphorylation of CRTC2. Liver-specific knockout of PHD3 (PHD3 LKO) or prolyl hydroxylase–deficient knockin mice (PHD3 KI) show attenuated fasting gluconeogenic genes, glycemia, and hepatic capacity to produce glucose during fasting or fed with high-fat, high-sucrose diet. Importantly, Pro615 hydroxylation of CRTC2 by PHD3 is increased in livers of fasted mice, diet-induced insulin resistance or genetically obese ob/ob mice, and humans with diabetes. These findings increase our understanding of molecular mechanisms linking protein hydroxylation to gluconeogenesis and may offer therapeutic potential for treating excessive gluconeogenesis, hyperglycemia, and type 2 diabetes.

hydroxylation | PHD3 | CRTC2 | gluconeogenesis | type 2 diabetes

Elevated gluconeogenesis and excessive glucose production in the liver contribute to hepatic insulin resistance in type 2 diabetes. Glucagon is a key hormone that promotes hepatic glucose production via stimulating cAMP-dependent protein kinase A (PKA) and phosphorylation of CREB. CREB associates with CREB-regulated transcriptional coactivator (CRTC) family and binds to the cAMP-response element (CRE) motif on promoters of rate-limiting gluconeogenic enzymes, such as phosphoenolpyruvate carboxy-kinase (PEPCK) and glucose 6 phosphatase (G6Pase), leading to enhanced gluconeogenesis (1, 2).

CRTC2, the most highly expressed member of CRTC family in the liver, is an essential mediator for the hormonal control of PKA-dependent activation of CREB–CRTC2 module (3). Various posttranslational modifications of CRTC2 have been shown to be involved in the regulation of gluconeogenesis. Phosphorylation plays important roles in the regulation of the cellular localization and protein stability of CRTC2. Under basal conditions, CRTC2 is phosphorylated at Ser171 via AMPK and salt-inducible kinases (SIKs) and sequestered in the cytoplasm through association with 14-3-3 (4, 5). In response to glucagon, PKA-mediated inhibition of AMPK and SIKs leads to dephosphorylation and nuclear translocation of CRTC2, which binds to phosphorylated CREB and activates the transcription of PEPCK and G6Pase (4, 6–8).

As an emerging modification, proline hydroxylation of protein is not well understood (9, 10). The HIF prolyl hydroxylase domain proteins, termed PHD1, PHD2, and PHD3 (also called EGLN2, EGLN1, and EGLN3, respectively) (11), are one class of enzymes which hydroxylate proline residues of substrates in the presence of oxygen. In response to oxygen, PHDs hydroxylate hypoxia-inducible factor α (HIFα) and increase the proteasomal degradation of HIFα (11). As the most inducible PHD in response to hypoxia, PHD3 appears to have broader substrate specificity and participates in various biological processes via hydroxylating non-HIF substrates or hydroxylation-independent activities. Although PHD3 has been reported to regulate insulin sensitivity in the liver in a HIF-dependent manner (12), whether PHD3-mediated hydroxylation of a non-HIF target affects gluconeogenesis and glucose metabolism under pathophysiological conditions remains unknown.

In this study, we provide insights into mechanisms that underline the importance of hydroxylation in regulating hepatic gluconeogenesis. These in vivo and in vitro studies

Significance

Unrestrained gluconeogenesis and glucose production in the liver contribute to hyperglycemia and type 2 diabetes. PHD-mediated protein hydroxylation is involved in the regulation of cellular adaptation to hypoxia. However, whether it is regulated by nutritional or hormonal signals and modulates hepatic gluconeogenesis remains unknown. Here, we identify a unique PHD3-dependent hydroxylation of CRTC2, a key regulator of gluconeogenesis, in the regulation of hepatic gluconeogenesis. Upon fasting, Pro129 and Pro615 hydroxylation of CRTC2 by PHD3 enhances its association with CREB and increases the nuclear translocation and transcriptional activity of CRTC2, leading to enhanced hepatic glucose production. Pharmacological and genetic approaches for the modulation of PHD3 activity or CRTC2 hydroxylation may provide therapeutic avenues for treating hyperglycemia and type 2 diabetes.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

[1]Y. Xue and A.C. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: liyu@sinh.ac.cn, jfang@qdu.edu.cn, or fuwg@swmu.edu.cn.

This article contains supporting information online at https://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2219419120/-/DCSupplemental.

Published May 30, 2023.

demonstrate that 1) PHD3 is an upstream hydroxylase of CRTC2; 2) PHD3-dependent hydroxylation of CRTC2 increases its association with CREB and nuclear translocation; 3) Pro129 and Pro615 hydroxylation of CRTC2 are essential for PHD3 to enhance CRTC2 activity and stimulate target gluconeogenic gene expression; 4) PHD3-dependent CRTC2 hydroxylation may represent a noncanonical CRTC2 pathway for hepatic gluconeogenesis.

Materials and Methods

Liver Specimens from Humans. Liver samples were obtained from adult patients undergoing resection of benign liver diseases such as liver hemangioma, liver trauma, or gallstone disease. Diabetic and nondiabetic control liver specimens were acquired from these patients diagnosed with or without diabetes, and patient characteristics are listed in *SI Appendix*, Table S3. Patients gave written consent for their samples to be collected. The study protocol was approved by the Ethics Committee of the Affiliated Hospital of Southwest Medical University (KY2022167) and was conducted in accordance with the 1975 Declaration of Helsinki.

Animal Model and Diets. Liver-specific knockout of PHD3 (PHD3 LKO) mice were generated by crossing floxed PHD3 mice containing the deleted exon 2 of PHD3 (13) with albumin-Cre recombinase transgenic mice. PHD3 KI mice were generated by crossing floxed alanine mutation in PHD3 exon 3 at His196 (14) with albumin-Cre recombinase transgenic mice. Mice were fed a high-fat, high-sucrose (HFHS) diet (D12327; Research Diets) for 16 wk. All mice were housed under a 12:12-h light/dark cycle at controlled temperature. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee at the Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences (SINH-2022-LY-1).

Statistical Analysis. Data were expressed as mean ± SEM. Statistical significance was evaluated using the unpaired two-tailed Student's *t* test and among more than two groups by analysis of one-way ANOVA. Differences were considered significant at a P value < 0.05.

Results

Hepatocyte-Specific Deletion of PHD3 Lowers Gluconeogenesis in Mice during Fasting. To investigate whether PHD3 is involved in regulating glucose metabolism, expression levels of PHD3 in livers of mice under fasted conditions were assessed. Interestingly, protein levels of PHD3 were markedly increased by fasting, which is positively correlated with activated PKA signaling and increased expression of the key gluconeogenic enzyme PEPCK (Fig. 1*A*). Consistently, upregulation of PHD3 was observed in mouse primary hepatocytes treated with forskolin, which activates cAMP signaling and mimics a fasting signal (Fig. 1*B*). The increased PHD3 was reversed by PKA inhibitor H89, and the direct association between PKA and PHD3 was further determined (Fig. 1*C* and *SI Appendix*, Fig. S1 *B* and *C*). Given that mRNA levels of PHD3 had little change under fasted state (*SI Appendix*, Fig. S1*A*), these findings suggest a posttranslational modification and activation of PHD3 via PKA signaling. Moreover, in single-cell transcriptome data from livers of mice on Tabula Muris (https://tabula-muris.ds.czbiohub.org/images/All-facs-Actb-tsne.png), expression levels of PHD3 and gluconeogenic genes PEPCK and G6Pase are all enriched in the hepatocyte cluster (*SI Appendix*, Fig. S1 *D–G*), which suggest that PHD3 might play a role in regulating hepatic glucose metabolism in response to fasting signals. To test this hypothesis, PHD3 LKO mice and their WT littermates were further assessed. Compared with WT mice, fasting glycemia and conversion of pyruvate to glucose as evidenced by pyruvate tolerance test were lowered in PHD3 LKO mice (Fig. 1 *D* and *E*). Consistently, gluconeogenic enzymes PEPCK and G6Pase were markedly reduced in PHD3 LKO mice under fasted conditions (Fig. 1*F*). Notably, plasma levels of glucagon and insulin were unaltered, suggesting that PHD3 deficiency–reduced gluconeogenesis is not mediated by regulating hormonal signals (Fig. 1 *G* and *H*). To further determine the effects of PHD3 on hepatic gluconeogenesis, in vivo luciferase reporter assay using cAMP-response element luciferase (CRE-Luc) reporter was performed. As shown in Fig. 1*I*, fasting-induced CRE-Luc activity in WT mice was markedly alleviated in PHD3 LKO mice. Notably, the knockout efficiency and specificity of PHD3 in the liver were verified by real-time PCR and immunoblots (Fig. 1 *J* and *K* and *SI Appendix*, Fig. S1*H*). Together, these data indicate that hepatocyte-specific deletion of PHD3 reduces hepatic gluconeogenesis in response to fasting signals.

Inhibition of CRTC2 Nuclear Translocation and Lowered Hepatocyte Glucose Production by PHD3 Deficiency. As shown in Fig. 2*A*, PHD3 deficiency caused a reciprocal downregulation of active form of dephosphorylated CRTC2 and upregulation of inactive form of phosphorylated CRTC2 in response to forskolin. PHD3 deficiency caused a significant reduction of forskolin-stimulated CRE-Luc reporter activity (Fig. 2*B* and *SI Appendix*, Fig. S2*A*). In consistent, forskolin-induced nuclear translocation of CRTC2 was diminished in PHD3-deficient primary hepatocytes and HEK293T cells (Fig. 2 *C* and *D* and *SI Appendix*, Fig. S2*B*). Furthermore, CRTC2 protein localizing in nucleus was reduced in livers of fasted PHD3 LKO mice (*SI Appendix*, Fig. S2*C*). Notably, protein levels of CRTC2 in livers were unaltered (*SI Appendix*, Fig. S2*D*). These data suggest a potential role of CRTC2 in mediating PHD3's effects on gluconeogenesis.

Next, chromatin immunoprecipitation assays were performed. Fasting caused a significant induction of the occupancy of CRTC2 over PEPCK and G6Pase promoters in livers of WT mice, whereas these effects were ablated in PHD3 LKO mice (Fig. 2*E*). Importantly, disruption of PHD3 in hepatocytes resulted in a potent reduction of forskolin- or glucagon-stimulated expression of gluconeogenic genes and glucose output, suggesting that PHD3 regulates gluconeogenic program in a hepatocyte-autonomous manner (Fig. 2 *F* and *G*). Expression levels of PGC-1α and phosphorylation and nuclear localization of FoxO1, the key regulator of hepatic glucose production (15–17), were unaltered in livers of PHD3 LKO mice under the fasted state (*SI Appendix*, Fig. S2 *C* and *E–H*). Together, these data demonstrate that PHD3 deficiency inhibits nuclear translocation of CRTC2 and lowers hepatocyte glucose production.

PHD3 KI Mice Are Resistant to Fasting-Induced Gluconeogenesis. To investigate whether prolyl hydroxylase activity is required for stimulatory effects of PHD3 on gluconeogenesis in vivo, liver-specific prolyl hydroxylase activity–deficient PHD3 knockin mice were generated with alanine mutation in PHD3 at His196 (PHD3 KI) (18). Interestingly, as shown in Fig. 3 *A–F*, PHD3 KI mice had lowered gluconeogenic profiles during fasting, including fasting glycemia, hepatic glucose production capacity, expression of gluconeogenic genes, and hepatic CRE-Luc reporter activity, which almost mimics the phenotypes observed in PHD3 LKO mice. Consistently, plasma levels of glucagon and insulin were comparable. Notably, gene-targeting strategy and the knockin efficiency for PHD3 H196A KI mice were verified by Sanger sequencing and real-time PCR (Fig. 3 *G* and *H* and *SI Appendix*, Fig. S3*A*). These results strongly demonstrate that prolyl hydroxylase activity of PHD3 is required for its effects on promoting hepatic gluconeogenesis.

Next, the importance of PHD3 hydroxylase activity in regulating gluconeogenesis in vitro was further determined. As shown in Fig. 3*I*, PHD3 induced CRE-Luc activity, whereas these effects were

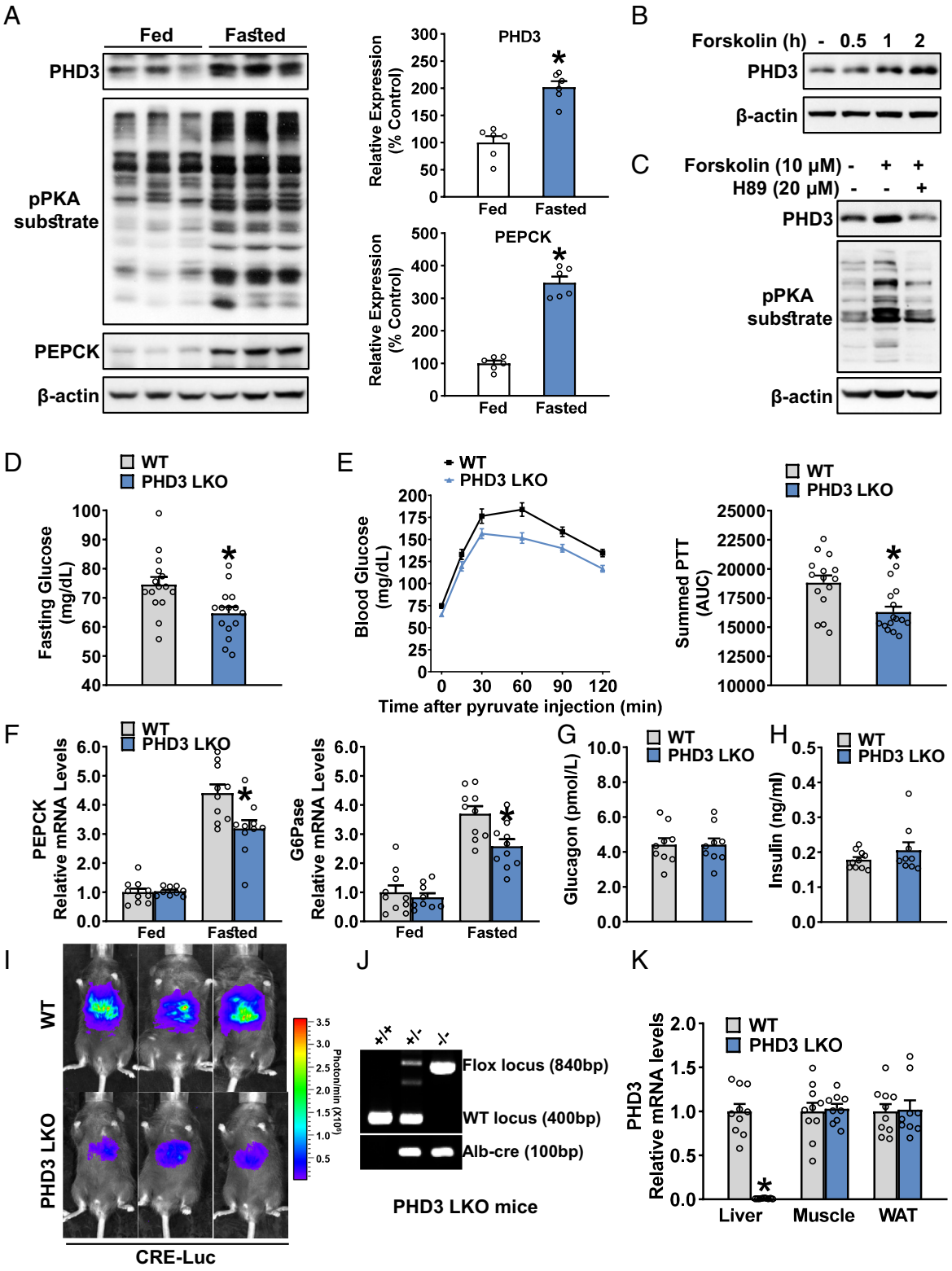

Fig. 1. Hepatocyte-specific deletion of PHD3 decreases fasting-induced hepatic gluconeogenesis in mice. (*A*) Eight-week-old male mice were fasted for 16 h. Protein levels of PHD3 and PEPCK in livers and the band intensity were quantified. n = 6. *$P < 0.05$ vs. fed. (*B*) Protein levels of PHD3 in mouse primary hepatocytes treated with forskolin. (*C*) Mouse primary hepatocytes were treated with forskolin for 4 h and H89 for 3 h, protein levels of PHD3 were determined. (*D–E*) PHD3 LKO mice and WT littermates at 8 wk old were fasted for 16 h. (*D*) Fasting blood glucose was decreased in PHD3 LKO mice. n = 15. (*E*) Pyruvate tolerance tests. n = 15. (*F*) Hepatic expression of gluconeogenic genes were decreased in fasted PHD3 LKO mice. n = 11 to 12. (*G* and *H*) Plasma glucagon (*G*) and insulin (*H*) levels. n = 9 to 10. *$P < 0.05$ vs. WT. (*I*) Hepatic CRE-Luc activity in 16-h-fasted PHD3 LKO and WT mice. (*J*) Genotypes of WT, PHD3 heterozygous, and LKO mice. (*K*) mRNA levels of PHD3 in mouse liver, muscle, and white adipose tissue (WAT). n = 9 to 10. *$P < 0.05$ vs. WT.

attenuated by PHD3 H196A mutant. Consistently, forskolin-stimulated CRE-Luc activity was repressed by prolyl-hydroxylase inhibitor dimethyloxaloylglycine (DMOG). Importantly, a potent reduction of forskolin- or glucagon-stimulated expression of gluconeogenic genes and glucose output was observed in PHD3 H196A primary hepatocytes (Fig. 3 *J* and *K*). Moreover, prolyl hydroxylase

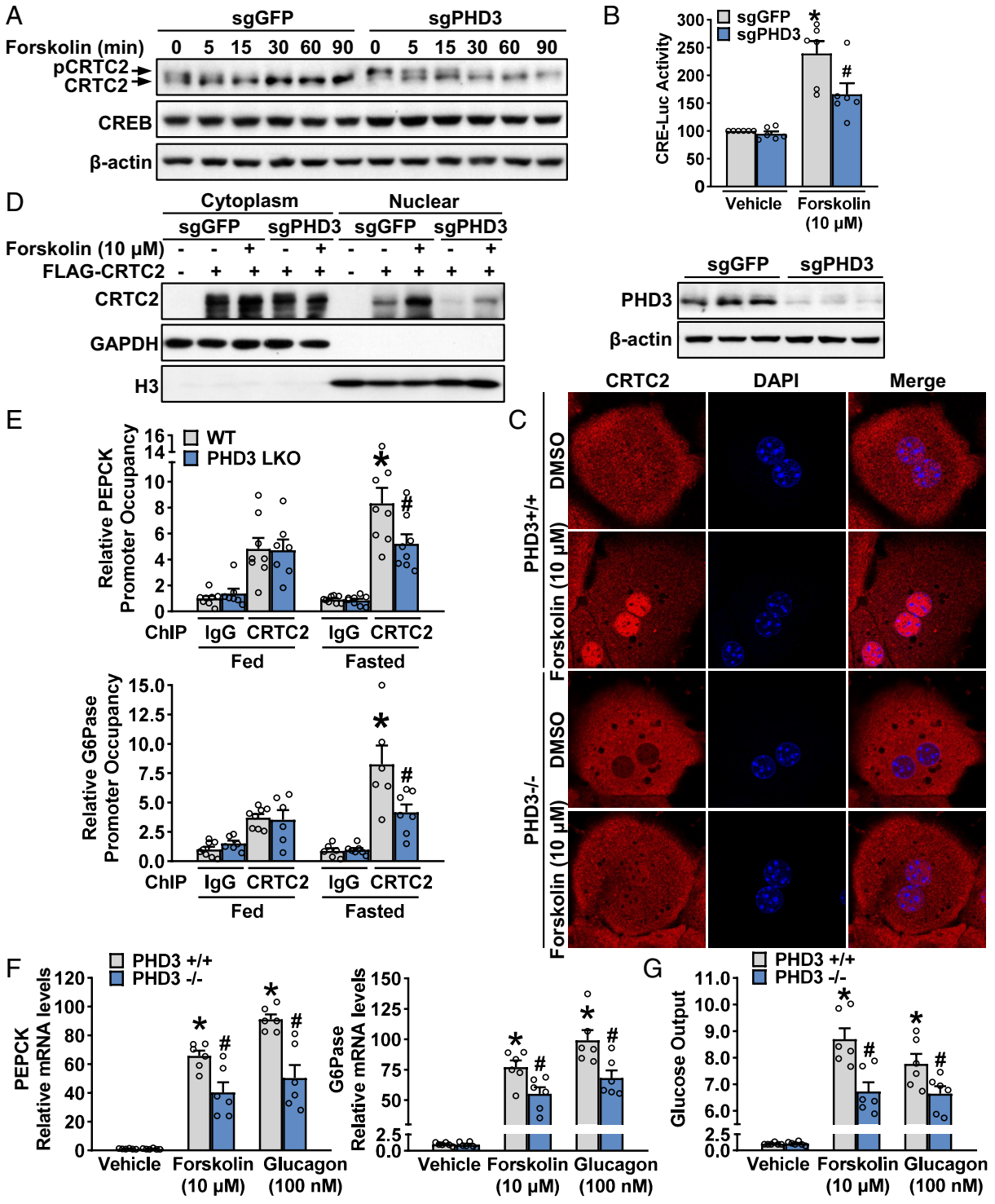

Fig. 2. PHD3 deficiency inhibits nuclear translocation of CRTC2 and lowers hepatocyte glucose production. (*A* and *B*) PHD3 deficiency decreases dephosphorylation and activity of CRTC2 induced by forskolin. (*A*) Immunoblot of CRTC2 in single-guide GFP control (sgGFP) and PHD3-deficient (sgPHD3) HEK293T cells treated with forskolin. (*B*) The luciferase activities of CRE-Luc in sgGFP and sgPHD3 HEK293T cells (*Top*) and the knockout efficiency of PHD3 (*Bottom*). n = 6. **P* < 0.05 vs. sgGFP and vehicle; #*P* < 0.05 vs. sgGFP and forskolin. (*C*) Immunofluorescence of endogenous CRTC2 (red) and DAPI (blue) in primary hepatocytes. (*D*) Distribution of CRTC2 in cytoplasmic and nuclear fractions in sgGFP and sgPHD3 HEK293T. (*E*) The occupancy of endogenous CRTC2 on the PEPCK and G6Pase promoters is abrogated by PHD3 deficiency in fasted mouse livers. n = 6 to 8. **P* < 0.05 vs. fed WT mice with CRTC2 immunoprecipitation; #*P* < 0.05 vs. fasted WT mice with CRTC2 immunoprecipitation. (*F* and *G*) mRNA levels of PEPCK and G6Pase (*F*) and glucose output (*G*) in primary hepatocytes. n = 6. **P* < 0.05 vs. PHD3+/+ and vehicle; #*P* < 0.05 vs. PHD3+/+ and forskolin or glucagon.

activity–deficient PHD3 also abolished endogenous CRTC2 nuclear entry in hepatocytes and livers but had little effects on protein levels of CRTC2 in liver lysates (*SI Appendix*, Fig. S3 *B–D*). Expression levels of PGC-1α and activation of FoxO1 were unaltered in livers of KI mice under the fasted state (*SI Appendix*, Fig. S3 *E–H*). Together, prolyl hydroxylase activity of PHD3 is necessary for its stimulatory effects on fasting-induced gluconeogenesis in vivo and in vitro.

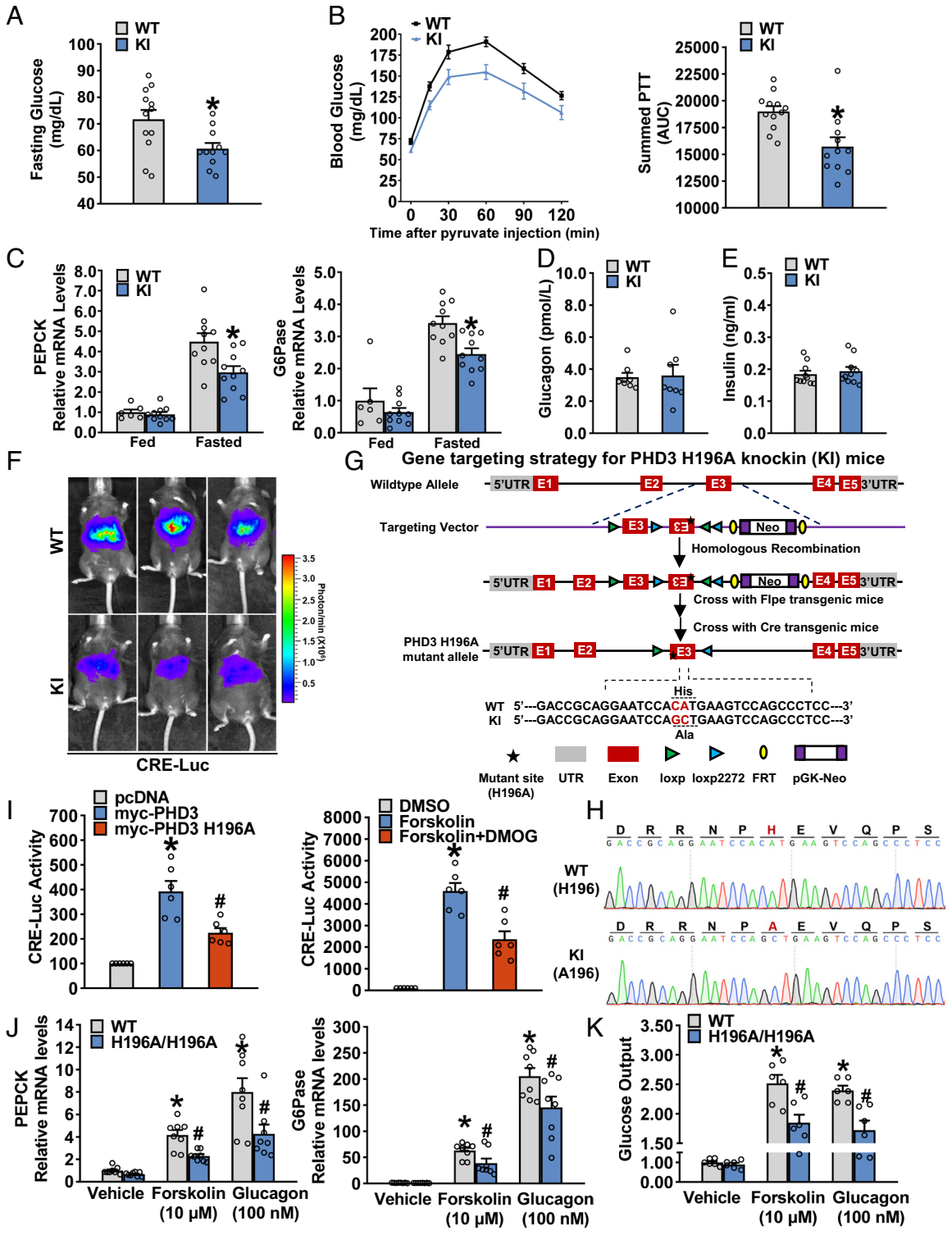

Fig. 3. PHD3 His196 is essential for stimulating gluconeogenesis in mice during fasting. Male PHD3 H196A knockin (KI) mice and WT mice at 8 wk old were fasted for 16 h. (*A*) Fasting blood glucose levels. n = 11 to 12. (*B*) Pyruvate tolerance tests. n = 11 to 12. (*C*) Hepatic expression of gluconeogenic genes. n = 10 to 11. **P* < 0.05 vs. WT. (*D* and *E*) Plasma glucagon (*D*) and insulin (*E*) levels. n = 8 to 10. (*F*) Hepatic CRE-Luc activity in 16-h-fasted PHD3 KI and WT mice. (*G* and *H*) Generation of PHD3 H196A knockin (KI) mice. Gene-targeting strategy (*G*) and sequencing verification (*H*) for KI mice are shown. (*I*) Relative luciferase activities of CRE-Luc in HEK293T cells transfected with PHD3 or PHD3 H196A (*Left*) or treated with forskolin and DMOG (*Right*). n = 6. **P* < 0.05 vs. pcDNA or DMSO; #*P* < 0.05 vs. myc-PHD3 or forskolin. (*J* and *K*) Transcriptional levels of PEPCK and G6Pase (*J*) and glucose output (*K*) in WT or H196A/H196A primary hepatocytes. n = 6. **P* < 0.05 vs. WT treated with vehicle; #*P* < 0.05 vs. WT treated with forskolin or glucagon.

Catalytic Domain of PHD3 Interacts with CRTC2. Next, the direct substrate of PHD3 was investigated. Among 388 proteins that bind to PHD3 identified by quantitative interaction proteomics (19), a group of proteins involved in the regulation of glucose metabolic process are enriched, including CRTC2 (Fig. 4 *A* and *B*). Given that PHD3 deficiency caused a significant reduction of nuclear translocation and transcriptional activity of CRTC2, we hypothesize that PHD3 may regulate gluconeogenic gene expression via interaction with CRTC2.

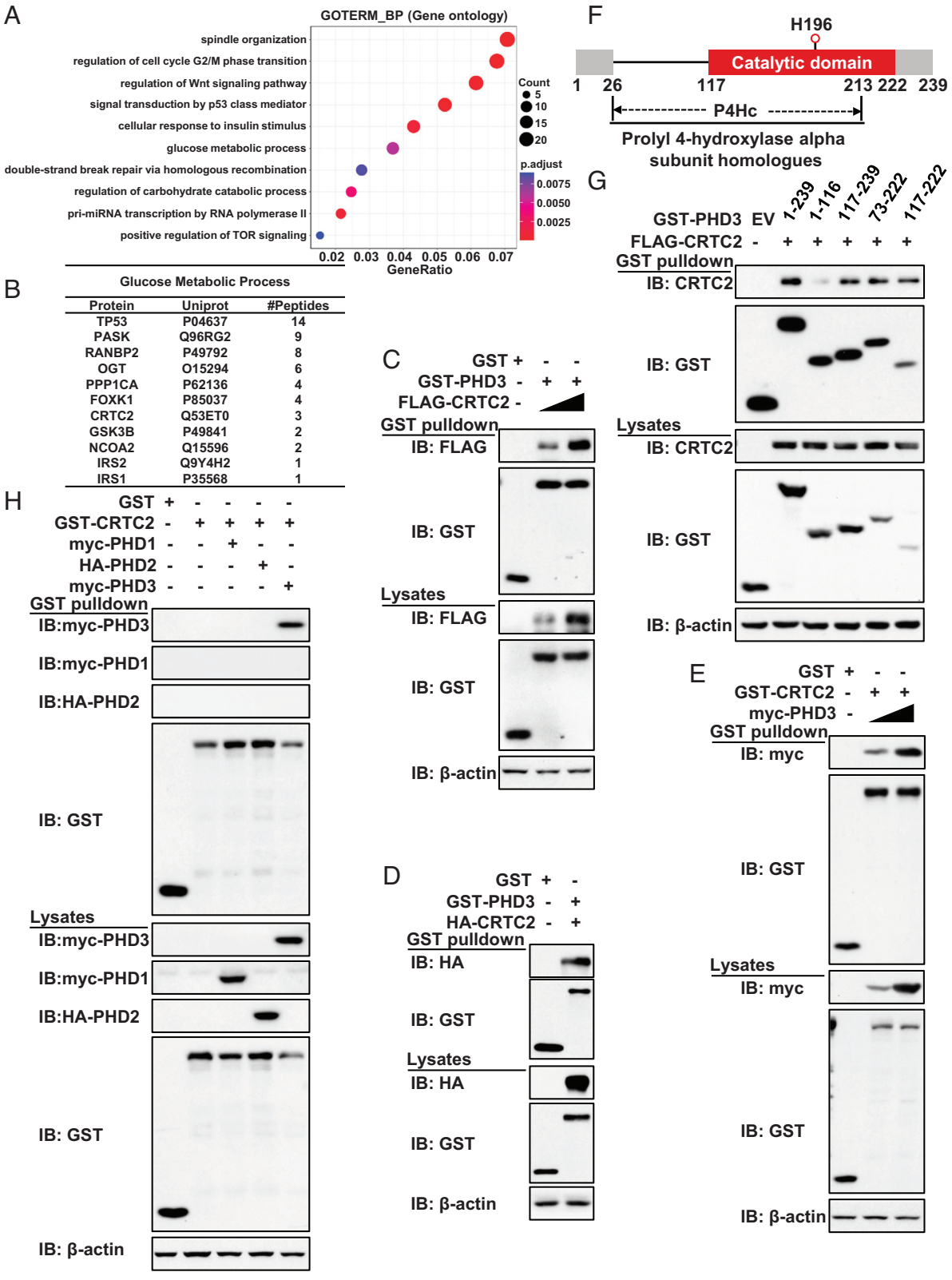

Fig. 4. PHD3 catalytic domain associates with CRTC2. (*A*) Gene ontology (GO) analysis of candidate PHD3–associated proteins identified by mass spectrometry analysis. (*B*) CRTC2 is involved in the PHD3-associated proteins that enriched in glucose metabolic process. (*C–E*) Immunoblot showing association between PHD3 and CRTC2. GST-PHD3 together with FLAG-CRTC2 (*C*) or HA-CRTC2 (*D*), GST-CRTC2, and myc-PHD3 (*E*) were transfected in HEK293T cells. (*F*) Schematic presentation of PHD3 domain structures. (*G*) FLAG-CRTC2 and the truncate mutants were cotransfected with GST-PHD3 in HEK293T cells. The lysates were analyzed by GST-pulldown analysis. (*H*) GST-CRTC2 was cotransfected with myc-PHD1, HA-PHD2, or myc-PHD3 in HEK293T cells, followed by GST-pulldown analysis.

As shown in Fig. 4 *C–E*, PHD3 and CRTC2 indeed associates with each other. Moreover, PHD3 mutant that does not contain catalytic domain (from amino acid 1-116) fails to interact with CRTC2 (Fig. 4 *F* and *G*), suggesting that the catalytic domain of PHD3 is required for the interaction with CRTC2. Interestingly, CRTC2 specifically binds to PHD3 but not PHD1 or PHD2 (Fig. 4*H*).

Given that PHD3 is involved in the cellular response to oxygen by hydroxylating HIFα (20), expression levels of HIFα were measured. As shown in *SI Appendix*, Fig. S4 *A* and *B*, expression levels of both HIF-1α and HIF-2α were unaffected by PHD3 deficiency, which are consistent with previous observations in PHD3-deficient colon epithelial cells (13), (14). Consistently, PHD3 deficiency caused a significant reduction of gluconeogenic gene expression in HIF-1α or HIF-2α knockdown primary hepatocytes (*SI Appendix*, Fig. S4 *C* and *D*). These data demonstrate that PHD3 may regulate gluconeogenic program through interaction with CRTC2, which is likely independent of HIF pathway.

PHD3-Mediated Hydroxylation of CRTC2 Enhances the Association between CRTC2 and CREB and Promotes Gluconeogenic Capacity. We hypothesize that PHD3 might increase CRTC2 activity through hydroxylation. As shown in Fig. 5 *A–C*, administration of forskolin and overexpression of PKA or PHD3 caused a potent induction of hydroxylation levels of CRTC2, whereas these effects were diminished by the inhibition of hydroxylase activity using DMOG. Consistently, hydroxylation of CRTC2 was stimulated by forskolin in PHD3+/+ MEFs, but not in PHD3−/− MEFs (Fig. 5*D*). Importantly, catalytically inactive PHD3 H196A mutant failed to increase the hydroxylation of CRTC2 (Fig. 5*E*). These results demonstrate that CRTC2 is regulated by PHD3 through hydroxylation.

To further investigate PHD3-mediated hydroxyproline in CRTC2, liquid chromatography–tandem mass spectrometry (LC–MS/MS) analysis was performed. Six candidate hydroxylated prolines were revealed, including Pro129, Pro132, Pro138, Pro139, Pro229, and Pro615 (Fig. 5*F* and *SI Appendix*, Fig. S5*A*). Strikingly, forskolin-caused hydroxylation of CRTC2 was eliminated by the mutation of P129A and P615A, but not of P132A, P138A, P139A, and P229A (Fig. 5*G*), suggesting that Pro129 and Pro615 sites are essential for PHD3's effects on hydroxylation of CRTC2. Notably, the conservation of these two residues in CRTC2 across different species is observed (*SI Appendix*, Fig. S5*B*).

As shown in Fig. 5*H*, nuclear translocation of CRTC2 by forskolin was markedly attenuated by P129A, P615A, or double mutant (P2A), suggesting that both sites are required for nuclear translocation of CRTC2. As shown in Fig. 5 *I* and *J*, treatment with WT PHD3, but not H196A mutant, increased the interaction between CRTC2 and CREB. Consistently, nonhydroxylatable CRTC2 P2A mutant had impaired association with CREB. Importantly, the stimulation of CRE-Luc activity by WT CRTC2 was significantly reduced by P2A mutant (Fig. 5*K* and *SI Appendix*, Fig. S5*C*). These data demonstrate that Pro129 and Pro615 hydroxylation of CRTC2 by PHD3 is required for its association with CREB and nuclear localization, and the potentiation of transcriptional activities of CRTC2–CREB complex.

Given that dephosphorylation of mouse CRTC2 at Ser171 and Ser275 (Ser274 in humans) facilitates nuclear entry and activation of CRTC2 (21–23), the correlation between hydroxylation and phosphorylation in regulating CRTC2 activity is investigated. The induction of CRE-Luc activity by nonphosphorylatable CRTC2 double-mutant S171A/S274A (S2A) was attenuated by S2A/P2A mutant but was further enhanced by PHD3 overexpression (Fig. 5 *L* and *M*). Consistently, the nonphosphorylatable S2A mutant was localized in the nucleus of WT cells, whereas these effects were attenuated by the nonphosphorylatable/nonhydroxylatable S2A/P2A mutant (*SI Appendix*, Fig. S5*D*). Furthermore, inhibition of SIK2, that mediates Ser171 phosphorylation and nuclear exclusion of CRTC2, induced PEPCK and G6Pase, which was abrogated by PHD3 deficiency (Fig. 5*N*). These results suggest that hydroxylation-mediated nuclear entry and activation of CRTC2 is likely independent of CRTC2 phosphorylation. Importantly, PHD3-caused stimulation of CRE-Luc activity was abrogated by CRTC2 deficiency in primary hepatocytes and 293T cells, suggesting a causal link between PHD3 and CRTC2 axis and their effects on gluconeogenesis (*SI Appendix*, Fig. S5 *E–G*). Taken together, these findings indicate that PHD3-mediated hydroxylation at Pro129 and Pro615 increases CRTC2 association with CREB, nucleus shuttling, and activation of hepatic gluconeogenesis, which may represent a noncanonical hydroxylation-dependent and phosphorylation-independent CRTC2 pathway.

PHD3 LKO and KI Mice Are Resistant to HFHS Diet–Increased Gluconeogenesis and Glycemia. As shown in Fig. 6 *A–D*, overexpression of WT CRTC2 increased fasting glycemia, capacity of gluconeogenesis, and expression of PEPCK and G6Pase in livers. However, these effects were remarkably impaired by P2A mutant. Consistently, the nuclear localization of P2A mutant was markedly reduced under fasted state compared with WT CRTC2 (*SI Appendix*, Fig. S6*A*). Moreover, the reduction of PEPCK and G6Pase by PHD3 deficiency was reversed by CRTC2 restoration in primary hepatocytes (Fig. 6*E* and *SI Appendix*, Fig. S6*B*). Consistently, nuclear localization of endogenous CRTC2 was decreased in PHD3−/− primary hepatocytes as measured by CRTC2 antibody. Exogenously overexpressed FLAG-tagged CRTC2 is detected by both CRTC2 and FLAG antibody and localized in the nucleus (*SI Appendix*, Fig. S6*C*).

Next, the effects of PHD3 were further investigated in diet-induced diabetogenic mice (24, 25). PHD3 deficiency lowered the fasting glycemia, hepatic glucose production capacity, and expression of gluconeogenic genes (Fig. 6 *F–H*), whereas plasma glucagon and body weight were not changed (Fig. 6 *I–J*). As shown in *SI Appendix*, Fig. S7 *A-D* and 6K, the improved glucose tolerance test, insulin tolerance test, HOMA-IR, plasma insulin, and increased hepatic phosphorylation of Akt and insulin receptor were observed in PHD3 LKO mice, which suggested that insulin sensitivity was improved. Consistently, as shown in Fig. 6 *L–Q* and *SI Appendix*, Fig. S7 *E–H*, PHD3 KI mice fed on HFHS diet showed similar effects. Notably, plasma levels of glucagon and body weight were comparable. Collectively, these data reveal that PHD3 LKO and KI mice are resistant to HFHS diet–increased gluconeogenesis and hyperglycemia likely via inhibition of CRTC2.

Elevation of CRTC2 Hydroxylation in Livers of Fasted Mice and Diabetic Hyperglycemic Mice and Humans. To further investigate hydroxylation levels of CRTC2 in livers of mice during physiological and pathological conditions, the prolyl hydroxyl–specific Pro615 antibody (CRTC2 Pro615-OH) was generated, whereas the antibody that recognizes CRTC2 Pro129-OH was failed during the development process. Interestingly, hydroxylated CRTC2 at Pro615 site and PHD3 were remarkably elevated in livers of fasted, HFHS diet–induced hyperglycemic mice or genetically driven hyperglycemic ob/ob mice (Fig. 7 *A–C*). Moreover, in human liver specimen, hydroxylation levels of CRTC2 at Pro615 were induced in livers of diabetic subjects compared with nondiabetic controls (Fig. 7 *D* and *E*), which are positively correlated with the induction of PHD3 and PEPCK. Notably, the efficiency and specificity of CRTC2 Pro615-OH were validated (Fig. 7*F*).

We further explored the relevance of PHD3 with human metabolic disorders. Out of 62 single-nucleotide polymorphisms with minor allele frequencies >0.01 in the 1,000 Genomes phase 3 population of Han Chinese in Beijing (CHB) (data release 2, May 2013, on National Center for Biotechnology Information GRCh37 assembly), located in the encoding and

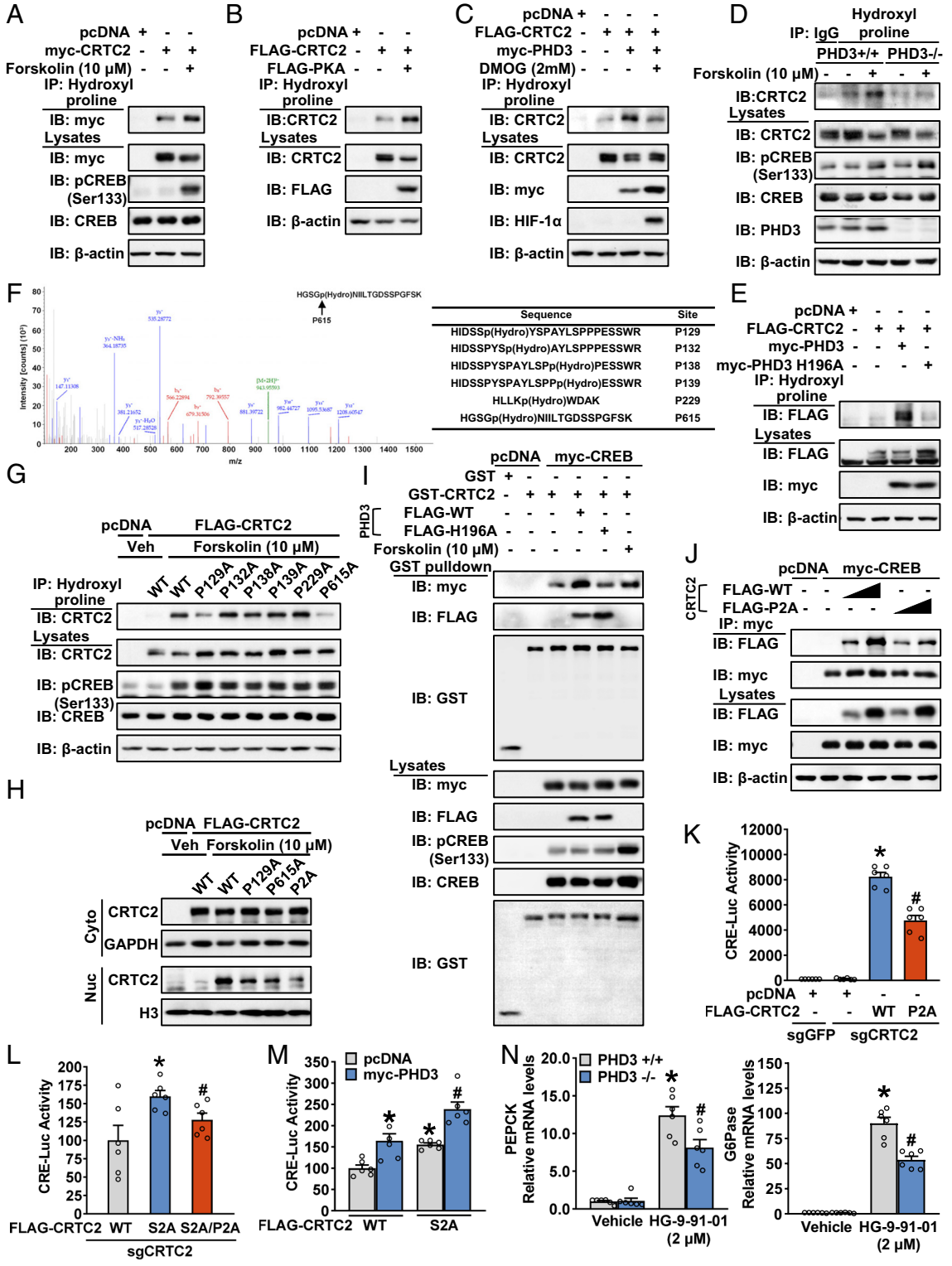

Fig. 5. Hydroxylation of CRTC2 by PHD3 is necessary for the association between CRTC2 and CREB and augmentation of gluconeogenesis. (*A*–*E*) PHD3 modulates CRTC2 hydroxylation. (*A*) HEK293T cells were transfected with myc-CRTC2, followed by treatment with forskolin. (*B*) FLAG-CRTC2 and FLAG-PKA were cotransfected. (*C*) FLAG-CRTC2 and myc-PHD3 were cotransfected, followed by treatment with DMOG. (*D*) PHD3+/+ or PHD3−/− MEFs were treated with forskolin. (*E*) FLAG-CRTC2 was cotransfected with myc-PHD3 or myc-PHD3 H196A mutants. Antihydroxyproline immunoprecipitates were analyzed. (*F*) Hydroxyproline residues of CRTC2 are identified by LC–MS/MS. Representative mass spectra for hydroxylated Pro615 in CRTC2 peptides and all identified hydroxyproline residues in CRTC2 are shown. (*G*) HEK293T cells were transfected with WT FLAG-CRTC2 or nonhydroxylatable mutants, followed by treatment with forskolin. Antihydroxyproline immunoprecipitates were analyzed for CRTC2. (*H*) The levels of CRTC2 in cytoplasm and nucleus were analyzed. P2A, P129A/P615A double mutant. (*I*) PHD3 facilitates the association between CRTC2 and CREB. (*J*) Hydroxylation deficiency of CRTC2 abrogates CRTC2–CREB complex formation. (*K*) Transcriptional activity of nonhydroxylatable CRTC2 is decreased. n = 6. *$P < 0.05$ vs. sgCRTC2 and pcDNA; #$P < 0.05$ vs. sgCRTC2 and WT CRTC2. (*L*) FLAG-WT human CRTC2, CRTC2 Ser171, and Ser274 nonphosphorylatable mutants (S2A) or S2A/P2A mutants were transfected in CRTC2-deficient HEK293T cells, and transcriptional activity of CRTC2 was analyzed by dual luciferase assay. n = 6. *$P < 0.05$ vs. WT CRTC2; #$P < 0.05$ vs. S2A CRTC2. (*M*) Overexpression of PHD3 further enhanced the activity of CRTC2 S2A mutant. n = 6. *$P < 0.05$ vs. pcDNA and WT CRTC2; #$P < 0.05$ vs. pcDNA and S2A CRTC2. (*N*) mRNA levels of PEPCK and G6Pase in primary hepatocytes treated with SIK inhibitor HG-9-91-01 as indicated. n = 6. *$P < 0.05$ vs. PHD3+/+ and vehicle; #$P < 0.05$ vs. PHD3−/− and HG-9-91-01.

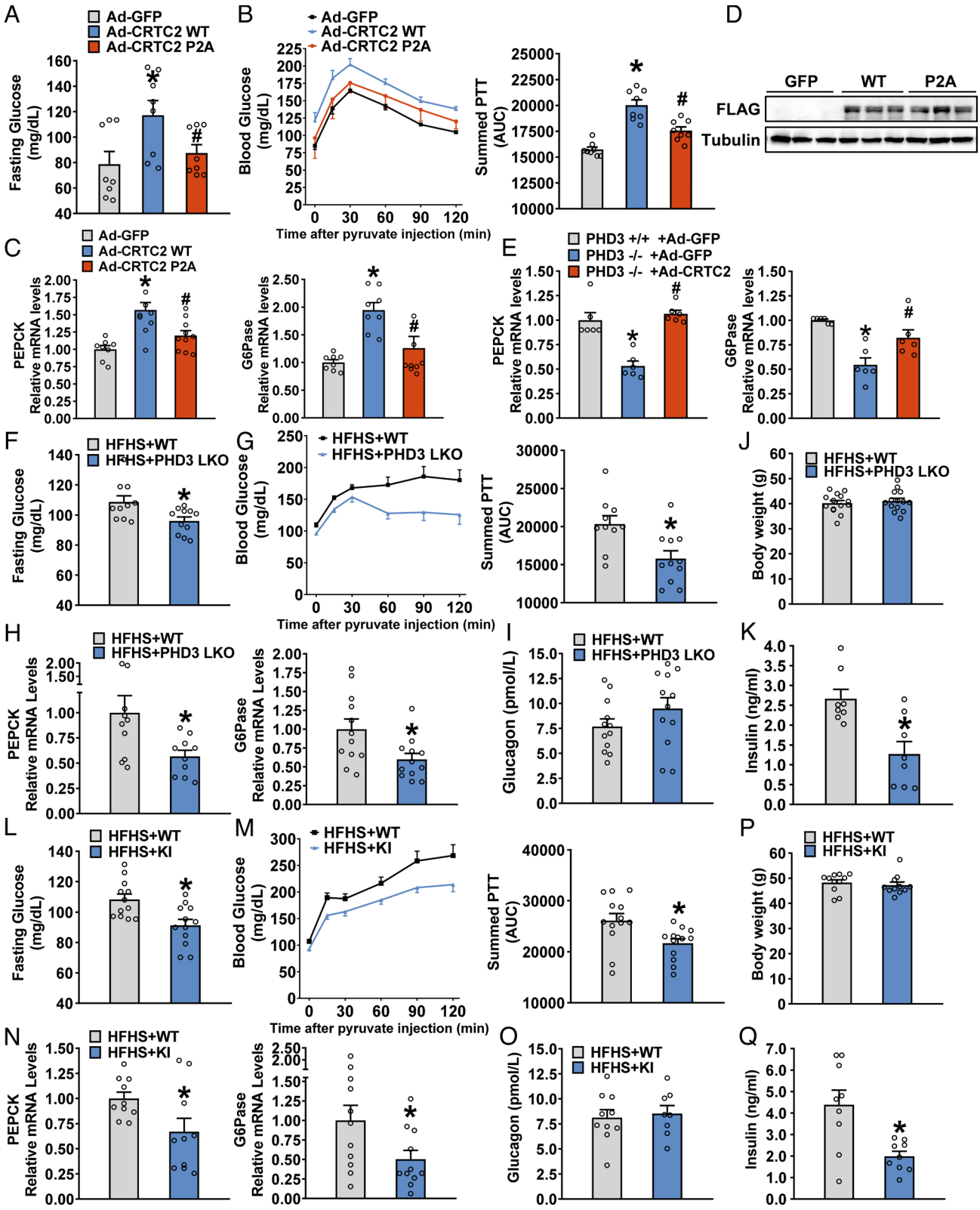

Fig. 6. PHD3 LKO and KI mice are resistant to increased gluconeogenesis and glycemia upon HFHS diet feeding. (*A–D*) Eight-week-old male C57BL/6 mice were treated with adenoviruses encoding WT CRTC2 (Ad-CRTC2 WT), CRTC2 P2A mutant (Ad-CRTC2 P2A), or GFP by tail-vein injection. (*A*) Fasting blood glucose levels. (*B*) Pyruvate tolerance tests. (*C*) mRNA levels of PEPCK and G6Pase in livers. n = 8 to 10. *$P < 0.05$ vs. Ad-GFP; #$P < 0.05$ vs. Ad-CRTC2 WT. (*D*) Overexpression of WT or P2A mutant CRTC2 in livers was verified. (*E*) mRNA levels of PEPCK and G6Pase in primary hepatocytes infected with adenoviruses encoding CRTC2 or GFP with forskolin treatment. n = 6. *$P < 0.05$ vs. PHD3+/+ and Ad-GFP; #$P < 0.05$ vs. PHD3−/− and Ad-GFP. (*F–K*) Male PHD3 LKO and WT mice at 8 wk old were fed with HFHS diet for 16 wk. (*F*) Fasting blood glucose levels. (*G*) Pyruvate tolerance test. (*H*) Expression levels of PEPCK and G6pase in livers. (*I*) Plasma glucagon levels. (*J*) Body weight. (*K*) Plasma insulin levels. n = 8 to 12. (*L–Q*) Male PHD3 KI and WT mice at 8 wk old were fed with HFHS diet for 16 wk. Fasting blood glucose (*L*), pyruvate tolerance test (*M*), mRNA levels of gluconeogenic genes (*N*), plasma glucagon levels (*O*), body weight (*P*), and plasma insulin levels (*Q*) were measured. n = 8 to 13. *$P < 0.05$ vs. HFHS + WT.

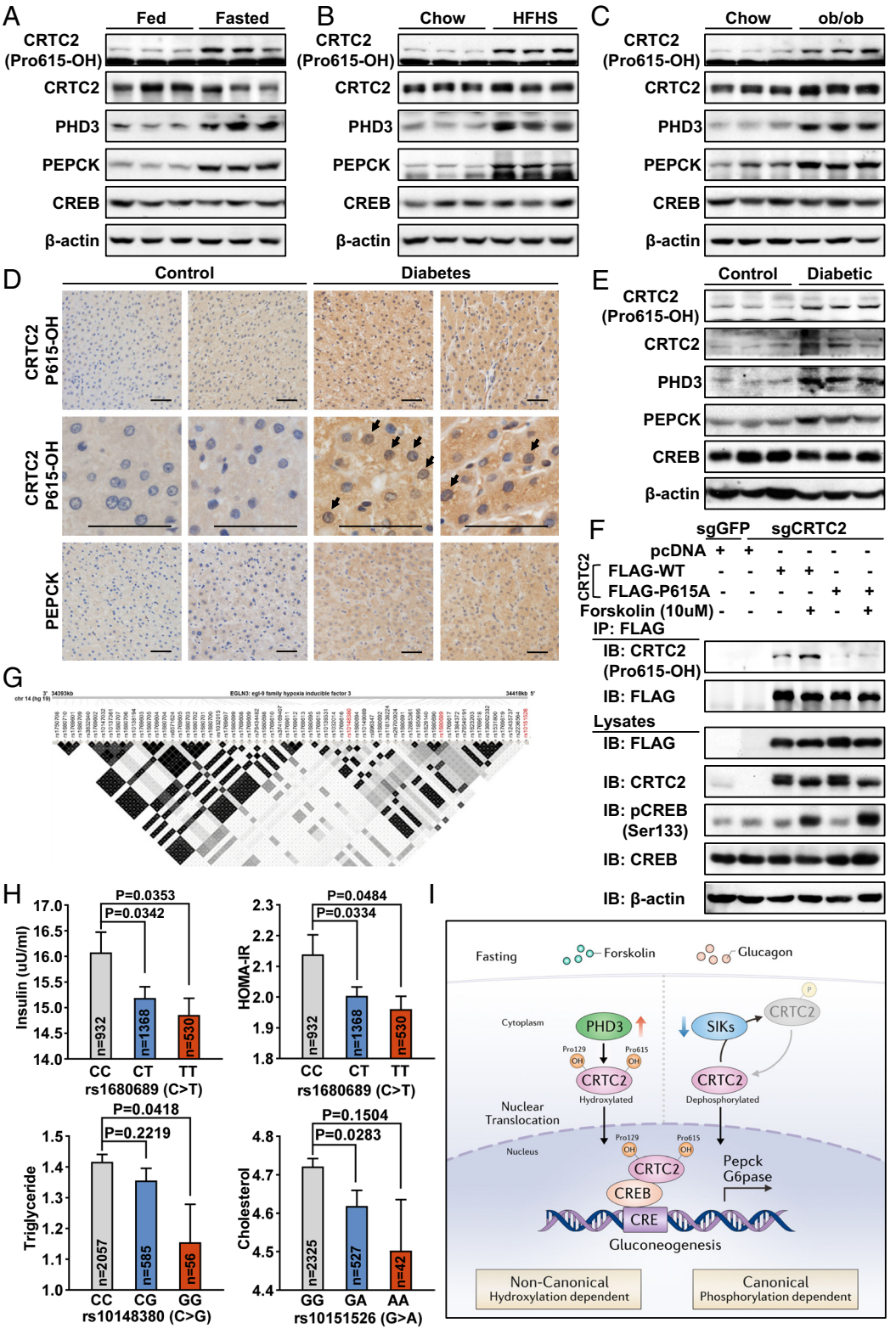

Fig. 7. Hydroxylation of CRTC2 is increased in livers of fasted mice and diabetic mice and humans. (*A*–*C*) Hydroxylation of CRTC2 at Pro615 residue and expression of PHD3 and PEPCK are increased in the livers of fasted (*A*), HFHS diet–fed (*B*), or ob/ob (*C*) mice. (*D* and *E*) Representative immunostaining (Scale bars: 50 μm) (*D*) and immunoblots (*E*) of CRTC2 Pro615-OH, PHD3, and PEPCK in livers from diabetic and nondiabetic control patients are shown. (*F*) The specificity of anti-CRTC2 Pro615-OH antibody is verified. (*G*) Linkage disequilibrium map of the PHD3 genomic region. See also *SI Appendix*, Table S1. (*H*) Comparison of the insulin, HOMA-IR, triglyceride, and cholesterol levels of people with different genotypes of rs1680689, rs10148380, and rs10151526. (*I*) The proposed model for PHD3-mediated noncanonical CRTC2 hydroxylation–dependent activation of hepatic gluconeogenesis. In response to fasting or glucagon signals, PHD3 associates with and hydroxylates CRTC2 protein at Pro129 and Pro615 residues, leading to CRTC2's nuclear translocation and CREB/CRTC2 pathway activation, which is likely independent of the canonical dephosphorylation–mediated CRTC2 activation. Reducing the hydroxylation of CRTC2 may improve hepatic glucose homeostasis and type 2 diabetes.

flanking regions of the PHD3 locus, 13 tagging single-nucleotide polymorphisms (r^2 threshold = 0.8) were genotyped in the Nutrition and Health of Aging Population in China study. We found that the minor allele T of rs1680689 is significantly correlated with lowered levels of insulin and HOMA-IR. The minor allele G of rs10148380 and the minor allele A of rs10151526 are related to lowered levels of triglyceride and cholesterol (Fig. 7 *G* and *H*). These results demonstrate that hydroxylation of CRTC2 by PHD3 may be linked to the pathogenesis of hyperglycemia, insulin resistance, and type 2 diabetes in rodents and humans.

Discussion

This report demonstrates that prolyl hydroxylase PHD3 acts as a unique regulator in regulating hepatic gluconeogenesis through a posttranslational modification. Mechanistically, catalytic domain of PHD3 specifically binds to and directly hydroxylates the CREB coactivator CRTC2, and hydroxylation of CRTC2 increases its nuclear translocation and association with CREB, which leads to enhanced transcription of gluconeogenic gene expression. PHD3-dependent hydroxylation of CRTC2 represents an unknown mechanism of hydroxylation-dependent pathway in the regulation of hepatic gluconeogenesis and glucose homeostasis (Fig. 7*I*).

The Prolyl Hydroxylase PHD3 Acts as a Unique Regulator for Hepatic Gluconeogenesis. This study characterizes PHD3 as a unique regulator for glucose metabolism in livers. Several lines of evidence support the uncovered role of PHD3 in the regulation of hepatic gluconeogenesis. First, PHD3 deficiency in the liver profoundly reduced fasting blood glucose, conversion of pyruvate to glucose, and expression of key gluconeogenic enzymes. Moreover, PHD3 deficiency decreased glucose output and expression of PEPCK and G6Pase in primary hepatocytes. Notably, these effects of PHD3 were observed in PHD3 prolyl hydroxylase activity–deficient knockin mice and primary hepatocytes, suggesting the importance of hydroxylase activity of PHD3 in regulating gluconeogenesis. Our findings suggest that the prolyl hydroxylase activity of PHD3 plays essential roles in coordinating cellular catabolic and anabolic processes.

Herein, we demonstrate that fasting or glucagon modulates PHD3 activity via PKA-mediated posttranslational modification. The findings broaden our views of the regulation of PHD3 activity and functions under different physiological conditions. First, hepatic protein levels but not transcriptional levels of PHD3 are highly up-regulated by fasting and forskolin. Second, glucagon-stimulated gluconeogenic genes and glucose production are attenuated in PHD3-deficient or catalytically defective primary hepatocytes. Furthermore, PKA is sufficient to associate with PHD3 and increases the hydroxylation of CRTC2. These evidence demonstrate that PHD3 mediates glucagon's effects on hepatic gluconeogenesis under fasting.

CRTC2 Serves as a Unique Substrate of PHD3. One of the major findings of this study is the characterization of CRTC2 as a critical substrate of prolyl hydroxylase PHD3. A number of assays have demonstrated the effects of PHD3–CRTC2 axis on gluconeogenesis. First, PHD3 is sufficient to associate with CRTC2. Second, PHD3 hydroxylates CRTC2 at Pro129 and Pro615 sites. Importantly, the nonhydroxylatable CRTC2 P2A mutant has reduced protein association with CREB and nuclear translocation, which causes a lesser extent of hepatic glucose production and expression of hepatic gluconeogenic genes in mice under the fasting conditions. Moreover, hydroxylation-mediated CRTC2 nuclear translocation and activation is likely independent of the effects of phosphorylation on CRTC2 activity (4), as the activation and increased nuclear entry of nonphosphorylatable CRTC2 was abrogated by P2A mutant and the inhibitory effects of PHD3 deficiency on gluconeogenic genes were unaltered after SIK inhibition.

Although previous studies showed the involvement of PGC-1α in mediating PHD's effects on gluconeogenesis using shRNA-mediated knockdown in hepatocytes (26), expression levels of PGC-1α were comparable in livers of WT and PHD3 LKO mice or KI mice under fasted state. The different gene-knockout strategies and in vivo or in vitro experimental settings may be the contributing factors to this difference. Although mRNA and protein levels of PGC-1α were unaltered in livers of PHD3 LKO and KI mice, we could not rule out the possibility of PGC-1α in mediating the beneficial effects of PHD3 deficiency on gluconeogenesis (27, 28). In accordance with previous reports that show unaltered protein levels of HIF-1α and HIF-2α in Cre-Loxp system– or siRNA-mediated PHD3-deficient colon epithelial cells, livers, and cell lines (13, 29–31), we discovered that the levels of HIF-1α and HIF-2α were not changed by PHD3 deficiency in cells and livers. These findings indicate the importance of non-HIF target in mediating gluconeogenic action of PHD3. Although these data appear to contradict the previous study showing that adenovirus-mediated knockdown of PHD3 increases HIF-2α and improves insulin sensitivity in mice (12), the chronic compensatory mechanisms that are invoked in germline knockout models may be a contributing factor to this difference.

Therapeutic Potential of Targeting Hydroxylation of CRTC2 in Type 2 Diabetes. The current findings indicate that hyperactivation of hydroxylation-dependent PHD3–CRTC2 axis under the nutrient-overload conditions could lead to sustained hepatic glucose production and progression of diabetes. First, PHD3 deficiency or PHD3 prolyl hydroxylase activity–deficiency significantly improved glucose homeostasis in HFHS diet–induced diabetic mice. Furthermore, hydroxylation of CRTC2 was up-regulated in livers of diet-induced and ob/ob diabetic mice. Importantly, increased hydroxylation of CRTC2 was also observed in livers of diabetic patients. The single-nucleotide polymorphisms (SNPs) in PHD3 are associated with insulin resistance and dyslipidemia in humans. These findings support the clinical relevance of CRTC2 hydroxylation during the progression of diabetes. Given that protein hydroxylation is critical in maintaining CREB/CRTC2 interaction, inhibition of CRTC2 hydroxylation or the association between PHD3–CRTC2 may provide unique strategies for treating excessive hepatic glucose production.

In summary, the current study demonstrates a hydroxylation-dependent network of gluconeogenesis. Upon fasting, hydroxylation and nuclear translocation of CRTC2 are augmented by PHD3, leading to enhanced activity of CRTC2 and hepatic gluconeogenesis. Pharmacological and genetic approaches for the modulation of PHD3 or CRTC2 hydroxylation may provide unique therapeutic avenues for the treatment of hyperglycemia in type 2 diabetes.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

ACKNOWLEDGMENTS. This work was supported by grants from National Key R&D Program of China (2019YFA0802502 and 2020YFA0803301), National Natural Science Foundation of China (81925008, 32130047, 82073061, 82170587, 32100943), Shanghai Municipal Science and Technology Major Project, Open Project Program of Metabolic Vascular Diseases Key Laboratory of Sichuan Province (2022MVDKL-K2), Shandong Natural Science Foundation (ZR2020MH206), and

China Postdoctoral Science Foundation (2021M693273). We are grateful to Institutional Center for Shared Technologies and Facilities of Shanghai Institute of Nutrition and Health (SINH), Chinese Academy of Sciences (CAS), for technical assistance.

Author affiliations: [a]CAS Key Laboratory of Nutrition, Metabolism and Food Safety, Shanghai Institute of Nutrition and Health, University of Chinese Academy of Sciences, Chinese Academy of Sciences, Shanghai 200031, China; [b]Department of Endocrinology and Metabolism, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, China; [c]Sirio Pharma Co., Ltd., Shantou 515041, China; [d]Department of Endocrinology and Metabolism, Zhongshan Hospital, Fudan University, Shanghai 200031, China; [e]CAS Engineering Laboratory for Nutrition, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, Shanghai 200031, China; [f]CAS Key Laboratory of Computational Biology, Shanghai Institute of Nutrition and Health, Chinese Academy of Sciences, Shanghai 200031, China; [g]Department of General Surgery, The Affiliated Hospital of Southwest Medical University, Luzhou, Sichuan 646000, China; [h]Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Fudan University, Shanghai 200032, China; and [i]Department of Oncology, Cancer Institute, The Affiliated Hospital of Qingdao University, Qingdao University, Qingdao 266071, China

Author contributions: Y. Xue, H.Z., H.L., Yi Liu, X.L., Y. Xu, W.F., J.F., and Y. Li designed research; Y. Xue, S.W., F.M., Z.L., X.F., S.H., X.S., W.L., Z.H., Yuxiao Liu, G.C., W.S., J.Z., X.Y., and J.W. performed research; X.F., C.G., J.W., Y. Xu, and W.F. contributed new reagents/analytic tools; Y. Xue, A.C., S.W., F.M., Z.L., X.S., W.L., Z.H., Yuxiao Liu, G.C., W.S., H.Z., H.L., Yi Liu, X.L., and Y. Li analyzed data; and Y. Xue, A.C., J.F., and Y. Li wrote the paper.

1. H. V. Lin, D. Accili, Hormonal regulation of hepatic glucose production in health and disease. *Cell Metab.* **14**, 9–19 (2011).
2. K. M. Habegger *et al.*, The metabolic actions of glucagon revisited. *Nat. Rev. Endocrinol.* **6**, 689–697 (2010).
3. M. C. Petersen, D. F. Vatner, G. I. Shulman, Regulation of hepatic glucose metabolism in health and disease. *Nat. Rev. Endocrinol.* **13**, 572–587 (2017).
4. R. A. Screaton *et al.*, The CREB coactivator TORC2 functions as a calcium- and cAMP-sensitive coincidence detector. *Cell* **119**, 61–74 (2004).
5. S. H. Koo *et al.*, The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* **437**, 1109–1111 (2005).
6. R. Dentin *et al.*, Insulin modulates gluconeogenesis by inhibition of the coactivator TORC2. *Nature* **449**, 366–369 (2007).
7. Q. Luo *et al.*, Mechanism of CREB recognition and coactivation by the CREB-regulated transcriptional coactivator CRTC2. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 20865–20870 (2012).
8. A. Salminen, K. Kaarniranta, A. Kauppinen, Age-related changes in AMPK activation: Role for AMPK phosphatases and inhibitory phosphorylation by upstream signaling pathways. *Ageing Res. Rev.* **28**, 15–26 (2016).
9. A. Ploumakis, M. L. Coleman, OH, the places you'll go! hydroxylation, gene expression, and cancer *Mol. Cell* **58**, 729–741 (2015).
10. S. B. Lee *et al.*, Proline hydroxylation primes protein kinases for autophosphorylation and activation. *Mol. Cell* **79**, 376–389.e8 (2020).
11. W. G. Kaelin, P. J. Ratcliffe, Oxygen sensing by metazoans: The central role of the HIF hydroxylase pathway. *Mol. Cell* **30**, 393–402 (2008).
12. C. M. Taniguchi *et al.*, Cross-talk between hypoxia and insulin signaling through Phd3 regulates hepatic glucose and lipid metabolism and ameliorates diabetes. *Nat. Med.* **19**, 1325–1330 (2013).
13. Y. Chen *et al.*, PHD3 stabilizes the tight junction protein occludin and protects intestinal epithelial barrier function. *J. Biol. Chem.* **290**, 20580–20589 (2015).
14. Y. Xu *et al.*, Prolyl hydroxylase 3 stabilizes the p53 tumor suppressor by inhibiting the p53-MDM2 interaction in a hydroxylase-independent manner. *J. Biol. Chem.* **294**, 9949–9958 (2019).
15. J. Nakae, T. Kitamura, D. L. Silver, D. Accili, The forkhead transcription factor Foxo1 (Fkhr) confers insulin sensitivity onto glucose-6-phosphatase expression. *J. Clin. Invest.* **108**, 1359–1367 (2001).
16. M. Matsumoto, A. Pocai, L. Rossetti, R. A. Depinho, D. Accili, Impaired regulation of hepatic glucose production in mice lacking the forkhead transcription factor Foxo1 in liver. *Cell Metab.* **6**, 208–216 (2007).
17. P. Puigserver *et al.*, Insulin-regulated hepatic gluconeogenesis through FOXO1-PGC-1alpha interaction. *Nature* **423**, 550–555 (2003).
18. S. Lee *et al.*, Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: Developmental culling and cancer. *Cancer Cell* **8**, 155–167 (2005).
19. J. Rodriguez *et al.*, Substrate-trapped interactors of PHD3 and FIH cluster in distinct signaling pathways. *Cell Rep.* **14**, 2745–2760 (2016).
20. A. C. Epstein *et al.*, C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43–54 (2001).
21. M. F. Hogan *et al.*, Hepatic insulin resistance following chronic activation of the CREB coactivator CRTC2. *J. Biol. Chem.* **290**, 25997–26006 (2015).
22. J. Han *et al.*, The CREB coactivator CRTC2 controls hepatic lipid metabolism by regulating SREBP1. *Nature* **524**, 243–246 (2015).
23. J. Y. Altarejos, M. Montminy, CREB and the CRTC co-activators: Sensors for hormonal and metabolic signals. *Nat. Rev. Mol. Cell Biol.* **12**, 141–151 (2011).
24. F. Zhang *et al.*, Hepatic CREBZF couples insulin to lipogenesis by inhibiting insig activity and contributes to hepatic steatosis in diet-induced insulin-resistant mice. *Hepatology* **68**, 1361–1375 (2018).
25. Q. Gong *et al.*, Fibroblast growth factor 21 improves hepatic insulin sensitivity by inhibiting mammalian target of rapamycin complex 1 in mice. *Hepatology* **64**, 425–438 (2016).
26. H. Yano *et al.*, PHD3 regulates glucose metabolism by suppressing stress-induced signalling and optimising gluconeogenesis and insulin signalling in hepatocytes. *Sci. Rep.* **8**, 14290 (2018).
27. S. Herzig *et al.*, CREB regulates hepatic gluconeogenesis through the coactivator PGC-1. *Nature* **413**, 179–183 (2001).
28. S.-H. Koo *et al.*, The CREB coactivator TORC2 is a key regulator of fasting glucose metabolism. *Nature* **437**, 1109–1111 (2005).
29. Y. A. Minamishima, W. G. Kaelin Jr., Reactivation of hepatic EPO synthesis in mice after PHD loss. *Science* **329**, 407 (2010).
30. L. J. Duan, K. Takeda, G. H. Fong, Hematological, hepatic, and retinal phenotypes in mice deficient for prolyl hydroxylase domain proteins in the liver. *Am. J. Pathol.* **184**, 1240–1250 (2014).
31. R. J. Appelhoff *et al.*, Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J. Biol. Chem.* **279**, 38458–38465 (2004).