Hepatic TET3 contributes to type-2 diabetes by inducing the HNF4α fetal isoform

Da Li1,2,10, Tiefeng Cao1,3,10, Xiaoli Sun1,4,10, Sungho Jin5,6, Di Xie1,7, Xinmei Huang1,8, Xiaoyong Yang6,6, Gordon G. Carmichael9, Hugh S. Taylor1, Sabrina Diano5,6 & Yingqun Huang1*.

Precise control of hepatic glucose production (HGP) is pivotal to maintain systemic glucose homeostasis. HNF4α functions to stimulate transcription of key gluconeogenic genes. HNF4α harbors two promoters (P2 and P1) thought to be primarily active in fetal and adult livers, respectively. Here we report that the fetal version of HNF4α is required for HGP in the adult liver. This isoform is acutely induced upon fasting and chronically increased in type-2 diabetes (T2D). P2 isoform induction occurs in response to glucagon-stimulated upregulation of TET3, not previously shown to be involved in HGP. TET3 is recruited to the P2 promoter by FOXA2, leading to promoter demethylation and increased transcription. While TET3 overexpression augments HGP, knockdown of either TET3 or the P2 isoform alone in the liver improves glucose homeostasis in dietary and genetic mouse models of T2D. These studies unmask an unanticipated, conserved regulatory mechanism in HGP and offer potential therapeutic targets for T2D.
The evolutionarily conserved, liver-enriched transcription factor HNF4α has been extensively studied for its role in hepatic differentiation and function. During fasting, hepatic expression of HNF4α and its coactivator PGC-1α both increase and transcriptionally activate the rate-limiting enzymes PEPCK and G6PC (encoded by PCK1 and G6PC, respectively), leading to gluconeogenesis. HNF4α contains two promoters, P2 and its downstream P1, which drive multiple HNF4α isoforms (a1–a9) via alternative splicing in a development-specific and tissue-specific manner. The P2-derived isoforms differ from P1-derived isoforms only in their N-terminal regions. The current dogma is that the P2 isoform predominates during fetal development, however after birth the P1 isoform takes over, directing a wide range of liver functions including gluconeogenesis. While many studies have focused on the P2-to-P1 promoter switch during hepatic differentiation, none has yet documented P2 promoter reactivation in the adult liver.

DNA methylation involves conversion of unmodified cytosine to 5-methylcytosine (5mC) by DNA methyltransferases. The Ten-eleven translocation (TET) family proteins (TET1, TET2, and TET3) facilitate active (replication-independent) and passive (replication-dependent) DNA demethylation by iteratively oxidizing 5mC to 5-hydroxymethylcytosines (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), followed by excision of 5fC or 5caC by thymine DNA glycosylase coupled with base excision repair. The enzymatic activity of TETs is regulated by co-factors including α-ketoglutarate (α-KG) generated through the TCA cycle and vitamin C, and by post-translational modifications. Besides being the key intermediate for TET-mediated DNA demethylation, 5hmC is a potential epigenetic mark; yet, the exact rules that govern how and when TETs proceed beyond 5hmC leading to DNA demethylation are unclear. Recently, non-catalytic functions of TETs have also been described. Mechanistically, TETs as general DNA-binding proteins appear to be recruited to specific gene loci by transcription factors. For example, during B cell differentiation TET2 and TET3 cooperate with PU.1 to regulate gene expression. PU.1 is a pioneer transcription factor (PTF), defined by its ability to directly associate with target sequences on condensed chromatin opening the local chromatin for other factors to bind. Among the numerous loci with which PU.1 associated was the Igκ enhancer. Using a TET2/TET3 double knockout (KO) mouse model and TET7/P1.1 co-immunoprecipitation (co-IP) and PU.1 shRNA knockdown in B cells, it was demonstrated that PU.1 interacts with and guides TET2/TET3 to the Igκ enhancer for 5hmC deposition and subsequent demethylation.

While much is known about the role of TETs in development, stem cells, and cancer, little is known about their role in energy metabolism. In the current study we report an unexpected finding of P2 promoter reactivation in adult liver by TET3 with an essential role in control of hepatic glucose production (HGP).

**Results**

**Fasting upregulates hepatic TET3.** We have previously documented that fasting upregulates hepatic expression of H19 long noncoding RNA contributing to increased expression of HNF4α. Overnight fasting expectedly increased levels of H19 as well as mRNAs for PGC-1α, HNF4α, PEPCK, and G6PC; curiously, fasting also increased mRNA expression of TET3, but not of its family members TET2 and TET1 (Supplementary Fig. 2a). To determine how TET3 is upregulated, primary hepatocytes from wild-type (WT) and H19 KO mice were stimulated with glucagon. In WT hepatocytes, H19 expression was readily induced by glucagon, as was TET3; however, in KO hepatocytes, glucagon no longer stimulated TET3 expression (Supplementary Fig. 2b). Next, H19 was expressed in WT primary hepatocytes with an adeno-associated virus-based vector (AAV-H19) in absence of glucagon. Exogenous H19 expression increased TET3 mRNA levels (Supplementary Fig. 2c). Consistently, livers from ad libitum-fed mice injected with AAV-H19 showed increased TET3 mRNA (Supplementary Fig. 2d). H19 contains multiple microRNA let-7-binding sites, acting as a molecular sponge for let-7. Recently, we identified TET3 as a target of let-7-mediated repression of expression at the posttranscriptional level (both human and mouse TET3 mRNAs contain let-7-binding sites) and demonstrated that H19 promotes TET3 expression by reducing the bioavailability of let-7. Thus, H19 enables glucagon-induced TET3 upregulation, likely via inhibiting let-7, in isolated hepatocytes.

**TET3 promotes HGP.** To determine whether TET3 expression in the absence of upstream stimulators (glucagon and H19) is sufficient to enhance glucose production, TET3 was expressed in primary hepatocytes from H19 KO mice. Hepatocytes were infected with viruses containing a cDNA encoding TET3 (Ad–TET3) or green fluorescent protein (Ad–GFP). When TET3 was overexpressed, increased expression of PCK1 and G6PC was evident (Fig. 1a, b). TET3 overexpression also increased glucose production (Fig. 1c). In contrast, when WT hepatocytes were infected with AAV-siTET3 (specific against mouse TET3, or a non-targeting siRNA control AAV-scr) in the presence of glucagon stimulation, it led to decreased expression of PCK1 and G6PC (Fig. 1d, e) and glucose production (Fig. 1f). TET3 knockdown did not affect the expression of TET2 and TET1 (Supplementary Fig. 3a), confirming the specificity of the TET3 siRNA. As stated in our “Methods” section, all primary hepatocyte experiments (e.g., glucagon stimulation and TET3 overexpression) were performed on cells maintained in a complete culture medium (CM) containing serum, insulin, and dexamethasone, conditions optimized and important for cell viability. These conditions allowed cell viability to persist to the end of the experiments (Supplementary Fig. 3b). Our additional rationale for performing glucagon stimulation in the presence of insulin was derived from the fact that insulin is present in the circulation during fasting, albeit at a lower level as compared to fed conditions. Taken together, our results show that TET3 augments glucose production, at least in part by increasing expression of key gluconeogenic genes in isolated hepatocytes.

To demonstrate that TET3 regulates HGP in vivo, Ad–TET3 (or Ad–GFP) viruses were infused through the tail vein into H19 KO mice. Systemic administration of recombinant adenosviruses into rodents resulted in expression of transgenes in the liver, with no detectable expression in other peripheral tissues and brain; nor was hepatotoxicity detected. Following 10 days of viral administration, mice fed ad libitum were sacrificed and liver and blood samples were harvested. Mice infused with Ad–TET3 had a significant increase in hepatic TET3 expression relative to mice infused with Ad–GFP, which was accompanied by increased expression of PCK1 and G6PC (Fig. 1g, h); there was also an increase in blood glucose and insulin levels (Fig. 1i).

To determine whether upregulation of TET3 is necessary for HGP during fasting, AAV-siTET3 or AAV-scr were injected via tail vein into WT mice followed by fasting 10 days later. Systemic infusion of recombinant AAVs into mice leads to liver-specific expression of transgenes. Mice infused with AAV-siTET3 showed a significant decrease in fasting blood glucose and fasting insulin, as compared to AAV-scr injected animals (Fig. 1j). Pyruvate tolerance tests (P TT, a readout for HGP) showed lower glucose levels following pyruvate injection (Fig. 1k). Protein analyses revealed decreased levels of TET3, PEPCK, and G6PC in...
livers of AAV-siTET3 relative to AAV-scr-injected animals (Fig. 1). Based on these results we conclude that TET3 is a regulator of HGP.

TET3 reactivates the P2 promoter. Increased H19 expression was detected in the liver during fasting and in livers of human and mouse with type-2 diabetes (T2D)22, conditions known to have physiological and pathological increase in gluconeogenesis, respectively. Consistent with the notion that H19 positively regulates TET3 expression23, elevated TET3 was evident in all conditions (Fig. 2a–d), where H19 expression was increased17. Importantly, mining of human liver database24,25 revealed a significant increase in expression of TET3 in the liver of T2D patients as compared to non-diabetic controls (Supplementary Fig. 3c). Together, these results suggest that the H19/TET3-mediated regulation of HGP is likely conserved between human and mouse.

Remarkably, conditions that resulted in increased TET3 expression also led to an increase in expression of the P2- (but not P1) specific HNF4α isoform at levels of both mRNA (Fig. 2c–h) and protein (Fig. 2i–l). TET3 overexpression (Fig. 2m) increased P2 isoform both at the mRNA (Fig. 2n) and protein (Fig. 2o) levels without affecting P1 isoform levels. In contrast, TET3 knockdown (Fig. 2p) selectively decreased the P2 isoform (Fig. 2q, r). These data led to our hypothesis that TET3-mediated reactivation of HNF4α P2 promoter and the derived isoform may reflect a previously unexpected mechanism of gluconeogenesis activation in adult liver.
Fig. 2 TET3 expression positively correlates with P2 isoform expression. a-d IB of TET3 from liver tissues isolated from mice infected with AAV-Vec/AAV-H19, ad libitum fed/fasted, exposed to normal chow (NC)/HFD for 11 days or 12 weeks. n = 3-4. e-h qPCR of HNF4α P2 and P1 isoforms in liver tissues from mice under the indicated conditions. n = 4. i-l IB of TET3 in liver tissues from H19 KO mice infected with Ad-GFP or Ad-TET3 for 10 days. n = 4. m and n qPCR and IB of HNF4α P2 and P1 isoforms in liver tissues from mice treated as in m. n = 4. All data are representative of at least two independent experiments. Two-tailed Student’s t tests were used and data are presented as mean ± SEM. *p < 0.05, **p < 0.01. Source data are provided as a Source Data File.
The P2 isoform mediates TET3-dependent HGP. Next, we used primary mouse hepatocytes to test effects of glucagon and H19 on P2 and P1 promoter usage. While glucagon increased P2 usage in WT hepatocytes, it failed to do so in H19 KO hepatocytes (Fig. 3a). On the other hand, without glucagon exogenous expression of either H19 or TET3 alone was sufficient to increase P2 usage in both WT and KO hepatocytes (Fig. 3b). Further, when TET3 was downregulated by siRNA, glucagon no longer induced P2 (Fig. 3c). Together, these results show that glucagon selectively induces the P2 isoform and that TET3 is required for this induction to occur in hepatocytes.

To address whether an increase in the P2 isoform is necessary for enhanced glucose production, we tested effects of P2-specific siRNA (AAV-siP2) knockdown on gluconeogenic gene expression. As seen in Fig. 3d, glucagon expectedly increased the P2 (but not P1) isoform in hepatocytes; it also increased expression of PCK1 and G6PC. Infection with AAV-siP2 downregulated the P2 isoform without affecting the P1 isoform (Fig. 3d). Importantly, knockdown of the P2 isoform not only decreased PCK1 and G6PC expression (Fig. 3d), but also abolished glucagon-induced glucose production (Fig. 3e). Thus, the P2 isoform is critical for glucagon-induced glucose production in isolated hepatocytes. To determine whether this regulation also occurs in vivo, mice were injected with AAV-scr or AAV-siP2 viruses. Ten days later, mice injected with AAV-siP2 showed decreased fasting glucose and fasting insulin (Fig. 3f) and decreased PTT (Fig. 3g), as compared to AAV-scr-injected animals. Protein analysis confirmed selective decrease in the P2 isoform, with a concomitant decrease in PCK1 and G6PC (Fig. 3h). To address whether HNF4α P2 is required for TET3-induced glucose production, TET3 was overexpressed in H19 KO hepatocytes in the presence of HNF4α P2 knockdown. P2 knockdown (Supplementary Fig. 3d) abolished TET3-induced glucose production (Fig. 3i), suggesting that HNF4α P2 is the major effector of TET3-mediated regulation of HGP.

Next, primary human hepatocytes were infected with AAV-scr, AAV-sihTET3 (siRNA against human TET3), or AAV-sihP2

**Fig. 3 The mechanism of TET3-induced P2 isoform is conserved.** a qPCR of HNF4α isoforms from WT and H19 KO hepatocytes treated with glucagon (20 nM) or vehicle for 24 h. The ratio of P2-to-P1 isoforms is arbitrarily set as 1 in vehicle-treated group. n = 3, Two-tailed Student’s t tests. b qPCR of HNF4α isoforms from hepatocytes infected with indicated viruses for 48 h. n = 3, One-way ANOVA with Tukey post-test. c qPCR of P2 and P1 isoforms from primary hepatocytes infected with AAV-scr or AAV-siTET3 for 48 h and treated with vehicle or glucagon for 24 h. n = 3, One-way ANOVA with Tukey post-test. d qPCR of HNF4α isoforms and PCK1 and G6PC from primary hepatocytes infected with AAV-scr or AAV-siP2 for 48 h, followed by treatment with glucagon. HNF4α isoforms were analyzed after 24 h of glucagon stimulation; PCK1 and G6PC were analyzed after 48 h of glucagon stimulation. n = 3, One-way ANOVA with Tukey post-test. e Glucose production from primary hepatocytes infected with AAV-scr or AAV-siP2 for 48 h and treated with vehicle or glucagon for 48 h. n = 3, One-way ANOVA with Tukey post-test. f Fasting blood glucose and insulin levels of mice infected with AAV-scr or AAV-siP2 for 10 days. n = 5–6, Two-tailed Student’s t tests. g PTT in mice treated as in f. n = 5–6, Two-way ANOVA with Sidak post-test. h Iβ of indicated proteins from liver tissues isolated from mice treated as in f. n = 4, Two-tailed Student’s t tests. i Glucose production from primary hepatocytes treated as in j. n = 3, One-way ANOVA with Tukey post-test. Data a–e are representative of at least two independent experiments; data f–k are representative of two independent experiments. All data are presented as mean ± SEM. *p < 0.05, **p < 0.01. Source data are provided as a Source Data File.
Fig. 4 TET3 induces epigenetic modification at the P2 promoter. a Left, DMR sequence in the P2 promoter of mouse Hnf4α. The three differentially methylated CpGs are highlighted. Numbers mark the starting and ending positions of nucleotides in the chromosome. Right, schematic of mouse Hnf4α showing TET3/FOXA2 ChIP and hMeDIP (red) and RNAP ChIP (green) regions, respectively. Numbers mark the starting and ending positions of nucleotides in the chromosome. Not drawn to scale. b Mouse primary hepatocytes were treated with vehicle (−) or 20 nM of glucagon (+) for 24 h, followed by ChIP-qPCR of P2 and P1 promoters. Data are presented as % input, with gray bars representing background IgG signal. n = 3. Left, mouse hepatocytes were treated as in b, followed by hMeDIP of P2 and P1 promoters. n = 3. Right, mouse hepatocytes were infected with Ad-GFP or Ad-TET3, followed by hMeDIP at 36 h post-infection. n = 3. d Left, mouse hepatocytes were treated as in b, followed by QMSP of P2. Right, H19 KO hepatocytes were infected with Ad-GFP or Ad-TET3, followed by QMSP of P2 at 48 h post-infection. n = 3. e Left, mouse hepatocytes were treated with vehicle or glucagon for 24 h, followed by RNAP ChIP of P2 and P1 promoters. Right, H19 KO mouse hepatocytes were infected with Ad-GFP or Ad-TET3, followed by RNAP ChIP at 48 h post-infection. n = 3. Data b–e are representative of two independent experiments and are presented as mean ± SEM, using Two-tailed Student’s t tests. *p < 0.05, **p < 0.01.

(sirNA specific for human HNF4α P2) and treated with vehicle or glucagon. Glucagon stimulated expression from P2 (but not P1) and also increased expression of PCK1 and G6PC; knockdown of either TET3 or the P2 promoter was sufficient to reduce PCK1 and G6PC mRNAs to the control levels (Fig. 3). Knockdown of the TET3 or the HNF4α P2 isoform also led to decreased glucose production (Fig. 3k). The lower than basal level of glucose production in the TET3 or P2 promoter knockdown group reflected persistent siRNA effects. Neither TET1 nor TET2 expression was affected by AAV-siTET3 (Supplementary Fig. 3e) and the P1 isoform knockdown was not altered by AAV-siHNF4α P2 (Fig. 3). Based on these data the mechanism of TET3-mediated HNF4α P2 isoform induction in HGP is likely conserved between human and mouse.

TET3 promotes demethylation of the P2 promoter. We previously reported a positive correlation between increased H19 expression and HNF4α promoter hypomethylation in human hepatoma cells and in livers of multiple mouse models, including fasting, high fat diet (HFD)-induced T2D, and liver-specific H19 overexpression. The differentially methylated region (DMR) mapped to the HNF4α promoter known to be highly conserved between human and mouse (Fig. 4a, left). As TET3 expression was also increased under these conditions (Fig. 2a–d), we speculated that TET3 may activate P2-specific transcription by binding and demethylating the P2 promoter. TET3 binds and demethylate DNA leading to transcription activation.

First, we tested whether glucagon promotes TET3 binding to the P2 promoter. Mouse hepatocytes were treated with glucagon or vehicle, followed by chromatin immunoprecipitation coupled with qPCR (ChIP-qPCR). We used a TET3-specific antibody to immunoprecipitate protein–DNA complexes from hepatocytes and qPCR amplified the P2 and P1 promoters (Fig. 4a, right). Glucagon stimulation dramatically increased TET3 binding to the P2 (but not P1) promoter (Fig. 4b). Next, we determined effects of glucagon and TET3 on promoter 5hmC deposition using hydroxymethylated DNA immunoprecipitation–qPCR (hMeDIP-qPCR). Mouse hepatocytes were treated with glucagon (vehicle as control) or infected with Ad-TET3 (Ad-GFP as control). Either glucagon stimulation or exogenous TET3 expression increased 5hmC at the P2 (but not P1) promoter (Fig. 4c), demonstrating that glucagon promotes direct binding of TET3 to the P2 promoter inducing 5hmC deposition. To address whether TET3 binding facilitates promoter demethylation, quantitative methylation-specific PCR (QMSP) was performed using previously described methods.

The QMSP primers were designed based on the differentially methylated cytosine residues within the DMR of the P2 promoter (Fig. 4a, left). Figure 4d shows that treatment of hepatocytes with glucagon or with exogenous TET3 expression significantly decreased methylation at the P2 promoter without affecting the P1 promoter. Next, we performed ChIP assays using anti-Ser-5(P)-RNP antibody that specifically recognizes activated RNA polymerase (RNAP). We compared binding of activated RNAP to HNF4α between glucagon and vehicle-treated hepatocytes to assess relative transcription activity of both P2 and P1 promoters (Fig. 4a, right). We observed an ~5-fold enrichment in RNAP at the P2 promoter in glucagon-stimulated versus vehicle-treated hepatocytes and no change in RNAP association with P1 (Fig. 4e). Similar results were obtained when TET3 was overexpressed in the absence of glucagon stimulation (Fig. 4e). Collectively, our results demonstrate that TET3 activates P2-specific transcription by directly binding and demethylating the P2 promoter.

TET3 functionally and physically interacts with FOXA2. FOXA2 is essential for liver development and function via acting as a PTF1 regulator. Using ChIP-seq FOXA2 was found to bind 45% of expressed genes in the adult mouse liver, acting either as an inducer or repressor of gene expression depending on its binding location.

FOXA2 is required for fasting-induced hepatic gluconeogenesis in part by enabling recruitment of cAMP response element-binding protein (CREB) and the glucocorticoid receptor (GR) to their respective target genes. In the mouse liver FOXA2 occupies its target sites constitutively regardless of metabolic state (i.e., fasted or fed), yet binding of activated CREB and GR (respectively, by glucagon and glucocorticoids) to their target
genes was significantly enhanced by fasting (based on ChIP-qPCR) and the effect was abolished in FOXA2-deficient hepatocytes. Given the proximity of FOXA2-binding sites to those recognized by activated CREB and GR on a subset of gluconeogenic genes, it has been proposed that binding of FOXA2 to its cis-acting elements opens the local chromatin structure thereby facilitating binding of other transcription factors such as CREB and GR. Because PU.1 (a PTF) interacts with and recruits TET2/TET3 to the Igk enhancer and because FOXA2 is a PTF required for activation of the hepatic gluconeogenic transcriptional program in response to fasting, we hypothesized that FOXA2 may play a role in TET3-dependent P2 promoter reactivation.

The first evidence supporting our hypothesis was the observation of the preferential enrichment of FOXA2 at the P2 versus P1 promoters of HNF4α in the human liver (Supplementary Fig. 4, and ENCODE ChIP-seq GSE105237 and GSE105487). This preferential binding of FOXA2 to the P2 promoter has not been previously reported in the literature. Next, we performed ChIP-qPCR experiments with mouse hepatocytes treated with glucagon in the presence of FOXA2 knockout. While glucagon expectedly increased TET3 association with P2 (but not P1) (Fig. 5a), FOXA2 knockout (Fig. 5b) abolished this effect (Fig. 5a), demonstrating that FOXA2 is required for glucagon stimulated association of TET3 to the P2 promoter. To test whether FOXA2 physically interacts with TET3, co-IP experiments were carried out using hepatocytes overexpressing TET3. FOXA2 was readily detected in TET3-containing complexes, and vice versa (Fig. 5c), supporting a physical interaction of TET3 and FOXA2.

Importantly, co-IP experiments showed increased interaction between FOXA2 and TET3 in response to glucagon stimulation as compared to no glucagon stimulation (Fig. 5e). Based on these data we propose that the constitutive association of FOXA2 with the P2 promoter maintains an open chromatin structure; upon glucagon stimulation TET3 rises above a critical threshold level and is effectively recruited to the P2 promoter via interaction with FOXA2.

As the P2 isoform is specifically induced during fasting, we hypothesized that this isoform is more potent than the P1 isoform in transactivation of gluconeogenic genes. Transcription activation of PCK1 and G6PC by HNF4α requires PGC-1α which by itself does not bind DNA. We used a luciferase reporter (gAF1) driven by a fragment of the PCK1 promoter harboring a HNF4α-binding site. Plasmids expressing human HNF4α (representing a P2-derived isoform) or HNF4αα (representing a P1-derived isoform) were transfected into U-2 OS cells, together with gAF1, a Renilla luciferase reporter for transfection normalization, and increasing amounts of a PGC-1α expression vector. The transcriptional activity of HNF4α was significantly higher than that of HNF4αα in increasing concentrations of PGC-1α, although HNF4αα and HNF4α were expressed at comparable levels (Supplementary Fig. 5). These results suggest that the P2 isoform, when co-activated by PGC-1α, is likely a more potent transactivator of gluconeogenic genes. It remains to be determined which of the three P2 isoforms (α7–α9) are responsible for gluconeogenic activation in vivo.

**Targeting TET3 or the P2 isoform improves glucose metabolism.** Excessive HGP in T2D is primarily a result of dysregulated gluconeogenesis, a major contributor to impaired glucose homeostasis. Mice with T2D exhibit increased hepatic expression of TET3 and HNF4α P2 isoform (Fig. 2). Human T2D patients showed increased TET3 expression in the liver.

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**Fig. 5 TET3 interacts with FOXA2.** a TET3 ChIP-qPCR of mouse primary hepatocytes infected with AAV-scr or AAV-siFOXA2 for 48 h, followed by glucagon (20 nM) stimulation for 48 h. n = 3, one-way ANOVA with Tukey post-test. b IB of FOXA2 of mouse primary hepatocytes infected with AAV-scr or AAV-siFOXA2 for 48 h, followed by glucagon stimulation for 48 h. n = 3, one-way ANOVA with Tukey post-test. c IB of immunoprecipitated FOXA2 and TET3 from H19 KO hepatocytes infected with Ad-TET3 for 24 h, showing that FOXA2 and TET3 are present in each other’s complexes. The secondary antibodies used were Rabbit IgG TrueBlot® and Goat IgG TrueBlot®, respectively. These HRP-conjugated monoclonal secondary antibodies enable detection of immunoblotted target proteins without hindrance by interfering immunoprecipitating immunoglobulin heavy and light chains. d FOXA2 ChIP-qPCR of mouse primary hepatocytes treated as in a, n = 3, one-way ANOVA with Tukey post-test. e Mouse primary hepatocytes were treated with glucagon (+) or vehicle (−) and co-IP using TET3 antibody was carried out 24 h later. IB results show increased association of FOXA2 with TET3 following glucagon stimulation. All data are representative of two independent experiments and are presented as mean ± SEM. *p < 0.05, **p < 0.01. Source data are provided as a Source Data File.
Fig. 6 The P2 isoform epigenetically induced by TET3 contributes to increased HGP in HFD mice. a Fasting blood glucose and insulin levels (two-tailed Student’s t tests), and PTT, GTT, and ITT in HFD mice infected with AAV-scr or AAV-siTET3 for 10 days. n = 7–8. Two-way ANOVA with Sidak post-test. b Fasting blood glucose and insulin levels (two-tailed Student’s t tests), and PTT, GTT, and ITT in HFD mice infected with AAV-scr or AAV-siTET3 for 10 days. n = 6–7. Two-way ANOVA with Sidak post-test. c IB of TET3, HNF4α P2 and P1 isoforms, and PEPCK and G6PC in liver tissues from HFD mice infected with AAV-scr or AAV-siTET3 for 10 days. n = 4. d–g Hyperinsulinemic-euglycemic clamp studies from mice fed HFD for 12 weeks and infected with AAV-scr or AAV-siP2 for 10 days. n = 7. Two-way ANOVA with Sidak post-test. Data a–c are representative of two independent experiments. All data are presented as mean ± SEM. *p < 0.05, **p < 0.01, AUC area under the curve. Source data are provided as a Source Data File.

( Supplementary Fig. 3c). We asked whether inhibition of TET3 or the P2 isoform would reduce HGP thereby improving glucose homeostasis, using both HFD and genetic (Lepob/ob) mouse models of T2D3. We infused the diabetic mice with AAV-scr, AAV-siTET3, or AAV-siP2, and the effects of gene knockdown on glucose metabolism were assessed 10 days later. In the HFD animals, knockdown of TET3 significantly decreased fasting blood glucose, fasting insulin, and PTT (Fig. 6a), suggesting decreased hepatic gluconeogenesis, which was further supported by decreased protein levels of HNF4α P2, PEPCK, and G6PC in the liver (Fig. 6c). Glucose tolerance tests (GTT) and insulin tolerance tests (ITT) showed significantly enhanced glucose tolerance and insulin sensitivity in TET3 knockdown as compared to control animals (Fig. 6a). Effects of HNF4α P2 knockdown mirrored those of TET3 knockdown (Fig. 6b, c). Similar observations were made using Lepob/ob mice (Supplementary Fig. 6a–d). To further examine the cause of alterations in blood glucose, hyperinsulinemic–euglycemic clamp studies were conducted in HFD mice infected with AAV-siP2. Compared to the AAV-scr-treated mice, the AAV-siP2-treated mice showed significantly higher glucose infusion rate (GIR) to maintain euglycemia (Fig. 6d, e), reflecting increased insulin sensitivity. This was not due to increased peripheral glucose uptake (Fig. 6f) but rather to increased insulin-stimulated suppression of endogenous glucose production (EGP) (Fig. 6g). These results further support the notion that inhibiting expression of the P2 isoform improves hyperglycemia and insulin sensitivity via reduction in HGP. While in the adult pancreas both P2 and P1 promoters of HNF4α were reported to be active and important for pancreas β-cell function17, the unchanged expression of P2 and P1 isoforms in mice infected with AAV-siP2 (Supplementary Fig. 6c) ruled out the possibility that the decreased fasting blood insulin was caused by altered HNF4α in the pancreas.

Discussion

As illustrated in Fig. 7, here we report HNF4α P2 promoter reactivation in adult liver and its critical role in control of HGP under both physiological and pathological conditions. The regulation involves an epigenetic mechanism mediated by a member of the TET family proteins not previously known to have a role in glucose homeostasis. Importantly, this TET3-mediated P2 promoter reactivation in HGP appears to be conserved between human and mouse. It is noteworthy that the preferential enrichment of FOXA2 at the P2 versus the P1 promoters as demonstrated by ChIP from both human (Supplementary Fig. 4) and mouse (Fig. 4i) livers does not exclude the possibility of
For information on animal numbers, refer to the given report. Mice were housed at 22 °C with a 12 h light/12 h dark cycle with normal chow (NC) (Harlan Teklad no. 2018, 18% calories from fat). Animals were fed ad libitum. The H19 KO and WT mice on a background of C57BL/6J were custom made by cloning a mouse full-length TET3 ORF PCR amplified from cDNA Flag-Tet3^ΔN (60940, Addgene) into an Ad-TBG vector that drives TET3 expression from a liver-specific thyroxine-binding globulin (TBG) promoter (Vigene Biosciences, Rockville, MD 20850, USA). The AAV-H19 virus was previously described. The negative control siRNA virus aav-scr was purchased (aava01500, Applied Biological Materials Inc.). The AAV-siTET3 (aava092333), AAV-siP2, AAV-siHNF4α (aava2441008), AAV-siFOXA2, and AAV-siHNF4α (60079, Addgene) viruses were purchased from Applied Biological Materials Inc. AAV-si2P and AAV-siHNF4α were custom made by Applied Biological Materials Inc. The targeted sequences for AAV-siP2 and AAV-siHNF4α are: 3′-CCTTCTGCCTGTGTTGCCCCCCTGCTGAG-3′ and 5′-ATGCTCAGCCTGAGACGCTCCCGGGC-3′, respectively. All AAV viruses were serotyped C57BL/6j. Mice were 8 weeks of age at the time of surgery. Mice were fed ad libitum, and food and water were provided ad libitum. The H19 KO and WT mice on a background of C57BL/6j have been described. To induce insulin resistance (Fig. 2C, D), WT mice were subjected to a high-fat diet (HFD) (Research Diets, D12451, 45% calories from fat) and water for 20 weeks. Male mice were fed a high-fat diet (HFD) for 12 weeks, with sc abdomen injection of polyethylene glycol (6000 Da) under general anesthesia. Mice were then kept for a recovery period of 5–7 days. For euthanasia, mice were injected with 100 μl of ketamine (100 mg/ml) and xylazine (20 mg/ml) followed by 1 ml of a mixture of 5.5 μl of ketamine and 1 μl of xylazine. The injection was made in the lateral vein, using 30-gauge needles. Primary hepatocytes were isolated using the Yale Liver Center according to previous protocols. Cells were maintained in complete CM (Williams’ Medium [GIBCO,12551] supplemented with 5% FBS, 1% M Hepes buffer [GIBCO, 15630-080], 1% L-glutamine [GIBCO, 25300-081], 1% SPA [GIBCO, 15240-062], 4 mg/l insulin [GIBCO, 12583-014], and 1 μM dexamethasone [Sigma, D9602]). For TET overexpression experiments, freshly isolated mouse primary hepatocytes were transfected in 12-well collagen I-coated plates (BD Biosciences Discovery Labware) at 4 × 10^4 cells/well and infected with Ad-GFP or Ad-TET3 at 4000 gc/cell at 3 h after seeding. For TET3 or HFN4a P2 isoform knockdown experiments, cells seeded in 12-well plates were infected with AAV-scr, AAV-siTET3, or AAV-siP2 at 6000 gc/cell at 3 h after seeding. Medium was changed the next day and glucagon was added at a final concentration of 20 nM in CM. RNA and protein were isolated and glucose production assays were carried out at the time points indicated in the figure legends. Human primary hepatocytes (M00995, Millipore, Sigma) were purchased, thawed, and maintained in InVitroGRO® CP Medium (Sigma-Aldrich, Z990003). Cells were used at passage number 3–5. The AAV-scr, aava-siH19, and AAV-siP2 were the next day after cell thawing and followed the same dosing protocol for the mouse primary hepatocytes.

**Luciferase reporter assays.** Human U-2 OS cell line (9202271) was purchased as authenticated from Sigma Aldrich and maintained in McCoy’s 5a medium supplemented with 1.5 mM glutamine and 10% FBS. The cells were propagated in a 10 cm tissue culture plate as previously described. For RNA and protein analyses, tissues were collected following euthanasia, snap frozen in liquid nitrogen, and stored at −80 °C until use. For information on animal numbers, refer to figure legends.
DNA Methylation-Gold Kit (Zymo, D5006). 100

Quantitative methylation-specific PCR. Genomic DNA was extracted from mouse primary hepatocytes grown in 12-well plates using Quick-gDNA MicroPrep (Zymo Research Corporation, D3021) according to the manufacturer’s instructions. For bisulfite treatment, 480 ng of DNA was used for each column using EZ DNA Methylation-Gold Kit (Zymo, D5006). 100 μl of elution buffer was used to elute DNA from each column. Real-time quantitative PCR was performed in a 15 μl reaction containing 5 μl of the eluted DNA using iQSYBRGreen in a Bio-Rad iCycler. Two sets of PCR primers were designed: one for unmethylated and one for methylated DNA sequences. The PCR primers for methylated DNA were used at a final concentration of 0.6 μM in each PCR reaction. PCR was performed by initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Specificity was verified by melting curve analysis. The threshold cycle (Ct) values of each sample were used in the post-PCR data analysis. The ratio of methylated versus unmethylated DNA sequences are presented. The primers used for qMSP are listed in Supplementary Table 1.

Chromatin immunoprecipitation-quantitative PCR. Experiments were performed in a 12-well plate scale using the Pierce Agarose ChiP Kit (Thermo Scientific, 26156) according to the manufacturer’s instructions with minor modifications. Briefly, agarose beads were used to pre-bind with antibodies (anti-TET3, anti-Ser-5P)-RNAP, anti-FOXA2, or pre-immune IgGs as negative controls overnight at 4 °C. The next day, hepatocytes were crosslinked with 1% formaldehyde at RT for 10 min, and the reaction was stopped by 1xglcyrine. ChiPs were carried out at 4 °C. Levels of ChIP-puriﬁed DNA were determined by qPCR (see Supplementary Table 1 for primer sequences). The relative enrichments of the indicated DNA regions were calculated using the Percent Input Method according to the manufacturer’s instructions and are presented as % input.

Hydroxymethylated DNA immunoprecipitation coupled with qPCR. The experiments were carried out using the EpiQuik histone Chip Kit (P-1038-48, Epitekgen) according to the manufacturer’s instructions. Briefly, mouse primary hepatocytes maintained in 12-well plates at 4 × 10^5 cells/well were stimulated with 20 μM of glucagon (vehicle as control) or infected with Ad-TET3 (Ad-GFP as control) at 24 h (glucagon) or 36 h later. gDNAs were sheared using a Qsonica Sonicators Q125 probe, with a setting of nine pulses of 10 s each at 35% amplitude followed by a 40 s rest period on ice between each pulse. Sheared DNA fragments (ranged in size from 200 to 600 bps as assessed by agarose gel electrophoresis) were immunoprecipitated using the SmnC rabbit polyclonal antibody from the kit. qPCR was performed in a 25 μl reaction containing 2.5 μl of the eluted DNA using iTAC SYBGreen in a Bio-Rad iCycler. The Ct values of each sample were used in the post-PCR data analysis. The relative enrichments (after normalization against control IgG) of the indicated DNA regions were calculated using the Percent Input Method according to the manufacturer’s instructions.

Glucose production assay. Glucose production assays were performed using Amplex Red Glucose/Glucose Oxidase Assay Kit (Molecular Probes, Invitrogen, A22189), according to the manufacturer’s instructions. Brieﬂy, human liver TET3 expression datasets, the Robust Rank Aggregation (RRA) method60 was used.

RNA extraction and RT-qPCR. Total RNAs were extracted from liver or pancreas tissues or from primary hepatocytes using PureLink RNA Mini Kit. cDNA was synthesized using PrimerScript RT Reagent Kit in a 20 μl reaction containing 0.5–1 μg of total RNA. Real-time quantitative PCR was performed in a 15 μl reaction containing 0.5–1 μl of cDNA using iTAC SYBRGreen in a Bio-Rad iCycler. PCR was performed by initial denaturation at 95 °C for 5 min, followed by 40 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. Specificity was veriﬁed by melting curve analysis. The Ct values of each sample were used in the post-PCR data analysis. Gene expression levels were normalized against house-keeping genes GAPDH and RPLP0. Real-time PCR primers are listed in Supplementary Table 1.

Immunoblot analysis. Primary hepatocytes in 12-well plates were dissociated with 0.25% trypsin. After centrifugation at 500 × g for 5 min, supernatants were discarded and cell pellets were homogenized in 2x SDS-sample buffer (100 μl/well), followed by heating at 100 °C for 5 min, with occasional vortexing. For liver tissue samples, 5 mg of tissues were minced and 200 μl of 2xSDS-sample buffer was added, followed by homogenization using an ice sonicator (Qsonica, Q125-110). Homogenized samples were heated at 100 °C for 5 min and then centrifuged at 12,000g for 5 min to remove insoluble materials before loading onto 12% SDS gels (5 μl/well). Followed by Western blot analysis. Bands on Western blot gels were quantified using ImageJ. GAPDH was used as a loading control. Antibodies used in Western blot analysis with dilutions are presented in Supplementary Table 2.

Immunoprecipitation. To prepare antibodies, 8 μl (packed volume) of ChIP grade Protein A/G beads (Pierce Agarose ChiP Kit, Thermo Scientific, 26156) were washed twice with 0.5 ml of IP buffer (1% Triton X-100, 300 mM NaCl, 10 mM Tris–HCl at pH 7.5, and 10 mM EDTA), followed by incubation with 5 μg of rabbit polyclonal anti-TET3 (Millipore Sigma, ABE290), goat polyclonal anti-FOXAX2 (R&D systems, AF2400) or preimmune rabbit or goat IgG in 200 μl of IP buffer at 4 °C overnight. Antibody bound beads were pelleted by brief centrifugation and kept on ice until use. To prepare cell lysate, H19 KO mouse hepatocytes (1 × 10^6 cells per IP) infected with Ad-TET3 for 24 h (or WT mouse hepatocytes stimulated with glucagon 24 h) were washed on plate once with ice-cold PBS, followed by incubation on ice for 10 min in 400 μl of freshly prepared gentle lysis buffer (GLB: 1% Triton X-100, 10 mM NaCl, 10 mM Tris–HCl at pH 7.5, 10 mM EDTA, 1 mM PMFS, 1 mM DTT, and 1x protease inhibitor cocktail [Calbiochem]). The cells together with the lysis buffer were manually scraped into a 1.5 ml tube, followed by incubation on ice for an additional 15 min. After centrifugation at 12,000 × g for 5 min to remove insoluble materials, the lysate was transferred to a new tube with addition of 5 μl of a 1:10 dilution of protein G agarose (Bio-Rad). Western analysis, anti-TET3 and anti-FOXAX2 were diluted at 1:200 and 1:500, respectively. The secondary antibodies used were Rabbit IgG TrueBlot* and Goat IgG TrueBlot*. These unconjugated monoclonal secondary antibodies enable detection of immunoblotted targeted proteins without hindrance by interfering immunoprecipitating immunoglobulin heavy and light chains.

Cell viability analysis. These were performed using the CellTiter-Glo 2.0 Assay (G9242, Promega) according to the manufacturer’s protocol. Results are presented as percentage of viability of freshly isolated cells which are arbitrarily set as 100%.

Statistical analysis. The number of independent experiments and the statistical analysis for each figure are indicated in the legends. All statistical analyses were performed using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla, CA, USA, www.graphpad.com) and are presented as mean ± SEM. Two-tailed Student’s t tests (or as otherwise indicated) were used to compare means between groups. p < 0.05 was considered significant. For integrative analysis of human liver TET3 expression datasets, the Robust Rank Aggregation (RRA) method60 was used.

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References
1. Walesky, C. & Apte, U. Role of hepatocyte nuclear factor 4alpha (HNF4alpha) isoforms in mouse liver development is regulated by sequential HNF4alpha isoform expression datasets, the Robust Rank Aggregation (RRA) method60 was used.

Data availability
The data that support the findings of this study are available from the corresponding author upon request. The source data underlying Figs. 1b, e, h, l;2a, d–i, l–m, o, p, r, s; 3b, 5b, c, e; 6c; 5d; S5b and S6d are provided as Dataset Source File.

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6. Briancon, N. et al. Expression of the alpha7 isoform of hepatocyte nuclear factor (HNF) 4 is activated by HNF6/OC-2 and HNF1 and repressed by HNF-alpha1 in the liver. J. Biol. Chem. 279, 33398–33408 (2004).

7. Ancy, P. B. et al. TET-catalyzed 5-hydroxymethylcytosine precedes HNF4A promoter choice during differentiation of bipotent liver progenitors. Stem Cell Rep. 9, 264–278 (2017).

8. Wu, X. & Zhang, Y. TET-mediated active DNA demethylation: mechanism, function and beyond. Nat. Rev. Genet. 18, 517–534 (2017).

9. Lio, C. J. & Rao, A. TET enzymes and 5hmC in adaptive and innate immune systems. Front. Immunol. 10, 210 (2019).

10. Chen, Q., Chen, Y., Bian, C., Fujiki, R. & Yu, X. TET2 promotes histone O-GlcNAcylation during gene transcription. Nature 493, 561–564 (2013).

11. Vella, P. et al. Tet proteins connect the O-linked N-acetylglucosamine transferase Ogt to chromatin in embryonic stem cells. Mol. Cell 49, 645–656 (2013).

12. Guan, W. et al. Methylthioselenoic acid TET3 interacts with thyroid hormone nuclear receptors and stabilizes their association to chromatin. Proc. Natl Acad. Sci. USA 114, 8229–8234 (2017).

13. Montalban-Loro, R. et al. TET3 prevents terminal differentiation of adult NSCs by a non-catalytic action at Snprn. Nat. Commun. 10, 1728 (2019).

14. Liu, C. W. et al. Tet2 and Tet3 cooperate with B-lineage transcription factors to drive differentiation. Cell Metab. 25, 101–112 (2017).

15. Zaret, K. S. & Carroll, J. S. Pioneer transcription factors: establishing transcriptional potentials above and beyond. Cell 164, 872–883 (2016).

16. Nilsson, E. et al. Epigenetic alterations in human liver from subjects with type 2 diabetes. Clin. Epigenetics 5, 25 (2013).

17. Zhang, L., Rubins, N. E., Greenbaum, L. E. & Kaestner, K. H. Tissue-specific integration and meta-analysis. Genes Dev. 23, 3429–3438 (2009).

18. Kolde, R., Laur, S., Adler, P. & Vilo, J. Robust rank aggregation for gene list integration and meta-analysis. Bioinformatics 28, 573–580 (2012).

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Author contribution
D.L., T.C., and X.S. performed most of the experiments. D.X. and X.H. performed 5hMIDP and co-IP experiments. S.J. and S.D. designed and performed the hyper-insulinemic/euglycemic clamp studies and analyzed and interpreted the data. Y.H. initiated, designed, and led the study, and D.L. and Y.H. wrote the manuscript. S.D., X.Y., G.G.C., and H.T.S. provided intellectual input and critical reading of the manuscript.

Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to Y.H.

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