Epigenomic and transcriptional profiling identifies impaired glyoxylate detoxification in NAFLD as a risk factor for hyperoxaluria

Graphical abstract

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In brief

Gianmoena et al. identify an altered glyoxylate pathway in both mouse and human NAFLD. Functional analysis reveals reduced capacity of the fatty liver to detoxify glyoxylate derived from hydroxyproline breakdown, causing overproduction of the harmful waste product oxalate. This study provides a potential mechanism underlying kidney complications in NAFLD patients.

Highlights

- Agxt is hypermethylated and downregulated in mouse and human steatotic hepatocytes
- Detoxification of mitochondrial glyoxylate is insufficient in the fatty liver
- Challenge with dietary hydroxyproline leads to higher oxalate in the fatty liver
- Rescuing Agxt expression in the fatty liver reduces hepatic oxalate production

Article

Epigenomic and transcriptional profiling identifies impaired glyoxylate detoxification in NAFLD as a risk factor for hyperoxaluria

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SUMMARY

Epigenetic modifications (e.g. DNA methylation) in NAFLD and their contribution to disease progression and extrahepatic complications are poorly explored. Here, we use an integrated epigenome and transcriptome analysis of mouse NAFLD hepatocytes and identify alterations in glyoxylate metabolism, a pathway relevant in kidney damage via oxalate release—a harmful waste product and kidney stone-promoting factor. Downregulation and hypermethylation of alanine-glyoxylate aminotransferase (Agxt), which detoxifies glyoxylate, preventing excessive oxalate accumulation, is accompanied by increased oxalate formation after metabolism of the precursor hydroxyproline. Viral-mediated Agxt transfer or inhibiting hydroxyproline catabolism rescues excessive oxalate release. In human steatotic hepatocytes, AGXT is also downregulated and hypermethylated, and in NAFLD adolescents, steatosis severity correlates with urinary oxalate excretion. Thus, this work identifies a reduced capacity of the steatotic liver to detoxify glyoxylate, triggering elevated oxalate, and provides a mechanistic explanation for the increased risk of kidney stones and chronic kidney disease in NAFLD patients.

INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) is the most common liver disease in Western countries with increasing incidence worldwide. NAFLD is frequently accompanied by obesity, hyperlipidemia, hypertension, and insulin resistance—all characteristics of the metabolic syndrome (Wainwright and Byrne, 2016). A major risk of NAFLD is the progression to more severe liver diseases, such as non-alcoholic steatohepatitis, cirrhosis, and he-

patocellular carcinoma. Additional consequences of NAFLD and the metabolic syndrome are extrahepatic complications, such as cardiovascular disease, chronic kidney disease, and kidney stone disease (Byrne and Targher, 2015; Targher et al., 2016; Wu et al., 2016). However, the underlying molecular mechanisms of these extrahepatic complications have been only partially elucidated. One proposed mechanism is that the secretion of various factors by the liver, including proteins, metabolites, and microRNA (miRNA) species, is able to influence

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metabolism in the peripheral organs (Santos et al., 2019; Watt et al., 2019).

Kidney stone disease is a frequent extrahepatic complication of NAFLD and metabolic syndrome, with an odds ratio ranging between 1.17 and 5 in different studies (Qin et al., 2018; Nam, 2016; Einollahi et al., 2013). Although usually not life threatening, kidney stones are highly recurrent, affect quality of life, pose an economic burden, and are reported to be predictive of chronic kidney disease (Rule et al., 2011; Alexander et al., 2012). Changes in the urinary concentration of substances that promote stone formation, e.g., oxalate, a metabolic terminal waste product cleared by the urine, were previously discussed as a potential explanation for the relationship between the metabolic syndrome and kidney stone disease (Kohjimoto et al., 2013; Eisner et al., 2010; Negri et al., 2008). Recently, increased 24-h urinary oxalate excretion was shown to be associated with a higher risk of chronic kidney disease progression (Waikar et al., 2019). However, the contribution of hepatic metabolism to the formation of lithogenic substances, such as oxalate, in NAFLD has not yet been explored. Glyoxylate metabolism in the liver is responsible for the formation of hepatic oxalate, which accounts for 85%–90% of the total oxalate circulating in blood (Brzica et al., 2013). Increased urinary concentration of oxalate—hyperoxaluria—enhances the risk of kidney stone disease (Brzica et al., 2013). In the present study, by generating integrated genome-wide maps of transcription and epigenetic marks comprising DNA methylation and chromatin accessibility, we identified modifications within the glyoxylate pathway in NAFLD hepatocytes. More specifically, we observed promoter methylation and reduced expression, as well as reduced chromatin accessibility of alanine-glyoxylate aminotransferase (Agxt), a key enzyme in glyoxylate detoxification. Because Agxt is known to prevent excessive conversion of glyoxylate to oxalate, we investigated whether these alterations may lead to increased hepatic oxalate production, thereby promoting hyperoxaluria in NAFLD.

RESULTS

Genome-wide analysis of expression, DNA methylation, and open chromatin in obese and lean mice identifies deregulated genes with epigenetic alterations

In a first step, we aimed to identify steatosis-associated alterations in the transcriptome and epigenome of control (ob/+) and obese (ob/ob) mice, a widely used model of NAFLD (Anstee and Goldin, 2006). Obesity and hepatic fat accumulation in ob/ob mice were confirmed by body and liver weight, liver hypertrophy, and triglyceride (TAG) quantification (Figure S1A). Lack of extensive inflammation and fibrosis was illustrated by immunohistochemistry of the Kupffer cell marker F4/80 and by Sirius Red staining, respectively (Figure S1B). Analysis of mRNA expression and DNA methylation was performed in bulk liver tissue and in isolated hepatocytes from control (ob/+) and obese (ob/ob) mice. Profiles of chromatin accessibility were determined in isolated hepatocytes. Principal-component analysis (PCA) of liver tissue (Ti) and hepatocytes (He) showed a clear separation of obese and control mice for gene expression, DNA methylation, and chromatin accessibility along principal component 1 (PC1) (Figure 1A). However, separation of samples based on the DNA methylation data was less clear for liver tissue than for isolated hepatocytes (Figure 1A). This indicates that isolation of hepatocytes enables the identification of DNA methylation changes that remain undetected in complex whole-tissue samples due to different methylation patterns of individual cell types. Therefore, further analysis focused on the isolated hepatocyte-enriched cell fraction.

Expression analysis revealed 3,270 differentially expressed genes (DEGs), 953 downregulated and 2,317 upregulated, in ob/ob hepatocytes compared to ob/+ hepatocytes (Figures 1B and 1C). Analysis of differentially methylated regions (DMRs) identified 8,079 DMRs in ob/ob hepatocytes, 5,898 hypo- and 2,181 hypermethylated (Figures 1B and 1C). Finally, 16,210 differential open regions (DORs), encompassing 11,322 regions with reduced and 4,888 regions with increased chromatin accessibility, were observed in ob/ob hepatocytes (Figures 1B and 1C). To integrate DNA methylation and chromatin accessibility with gene expression changes, DMRs and DORs were annotated to the closest gene and matched with DEGs. In total, 649 DEGs were associated with DMRs and DORs (Figure 1D). As shown by gene set enrichment analysis, these genes were overrepresented for KEGG pathways related to tissue architecture (e.g., “focal adhesion” and “ECM-receptor interaction”), signaling (e.g., “Rap1, phosphatidylinositol 3-kinase [PI3K]-Akt, Ras signaling pathway”), and “metabolism” (Figure 1E). We next focused on DEGs with epigenetic changes in close proximity, namely DMRs and DORs overlapping at least by 1 bp. For the

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Figure 1. Genome-wide analysis of expression, DNA methylation, and open chromatin in obese and lean mice identifies deregulated genes with epigenetic alterations

(A) Principal-component analysis (PCA) of gene expression data (RNA-seq of liver tissue and isolated hepatocytes from ob/+ and ob/ob mice; $n = 4$), DNA methylation (RRBS of liver tissue and isolated hepatocytes of ob/+ and ob/ob mice; $n = 6$; only tiles present both in Ti and He), and chromatin accessibility (DNase-seq of hepatocytes; $n = 2$). He, hepatocytes; Ti, liver tissue; PC1, principal component 1 (related to disease state); PC2, principal component 2 (related to cell type). (B and C) Table (B) summarizing the number of differentially expressed genes (DEGs), differentially methylated regions (DMRs), and differential open chromatin regions (DORs) between ob/ob and ob/+ hepatocytes and the thresholds used and illustration (C) by heatmaps. (D) Venn diagram of DEGs and genes with DMRs and DORs in hepatocytes from ob/ob mice with an overlap of 649 genes. (E) Gene set enrichment analysis results showing the top 10 significantly overrepresented pathways in the 649 genes (FDR adj. $p < 0.01$). (F) Integrative heatmap of RNA expression, DNA methylation, and DNA accessibility showing 133 DEGs of the overlap with co-localized DMRs and DORs. 7 clusters are illustrated according to the relationship between gene expression (E), chromatin accessibility (C), and DNA methylation (M). Arrows represent the direction of the changes, in red (higher in ob/ob) or blue (lower in ob/ob) color. (G) Genomic annotation of the DMR-DOR pairs with the genomic region of the gene to which they have been assigned. (H) Integrative Genomics Viewer (IGV) screenshot of the genomic region of Agxt in ob/+ and ob/ob hepatocytes showing RNA expression, chromatin accessibility, and DNA methylation results (replicates overlaid) and positions of DOR, DMR, and amplicons for targeted deep sequencing relative to genomic coordinates. See also Figure S1.

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vast majority of the 155 DMR-DOR pairs associated with 135 DEGs, the direction of gene expression alteration was positively correlated with chromatin accessibility and inversely correlated with the direction of methylation changes (clusters 1 and 7; [Figure 1F](#)). DMR-DOR pairs were predominantly located in introns (34%) and promoters (20%; [Figure 1G](#)).

Altogether, these results show that liver steatosis in ob/ob mice is associated with marked global expression changes from which a relatively large fraction is associated with differentially methylated regions (30.3%) and differential chromatin accessibility (51.3%). Moreover, the positive correlation of the direction of gene expression alterations with chromatin accessibility and its inverse correlation with the direction of DNA methylation changes for a large fraction of genes point toward epigenetic modifications as possible mechanisms underlying transcriptional regulation in steatosis.

Genes involved in hepatic glyoxylate metabolism show steatosis-associated expression and epigenetic alterations

To support the relevance of the deregulated genes for human liver disease and to identify candidates for further evaluation, we considered publicly available genome-wide datasets of human liver tissue from patients with NAFLD, cirrhosis, and hepatocellular cancer ([Data S1](#); [Grinberg et al., 2014](#)). We compared deregulated genes in human liver disease ([Figure S2A](#)) to the 649 and 135 genes of interest identified in ob/ob hepatocytes ([Figure S2B](#)). Alanine-glyoxylate aminotransferase (AGXT) was one of the two genes found in the overlap between the 135 genes presenting DMR-DOR pairs and the human liver disease genes ([Figure S2B](#)). In ob/ob hepatocytes, *Agxt* showed reduced RNA expression (1.8-fold; adj. $p = 1.23E-19$), hypermethylation (7.97%; adj. $p = 2.68E-22$) and reduced chromatin accessibility (2.1-fold; q value = $2.02E-03$; [Figure 1H](#); [Data S2](#)). In all human liver diseases, AGXT was downregulated (more than 1.3-fold; adj. $p < 0.05$; [Data S1](#); [Figure S2A](#)).

AGXT encodes a liver-specific enzyme that transaminates glyoxylate to glycine, preventing its conversion to the harmful waste product oxalate. Loss-of-function mutations of AGXT cause the heritable human disease, primary hyperoxaluria type I (PH1) (OMIM entry 259900), characterized by hyperoxaluria, nephrocalcinosis, and kidney stone disease, which upon kidney injury can progress to systemic calcium oxalate deposition (oxalosis). Importantly, homozygous *Agxt*^{−/−} mice also exhibit key features of PH1 ([Salido et al., 2006](#)). Thus, we further investigated the impact of the observed NAFLD-associated alter-

ations of *Agxt* on the oxalate production by the liver. We extended the analysis to include further genes involved in glyoxylate metabolism ([Figure 2A](#)). These include: proline dehydrogenase (oxidase) 2 (*Prodh2*) and 4-hydroxy-2-oxoglutarate aldolase 1 (*Hoga1*) that catalyze the first and last step in mitochondrial hydroxyproline degradation to glyoxylate, respectively ([Jiang et al., 2012](#)); hydroxyacid oxidase 1 (*Hao1*) that converts glycolate to glyoxylate in hepatic peroxisomes ([Dutta et al., 2016](#)); glyoxylate reductase/hydroxypyruvate reductase (*Grhpr*) that reduces glyoxylate to glycolate in mitochondria and cytosol; and lactate dehydrogenase (subunits encoded by *Ldha*, *Ldhb*, *Ldhc*, and *Ldhd*), of which the liver-specific *Ldha* has recently been shown to play a key role in hyperoxaluria ([Lai et al., 2018](#)), that converts cytosolic glyoxylate to oxalate.

Similar to *Agxt*, *Hao1* was also significantly downregulated in ob/ob hepatocytes in the RNA sequencing (RNA-seq) analysis ([Figure 2A](#); [Data S2](#)). In addition to *Agxt*, DNA methylation changes were observed in *Got2* ([Figure 2A](#); [Data S2](#)) and chromatin rearrangements in *Hao1*, *Grhpr*, *Aldh4a1*, and *Hoga1* ([Figure 2A](#); [Data S2](#)). qPCR analysis in independent mice confirmed the approximately 2-fold reduction of *Agxt* mRNA levels in liver tissue of ob/ob mice compared to control mice ([Figure 2B](#)). This was also confirmed at the protein level by western blots of liver tissue extracts ([Figure 2C](#)) and by immunohistochemistry (IHC), where a zonated expression pattern was observed ([Figure 2D](#)). qPCR confirmed no change in gene expression for *Grhpr*, *Prodh2*, and *Hoga1* and a slight downregulation for *Hao1* mRNA. Moreover, a 2-fold upregulation of *Ldha*, which was not observed in the RNA-seq data, was revealed ([Figure 2B](#)). These changes indicate an affected glyoxylate metabolism in NAFLD.

Agxt transcription in response to glucagon is reduced by promoter hypermethylation in ob/ob hepatocytes

DNA methylation changes in the promoter of *Agxt* were validated by targeted deep sequencing. Two regions in the promoter of *Agxt* (*Agxt*(1) and *Agxt*(2)), the latter of which overlaps with the DMR ([Figure 1H](#)), confirmed a significant increase in methylation in ob/ob hepatocytes ([Figure 2E](#)), as also illustrated in the pattern maps ([Figure S2C](#)). One methylated cytosine in the DMR *Agxt*(2) is located within a half-site cyclic AMP (cAMP) response element (CRE), 5'-TGACG-3' ([Figure S2D](#)). CRE is predicted to bind to the phosphorylated CRE-binding protein (CREB) in response to hormones ([Iguchi-Ariga and Schaffner, 1989](#)). Because the mouse mitochondrial *Agxt* transcript in the liver has been shown to be induced by glucagon ([Li et al., 1999](#)), and glucagon signaling

Figure 2. Genes involved in hepatic glyoxylate metabolism show steatosis-associated expression and epigenetic alterations

(A) Pathway of glyoxylate production and detoxification in mouse hepatocytes. Changes in gene expression, chromatin accessibility, and DNA methylation of the genes catalyzing the corresponding reactions are illustrated in boxes, in red (higher in ob/ob) or blue (lower in ob/ob) color; darker color indicates a promoter-localized alteration.

(B) qPCR in ob/+ and ob/ob livers (n = 5).

(C) *Agxt* protein levels in ob/+ and ob/ob livers shown by western blot and densitometric quantification (n = 6).

(D) Representative images of H&E staining and immunohistochemistry of the pericentral marker glutamine synthetase (GS) and of *Agxt* in liver tissue (scale bars: 200 μ m except for rightmost panel: 20 μ m). CV, central vein; PV, portal vein.

(E) Targeted deep sequencing showing average methylation of the two analyzed amplicons in the *Agxt* promoter region (n = 4).

(F and G) qPCR showing (F) expression of *Agxt* mRNA in the liver in response to glucagon administration to ob/+ and ob/ob mice *in vivo* (n = 3) and qPCR analysis showing (G) expression of *Agxt* mRNA in response to glucagon treatment in cultivated primary mouse hepatocytes isolated from ob/+ and ob/ob livers (n = 3). All bar diagrams represent the mean $\pm$ SD. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Student's t test, unpaired, two sided. See also [Figure S2](#) and [Data S2](#).

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enhances CREB-dependent gene expression (Altarejos and Montminy, 2011; Figure S2E), we hypothesized that methylation at the CRE site of the *Agxt* promoter in ob/ob hepatocytes may affect its transcriptional response to glucagon. Accordingly, livers from ob/+ mice showed an approximately 11-fold glucagon-induced upregulation of *Agxt* mRNA, compared to only 2.9-fold in ob/ob mice (Figure 2F). Also, when cultivated *in vitro*, ob/ob hepatocytes showed a much weaker *Agxt* response to glucagon over time (Figure 2G). Thus, CpG methylation in the CRE site of *Agxt* is accompanied by a reduced transcriptional upregulation in response to glucagon.

Steatotic ob/ob hepatocytes generate more oxalate from hydroxyproline catabolism *in vitro*

We next investigated oxalate generation by steatotic hepatocytes. The release of oxalate into the culture medium by *in vitro* cultivated primary mouse hepatocytes (PMHs) from ob/ob and ob/+ control mice was analyzed upon exposure to glyoxylate (Glx), as well as to hydroxyproline (Hyp) and glycolate (Glc), which are precursors metabolized to glyoxylate in mitochondria or peroxisomes, respectively (Figures 3A and S3A–S3C). Increased TAGs in ob/ob hepatocytes were confirmed (Figure 3B). In addition, because the loss of liver-specific gene expression upon cultivation has been reported (Godoy et al., 2016), we first ensured stability of the key genes involved in the expected metabolic reactions in PMHs for the duration of our experiments (Figures S3D and S3E). In the absence of a precursor (0 mM), ob/ob hepatocytes produced similar or slightly lower amounts of oxalate than control hepatocytes (Figure 3C). Both ob/+ and ob/ob hepatocytes metabolized the three precursors and produced oxalate in a concentration-dependent manner (Figure 3C). However, the amount of oxalate generated from Hyp was higher for ob/ob than for control hepatocytes at Hyp concentrations higher than 3.16 mM (Figure 3C). Generation of glycolate from Hyp was also significantly higher in the ob/ob hepatocytes (Figure 3D). In contrast, the response to exogenously added glycolate and glyoxylate was similar for both ob/+ and ob/ob hepatocytes (Figures 3C and 3D). Thus, the observed steatosis-associated changes in the glyoxylate pathway only result in increased release of oxalate after metabolism of Hyp, suggesting that mitochondrial glyoxylate detoxification is selectively affected in murine steatosis.

The influence of the precursors on protein expression was explored by immunoblotting (Figures 3E and 3F). Baseline *Agxt* and *Hao1* levels were lower in ob/ob than in ob/+ hepatocytes, as expected. *Agxt* was not altered in response to glycolate and glyoxylate (Figure 3E). However, in response to Hyp, *Agxt*

increased exclusively in ob/+ and not in ob/ob hepatocytes (Figure 3E). *Hao1* protein increased in response to all three precursors (Figure 3F). Thus, the constitutively lower protein levels of *Agxt* together with its failure to increase in response to Hyp, in conjunction with an enhanced *Hao1* translation in presence of substrate, may contribute to the higher oxalate production from Hyp in fatty hepatocytes.

Increased oxalate formation from Hyp catabolism is validated in a Western diet NAFLD mouse model

To validate our results in a second mouse model of NAFLD, mice received a Western-type diet (WD) and were compared to age-matched mice receiving a normal chow diet (NCD). 6 weeks after the onset of the diet, body weight significantly increased (Figure S4A) and mild steatosis but no inflammation or fibrosis was observed (Figure S4B). Long-term (30 weeks) WD augmented obesity (Figure S4C), and massive hepatic fat accumulation was accompanied by inflammation and fibrosis, indicating steatohepatitis (Figure S4D). Transcription of *Agxt* decreased in livers of 6 weeks WD-fed mice while *Ldha* expression significantly increased (Figure 4A). *Agxt* downregulation was maintained and became even more pronounced after 30 weeks of WD (Figure 4B), which was accompanied by a downregulation of *Hao1* and *Hoga1*. Decreased *Agxt* protein levels in liver tissue were confirmed by western blot and by immunohistochemistry (Figures 4C and 4D), and a significant increase in *Agxt* promoter methylation was observed in the DMR *Agxt*(2) in hepatocytes isolated from 6 weeks WD-fed mice (Figure 4E). After confirmation of lipid content (Figure 4F), hepatocytes were cultivated with hydroxyproline, glycolate, and glyoxylate to monitor excretion of oxalate and glycolate into the medium. Similar to ob/ob hepatocytes, hepatocytes from WD-fed mice generated significantly higher amounts of oxalate and glycolate when Hyp was added as a precursor (Figures 4G and 4H) but less or similar amounts of oxalate after incubation with glycolate and glyoxylate (Figures 4G and 4H). Western blot analysis confirmed decreased *Agxt* expression in the WD-hepatocyte cultures (Figure S4E), as well as an upregulation of *Hao1* after exposure to glyoxylate and its precursors (Figure S4F), as observed in ob/ob hepatocytes (Figure 3). Thus, glyoxylate metabolism is similarly affected in two independent mouse models of NAFLD.

Inhibition of Hyp catabolism and *Agxt* gene transfer reduces oxalate levels in steatotic hepatocytes

We next investigated the consequences of complete *Agxt* deficiency by analyzing *Agxt* knockout (KO) mice (Salido et al., 2006). Hyperoxaluria and oxalemia were confirmed by increased

Figure 3. Steatotic ob/ob hepatocytes generate more oxalate from hydroxyproline catabolism *in vitro*

(A) Experimental outline.
(B) Representative images of hepatocytes isolated from ob/+ and ob/ob mice and cultivated in sandwich on top (SOT) cultures (scale bars: 25 μ m) and quantification of their triacylglyceride (TAG) content (n = 3).
(C and D) Quantification of oxalate (C) and glycolate (D) levels in supernatants of ob/+ and ob/ob hepatocyte cultures after the addition of glycolate, hydroxyproline, and glyoxylate (n = 6).
(E and F) Representative western blots showing the impact of precursor treatments on *Agxt* (E) and *Hao1* (lower band; F) protein expression in ob/+ and ob/ob hepatocytes with accompanying densitometric quantification (n = 3). Glc, glycolate; Glx, glyoxylate; Hyp, hydroxyproline. The mean $\pm$ SD is shown.
*p < 0.05, **p < 0.01, and ***p < 0.001 for comparison between ob/ob and ob/+. *p < 0.05 and ****p < 0.001 for comparison between treated and untreated (0 mM). Student's t test, unpaired, two sided. See also Figure S3.

Figure 4. Increased oxalate formation from Hyp catabolism is validated in a Western diet NAFLD mouse model

(A) qPCR analysis showing mRNA expression of glyoxylate metabolism genes in the liver of mice fed a Western diet (WD) for 6 weeks compared to their normal chow diet (NCD)-fed age-matched controls (n = 5).

(B) qPCR analysis of mRNA expression in liver tissue of WD-fed (n = 10) and NCD-fed mice (n = 9) for 30 weeks.

(C and D) Western blot analysis of *Agxt* with accompanying densitometric quantification (n = 5) together with representative images of *Agxt* immunohistochemistry in the liver of 6 weeks (C) and 30 weeks (D) NCD- and WD-fed mice. Scale bars: 50 μ m.

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oxalate levels in the 24-h urine and plasma, respectively (Figure S5A; Table S1). Isolated PMHs of Agxt KO mice, which are non-steatotic (Figure S5B), produced significantly higher levels of oxalate from all three precursors (Figure 5A), confirming that complete deficiency of Agxt (Figure 5B) results in oxalate overproduction from these precursors.

Given that in steatotic hepatocytes, which express lower levels of Agxt, only the addition of Hyp exacerbates oxalate production, we reasoned that either impairing Hyp catabolism or restoring expression of Agxt (Figure 5C) would normalize oxalate levels. Accordingly, blocking the first and rate-limiting step of Hyp degradation with the Prodh2 inhibitor tetrahydro-2-furoic acid (THFA) reduced the oxalate burden in both ob/ob and WD hepatocytes (Figure 5D). Also, transduction of PMHs with adeno-associated virus (AAV) carrying an Agxt expression vector limited oxalate formation from Hyp in both ob/ob and WD hepatocytes (Figure 5E). Overexpression of Agxt after Agxt AAV delivery was confirmed by qPCR and western blot compared to the untransduced or EGFP-transduced hepatocytes (Figures 5F, 5G, S5C, and S5D). These results demonstrate that insufficient Agxt expression in steatosis sensitizes hepatocytes to oxalate overproduction from Hyp.

Increased hepatic oxalate formation in ob/ob mice fed a 1% Hyp diet is partially prevented by Agxt gene transfer

To explore whether the increased oxalate production in the isolated hepatocytes of ob/ob mice also occurs *in vivo*, ob/ob and control ob/+ mice were fed either a NCD or a diet containing 1% Hyp for 10 days (Figure 6A). Oxalate was analyzed in plasma obtained from the right heart chamber, as well as from the portal and the hepatic veins, which correspond to the liver inflow and outflow, respectively (Schenk et al., 2017), to estimate the liver-derived oxalate released into the blood (Figure S6A). Secretion of oxalate into the bile was explored as well but was below the level of quantification. Daily urinary excretion of oxalate was studied in 24-h urine collections (Figure S6A).

The hepatic TAG content and histopathology (Figure S6B) did not change upon addition of Hyp. In the absence of dietary Hyp, there was no significant difference in plasma oxalate concentration between the ob/+ and ob/ob genotypes. However, after 10 days on Hyp-rich diet, the oxalate concentration in plasma obtained from the hepatic vein, as well as the ratio of oxalate in the liver outflow (hepatic vein) to the liver inflow (portal vein), were significantly increased in the ob/ob plus Hyp group compared to the other groups (Figure 6B). The oxalate concentration in the systemic circulation (blood derived from the heart) was not significantly increased (Figure 6B), possibly due to the dilution of hepatic vein blood by venous blood from the general circulation and by the renal excretion of oxalate, which was analyzed next.

Both the total oxalate amount per day ($\mu\text{mol}/24\text{ h}$) and the oxalate:creatinine ratio were higher in ob/ob than in ob/+ mice (Fig-

ure 6C), which increased upon consumption of dietary Hyp in the ob/+, as well as in the ob/ob genotype (Figure 6C), but were significantly higher in the ob/ob plus Hyp group (Figure 6C), even after correcting for the higher food (and Hyp) intake (Table S1) in ob/ob mice (oxalate/24 h g ingested food [i.f.]; Figure 6C). The more diluted oxalate (Figure 6C) and creatinine concentrations (Table S1) in ob/ob mice were attributed to their higher 24-h urine volume (Table S1). The total amount of creatinine excreted per day (mg/24 h) was, however, similar (Table S1).

The influence of dietary Hyp on gene expression in the liver was also analyzed (Figure S6C), revealing a slight decrease in Agxt mRNA in the ob/ob plus Hyp group. At the protein level, Hao1 was significantly augmented in response to Hyp in both ob/+ and ob/ob mice (Figure S6D), as observed *in vitro* (Figure 3F). This posttranscriptional upregulation of Hao1 may also contribute to higher hepatic oxalate generation.

We then investigated whether Agxt gene transfer by AAV *in vivo* has the potential to reduce the excess oxalate released by the fatty liver when challenged with Hyp. The conditions for an efficient Agxt-AAV delivery were first determined in Agxt KO mice (Figure S5E), where oxalate levels in plasma and urine decreased to normal levels (Figure S5F) after expression of Agxt in the liver was recovered, as demonstrated by immunohistochemistry and western blot (Figure S5G). 2 weeks after transduction with Agxt-AAV or control EGFP-AAV, ob/ob mice were given NCD or 1% Hyp for further 2 weeks and urinary and plasma oxalate levels were analyzed (Figure 6D). In ob/ob mice injected with the Agxt AAV, a significantly smaller increase in oxalate was measured in both plasma and urine with Hyp compared to the EGFP AAV-injected ob/ob mice (Figure 6E). Analysis of Agxt protein in the liver by western blot (Figure 6F) and immunohistochemistry (Figure 6G) revealed rescued Agxt expression in Agxt AAV-injected ob/ob mice.

Taken together, these results show that the fatty liver of ob/ob responds to a dietary challenge with Hyp by releasing more oxalate compared to control livers. This is reflected by higher oxaluria in the Hyp-fed ob/ob mice, which can be partially prevented by AAV-mediated Agxt transfer, and matches our *in vitro* studies (Figures 3C and 5E).

WD-fed mice accumulate oxalate in the liver

Next, oxalate excretion *in vivo* was investigated in mice on a WD, comparing short-term (6 weeks) with long-term (30 weeks) effects. In 6 weeks WD-fed mice, plasma oxalate was significantly elevated in the hepatic vein and in the heart blood, and a higher ratio of hepatic vein to portal vein oxalate was also observed (Figure S6E). After 30 weeks on WD, plasma oxalate concentrations remained similar to those measured in 6 weeks WD-fed mice (Figure S6E). Strikingly, mice on NCD for 30 weeks showed an aging-dependent increase in oxalate in the hepatic vein (Figure S6E). Despite these changes, the total urinary oxalate ($\mu\text{mol}/24\text{ h}$) and oxalate:creatinine ratio ($\mu\text{g}/\text{mg}$) did not differ

(E) Average methylation (%) of the two analyzed amplicons in the Agxt promoter region in hepatocytes isolated from 6 weeks NCD- and WD-fed mice ($n = 3$).

(F) Representative images of cultivated hepatocytes isolated from 6 weeks WD-fed and NCD-fed mice (scale bars: 25 μm) and quantification of their TAG content ($n = 3$).

(G and H) Quantification of oxalate (G) and glycolate (H) in the supernatants of hepatocyte cultures after addition of the precursors Glc, Hyp, and Glx ($n = 3$).

All diagrams represent the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Student's *t* test, unpaired, two sided. See also Figure S4.

Figure 5. Inhibition of Hyp catabolism and *Agxt* gene transfer reduce oxalate levels in steatotic hepatocytes

(A) Quantification of oxalate and glycolate in supernatants of hepatocytes isolated from *Agxt* knockout (KO) and age-matched wild-type (WT) mice and cultured in the presence of different concentrations of the precursors Glc, Hyp, and Glx (n = 3). Diagrams represent the mean $\pm$ SD.

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significantly between the NCD and WD-fed groups (Figure S6F) and could not be explained by parameters like urine volume, water consumption, or food intake (Table S1). This prompted us to extract and quantify oxalate from liver tissue. Mice fed a WD for 6 weeks showed higher hepatic oxalate content than their NCD-fed counterparts, which further increased after 30 weeks on WD (Figure S6G). Moreover, focal CaOx crystal deposition in the liver was detected with Pizzolato staining in the majority of the WD livers (Figure S6G). These results suggest that the excess of oxalate generated in the fatty liver of WD-fed mice is not efficiently excreted.

In conclusion, NAFLD induced by a hypercaloric WD resulted in similar expression changes in the glyoxylate pathway as in ob/ob mice, which provoke higher hepatic oxalate formation when Hyp is added. In the absence of dietary Hyp *in vivo*, the increased hepatic generation of oxalate is not reflected by hyperoxaluria but rather in CaOx crystal deposition within liver tissue, a condition that becomes aggravated in steatohepatitis.

Alterations in glyoxylate metabolism genes and urinary oxalate excretion correlate with the hepatic lipid content in human NAFLD

To investigate the translational relevance, we analyzed primary human hepatocytes (PHHs) isolated from 17 male steatotic and lean donors (Table S2). Pathological examination of the corresponding liver tissue revealed steatosis in approx. 60% (10/17) of the cases (Table S2), which was confirmed by the quantification of the TAG content in the hepatocytes and H&E staining of available samples in the majority of the cases (Table S2; Figures S7A and S7B).

Expression of AGXT mRNA, determined by qPCR, displayed a significant inverse correlation with the TAG content (Spearman $\rho = -0.642$; $p = 0.007$), while analysis of DNA methylation by targeted deep sequencing of the human AGXT promoter (amplicon AGXT(1)) revealed a positive correlation with the TAG content ($\rho = 0.601$; $p = 0.0122$; Figure 7A). When stratified into low- and high-TAG content, expression of AGXT mRNA was significantly lower (Figure 7B), and DNA methylation was significantly higher in the high-TAG-content hepatocytes in all three amplicons studied (Figure 7C), two of which were located in the promoter and one in an ~11-kb upstream region (Figure S7C), as illustrated in the pattern maps (Figure S7D). DNA methylation appears to affect recognition sites for hepatic nuclear factor 4 (HNF-4), transcription factor Sp1, and transcription factor AP-2-alpha (Figure S7C), from which at least AP-2-alpha is involved in basal promoter activity of AGXT (Uchida et al., 2002). Immunohistochemistry of AGXT in human liver tissue revealed that, un-

like the pericentral marker glutamine synthetase (GS), human AGXT is expressed in all zones (Figure 7D) and displayed a punctate staining pattern consistent with its reported peroxisomal localization (Salido et al., 2006).

We extended the analysis to include key genes involved in human glyoxylate metabolism (Figure 7E). Besides AGXT, two further genes showed significant inverse correlations with TAG content: *GRHPR* ($\rho = -0.527$; $p = 0.0318$) and *HOGA1* ($\rho = -0.515$; $p = 0.0366$; Figure S7E). Also, when stratified into low- and high-TAG content, expression of *GRHPR* and *HOGA1* mRNA was significantly lower in the high-TAG content hepatocytes (Figure 7F), indicating more complex alterations, involving all three primary hyperoxaluria-related genes (AGXT, *GRHPR*, and *HOGA1*) in human NAFLD.

The relationship between NAFLD and oxalate excretion was analyzed in a cohort of overweight or obese children and adolescents with biopsy-proven NAFLD (Table S3). A positive correlation was observed between the percentage steatosis and the urinary oxalate to creatinine ratio ($\rho = 0.366$; $p = 0.047$; Figure 7G), as well as between the percentage steatosis and the total amount of oxalate excreted in the urine when this was corrected for body surface area (BSA), as recommended for the interpretation of urine oxalate results in children (Clifford-Mobley et al., 2015; $\rho = 0.427$; $p = 0.019$; Figure 7G). In two samples, the normal reference value of oxalate excretion (<0.50 mmol/1.73 m²/day) was exceeded. No association was observed between the daily oxalate excretion and fibrosis, inflammation (portal or lobular), ballooning, or NAFLD activity score (NAS), indicating that hepatic fat accumulation is the key factor (Figure S7F).

The generation of oxalate and glycolate from metabolic precursors was also studied *in vitro* in cultivated PHHs (Figure 7H). Oxalate levels increased significantly when glycolate and glyoxylate were added (Figure 7H). In response to Hyp exposure, only glycolate, but not oxalate, was significantly increased (Figure 7H). Thus, in PHHs, the contribution of mitochondrial Hyp catabolism to hepatic oxalate seems less important compared to peroxisomal glycolate oxidation.

In conclusion, alterations in genes involved in glyoxylate metabolism are observed in human NAFLD in relation to the hepatic lipid content and may be causally linked to increased urinary oxalate excretion from the metabolism of precursors.

DISCUSSION

The present study comprising an integrated epigenome and transcriptome analysis of fatty hepatocytes from leptin-deficient

(B) Western blot confirming lack of Agxt expression in Agxt KO hepatocytes (n = 3).

(C) Schematic illustration of Hyp catabolism and interventions.

(D) Excretion of oxalate after the addition of Hyp to hepatocytes isolated from ob/+ and ob/ob mice (left panel) (n = 3) and from NCD- and WD-fed mice (right panel; n = 4) and cultured in the presence of the Prodh2 inhibitor tetrahydro-2-furoic acid (THFA) (10 mM).

(E) Excretion of oxalate after AAV-mediated delivery of control EGFP (pAAV-CMV-EGFP) or murine Agxt (pAAV-CMV-Agxt) in ob/ob (left panel; n = 5) and WD-fed hepatocytes (right panel; n = 4).

(F and G) Expression of Agxt measured with qPCR in hepatocytes of ob/ob mice (F; n = 5) and in hepatocytes of mice fed a WD (G; n = 4) that were untransduced or transduced with the control virus (pAAV-CMV-EGFP) or with the Agxt-delivering virus (pAAV-CMV-Agxt), together with representative western blots and densitometric quantification (n = 2). CMV: cytomegalovirus promoter.

For transduction of control hepatocytes, see Figure S5. Bar diagrams represent the mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Student's t test, unpaired, two sided. See also Figure S5.

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(ob/ob) mice identified glyoxylate metabolism as an affected pathway in steatosis. A key enzyme in this pathway, *Agxt*, was downregulated and hypermethylated in mouse steatotic hepatocytes. Because *Agxt* is crucial for the detoxification of glyoxylate, we hypothesized that glyoxylate detoxification capacity is compromised in steatosis, resulting in higher oxalate generation.

Our experiments with *in vitro* cultivated hepatocytes isolated from two different mouse models of NAFLD showed that the identified steatosis-associated changes lead to higher oxalate generation when challenged with the precursor hydroxyproline. After the addition of high but still physiologically relevant Hyp levels, glyoxylate detoxification capacity was overwhelmed, leading to increased oxalate formation. The increased production of oxalate after the addition of Hyp (but not glycolate or glyoxylate) in steatotic hepatocytes compared to controls showed that the mitochondrial detoxification of glyoxylate derived from the catabolism of Hyp is insufficient in this organelle in NAFLD. Our observation that *Agxt* is not induced in response to glucagon in ob/ob hepatocytes is consistent with this finding. Glucagon-induced upregulation of mouse *Agxt* affects only the mitochondrial *Agxt* mRNA (Li et al., 1999). This is relevant in glyoxylate metabolism because it has been shown to prevent oxalate formation from Hyp (Takayama et al., 2003). The observed phenotypical correction by blocking Hyp catabolism, and after viral transfer of *Agxt* in hepatocytes of both mouse models of NAFLD, confirmed the limitation of *Agxt* in steatosis.

The presented animal studies involving dietary challenges with Hyp in ob/+ and ob/ob mice confirmed the results obtained *in vitro*. Higher hepatic generation and higher urinary excretion of oxalate were observed in ob/ob mice under these conditions, and viral transfer of *Agxt* *in vivo* was able to reduce the oxalate overproduction, in agreement with the *in vitro* experiments. These findings demonstrate the importance of downregulated *Agxt* in the elevated oxalate production in steatosis. Other possible contributing mechanisms, such as increased mitochondrial localization of *Ldha* (Brooks et al., 1999), which would readily convert the mitochondrial glyoxylate pool to oxalate, are not excluded and deserve further investigation. Moreover, our finding that mice under WD show oxalate depositions in the liver points toward a possible deficiency of the hepatocytes to excrete oxalate, which also requires further research.

The addition of 1% Hyp to the diet is of human relevance, considering its high content in meat (0.3%–0.7%) and gelatin (9%–15%; Knight et al., 2006; Miyada and Tappel, 1956). In addition

to its dietary source, the endogenous Hyp pool derived from the turnover of body collagen contributes considerably to the daily excretion of oxalate (Knight et al., 2006; Li et al., 2016; Fargue et al., 2018). Therefore, aging (Grote et al., 2019; Laurent et al., 2019) and NAFLD-related perturbations of lean body mass (Lindström, 2007; Abrigo et al., 2016; Poggiogalle et al., 2017) likely influence the circulating concentration of endogenous Hyp and, consequently, oxalate excretion. This may be responsible for the differences observed between the mouse models and for the aging-dependent rise in hepatic oxalate release in lean mice in the absence of dietary Hyp. Interestingly, in patients with primary hyperoxaluria, a decreased body collagen turnover in response to increased oxalate generation has been reported (Fargue et al., 2018), indicating a complex adaptive organ cross-talk that remains poorly understood.

An important question is whether the results obtained in the rodent models are also relevant in human NAFLD, considering the well-known species divergences in glyoxylate metabolism (Ichiyama, 2011). Importantly, human AGXT is peroxisomal, whereas mouse *Agxt* has both mitochondrial and peroxisomal localization. This peroxisomal-only localization of human AGXT implies that it may not be directly involved in the detoxification of Hyp-derived glyoxylate in the mitochondria, in contrast to what is observed in mice. Our studies exposing human hepatocytes to glyoxylate precursors argue for glycolate being a more important source of oxalate than Hyp. Previous human studies investigating the ingestion of gelatin or infusion of a Hyp isotope (Knight et al., 2006; Fargue et al., 2018) revealed that Hyp contributed 15% to the daily excreted oxalate in control subjects. This contribution increased to 18%, 47%, and 33% in human primary hyperoxalurias (PHs) 1, 2, and 3 with deficiencies in AGXT, GRHPR, or HOGA1, respectively (Fargue et al., 2018). Accordingly, Hyp metabolism contributed the least in PH1 patients with AGXT deficiency, who have the highest urinary oxalate excretion (Fargue et al., 2018). However, our study showed that not only AGXT but also GRHPR and HOGA1 were downregulated in the human steatotic hepatocytes, which suggests that both high dietary Hyp and glycolate intake may favor hyperoxaluria in human NAFLD. Altogether, two results of our study are supportive of a risk for hyperoxaluria in human NAFLD: (1) downregulation and hypermethylation of AGXT together with downregulation of GRHPR and HOGA1 in human steatotic hepatocytes and (2) the significant correlation of excreted oxalate with the severity of steatosis in overweight children and adolescents

Figure 6. Increased hepatic oxalate formation in ob/ob mice fed a 1% Hyp diet is partially prevented by *Agxt* gene transfer

(A) Experimental outline.

(B) Oxalate concentration in plasma obtained from the portal vein, the hepatic vein, and the right heart chamber, as well as the hepatic vein to portal vein (hv/pv) ratio of oxalate of ob/+ and ob/ob fed a NCD or a 1% Hyp diet for 10 days (n = 5, each).

(C) Oxalate in the 24 h urine collected from the same animals. Concentration of oxalate, total amount excreted per day, oxalate to creatinine ratio, and total amount of oxalate per g ingested food (i.f.) are presented (n = 5).

(D) Experimental outline.

(E) Oxalate in plasma obtained from the right heart chamber and in the 24 h urine (oxalate to creatinine ratio) of ob/ob mice injected with control EGFP-AAV (pAAV-CMV-EGFP) or with *Agxt*-AAV (pAAV-TBG-*Agxt*), as well as control ob/+ mice fed a NCD or a 1% Hyp diet for 2 weeks (n = 5, each group). TBG: thyroxine-binding globulin promoter.

(F) Western blot showing *Agxt* expression in the liver (n = 3).

(G) Representative images of the *Agxt* immunohistochemistry in the liver. Scale bars: 50 μ m.

All bar diagrams represent the mean $\pm$ SEM. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. Student's t test, unpaired, two sided. See also Figures S5 and S6 and Table S1.

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with biopsy-proven NAFLD. We are, however, aware of the limitations of this cohort, more specifically its sample size, and the impossibility of implementing a controlled challenge, which hampers us from estimating the contribution of dietary precursors to urinary oxalate in NAFLD.

Elevated synthesis of oxalate by the fatty liver has important implications. Increased oxalate represents an initial step in the multifactorial process of calcium oxalate kidney stone formation (Alelign and Petros, 2018). However, the pathogenesis of kidney stone formation is complex and regulated by multiple factors, in addition to oxalate, that could not be addressed in our study. ob/ob mice showed an increased urine volume, possibly due to hyperglycemia- and glucosuria-induced osmotic diuresis (Lindström, 2007, 2010), a factor known to prevent calcium oxalate supersaturation (Mulay and Anders, 2017). Thus, hyperdiuresis antagonizes CaOx stone formation in this model, unless rather unphysiological challenges, such as intraperitoneal glyoxylate injection, are employed (Fujii et al., 2013). Despite this complexity, the toxicity of oxalate is well documented (Knauf et al., 2013; Mulay et al., 2013; Khan, 2004; Jonassen et al., 2003). In addition, oxalate was recently identified as a novel factor for chronic kidney disease (CKD) progression (Waikar et al., 2019). Therefore, the increased generation of oxalate by the fatty liver—as recognized in our study—is a plausible explanation, not only for the high risk of kidney stone disease (KSD) (Qin et al., 2018; Nam, 2016; Einollahi et al., 2013) but also for CKD (Musso et al., 2014; Mantovani et al., 2018) in NAFLD patients.

In conclusion, NAFLD leads to epigenomic and transcriptional changes in the liver. Functional analysis of one affected pathway, glyoxylate detoxification, showed that the metabolic network is compensated for under baseline conditions, but the capacity of the steatotic liver to cope with high concentrations of specific precursor metabolites seems to be limited. This finding agrees with a previous study showing that fat accumulation in the liver compromised the metabolic adaptability of patients with NAFLD (Hyötyläinen et al., 2016). Consequently, steatotic hepatocytes cannot efficiently detoxify and thus release higher amounts of the harmful terminal waste product oxalate, which contributes to extrahepatic complications. Therefore, our study not only provides one mechanistic explanation for the higher risk of renal diseases in NAFLD but may be

of clinical relevance to NAFLD patients, who should avoid high consumption of food containing glyoxylate precursors in order to prevent hyperoxaluria.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
 - Lead contact
 - Materials availability
 - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
 - Animals
 - Primary human hepatocytes and human liver tissue from patients with liver resection
 - Pediatric NAFLD cohort
- METHOD DETAILS
 - Sample collection
 - Isolation and cultivation of primary mouse hepatocytes
 - Vector production, virus packaging and transduction of primary hepatocytes
 - Gene transfer in mice
 - RNA sequencing (RNAseq) and identification of differentially expressed genes (DEGs)
 - Reduced Representation Bisulfite Sequencing (RRBS) and identification of differentially methylated regions (DMRs)
 - DNase I sequencing and identification of differential open regions
 - Integrative analysis and visualization of genome-wide data
 - Targeted deep sequencing
 - Analysis of publicly available human liver disease datasets
 - Tissue fixation, histopathology and immunohistochemistry
 - Quantitative real-time PCR
 - Western Blot analysis

Figure 7. Alterations in Glx metabolism genes and urinary oxalate excretion are correlated with the hepatic lipid content in human NAFLD

- (A) Scatterplot illustrating the correlation between the TAG content of hepatocytes and the AGXT mRNA, as well as the % DNA methylation in the AGXT promoter (amplicon 1) and the Spearman correlation coefficients.
- (B) Expression of AGXT in non-steatotic (n = 5) and steatotic (n = 12) hepatocytes dichotomized according to their TAG content.
- (C) Average DNA methylation of AGXT in the three amplicons studied in the same hepatocytes.
- (D) Representative images of H&E stainings and IHC of the pericentral marker glutamine synthetase (GS) and of AGXT in liver tissue from control and steatotic donors. Scale bars: 200 μ m; inset: 20 μ m.
- (E) Glx pathway in human hepatocytes.
- (F) Expression of HAO1, LDHA, GRHPR, PRODH2, and HOGA1 in non-steatotic (n = 5) and steatotic (n = 12) hepatocytes dichotomized according to their TAG content. All bar diagrams represent the mean $\pm$ SD. *p < 0.05 and ***p < 0.001; Mann Whitney test.
- (G) Scatterplots showing correlation of the % steatosis of the liver biopsies and the urinary oxalate excretion of the corresponding donors, expressed as oxalate concentration (μ M), total amount of oxalate per day (μ mol/24 h), ratio of oxalate to creatinine (mmol/mol), and total amount of oxalate per day normalized to body surface area (BSA) (mmol/1.73 m²/day) with Spearman correlation coefficients and corresponding p values.
- (H) Increase of oxalate and Glc in supernatants of human hepatocyte cultures after addition of the precursors Glc, Hyp, and Glx (n = 3) compared to non-exposed hepatocytes.

Values represent the mean $\pm$ SEM. *p < 0.05, **p < 0.01, and ****p < 0.0001 for comparisons in oxalate increment; #p < 0.05 and ##p < 0.01 for comparison in glycolate increment. Student's t test, unpaired, two sided. See also Figure S7 and Tables S2 and S3.

- Quantification of triglycerides
- Quantification of metabolites in biological samples and normalization
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2021.109526>.

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AUTHOR CONTRIBUTIONS

Conceptualization, K. Gianmoena, N.G., A.J., J.W., J.G.H., and C.C.; methodology, K. Gianmoena, A.G., J.R., A.S., and H.H.; validation, K. Gianmoena, N.G., A.J., and G.G.; formal analysis, N.G., G.G., K.N., and C.C.; investigation, K. Gianmoena, N.G., A.J., J.R., G.G., M.M., M.F.-P., P. Gabrys, and K. Grgas; resources, A.G., P. Godoy, P.R., C.A.H., G.D., D.S., T.S.W., T.S.S., E.S., and C.L.; data curation, K.N.; writing – original draft, K. Gianmoena, N.G., A.J., and C.C.; writing – review and editing, N.G., A.J., K.E., P.B., H.-J.A., E.S., C.L., R.M., and J.G.H.; visualization, N.G. and C.C.; supervision, C.C., J.G.H., and J.W.; project administration, J.W. and P.J.; funding acquisition, A.G., P.J., P.R., J.W., and J.G.H.

DECLARATION OF INTERESTS

K.N. is currently hired by AstraZeneca. P. Godoy is currently hired by Hoffman-La Roche, Ltd. A.S. is currently hired by Swiss-BioQuant AG. The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-AGXT antibody produced in rabbit	Sigma-Aldrich	Cat# HPA035370; RRID: AB_10673218
Recombinant Anti-AGXT antibody	Abcam	Cat# ab261910
LDHA antibody (2E2G6)	Proteintech	Cat# 66287-1-Ig; RRID: AB_2881670
Anti-HAO1 antibody produced in rabbit	Sigma Aldrich	Cat# AV42480, RRID: AB_1850490
Anti-GRHPR antibody produced in rabbit	Sigma Aldrich	Cat# HPA022971, RRID: AB_1849975
PRODH2 Polyclonal Antibody	Thermo Fisher Scientific	Cat# PA5-42039, RRID: AB_2576740
Anti-Glutamine Synthetase antibody produced in rabbit	Sigma Aldrich	Cat# G2781, RRID: AB_259853
Rat Anti-Mouse F4/80 ANTIGEN Monoclonal antibody, Unconjugated, Clone Cl:A3-1	Bio-Rad	Cat# MCA497R, RRID: AB_323279
Rabbit Anti-GAPDH Monoclonal Antibody, Unconjugated, Clone 14C10	Cell Signaling Technology	Cat# 2118, RRID: AB_561053
Mouse Anti-beta-Actin Monoclonal Antibody, Unconjugated, Clone AC-74	Sigma-Aldrich	Cat# A5316, RRID:AB_476743
Anti-mouse IgG, HRP-linked Antibody	Cell Signaling Technology	Cat# 7076, RRID:AB_330924
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling Technology	Cat# 7074, RRID:AB_2099233
Bacterial and virus strains		
pAAV[Exp]-CMV > EGFP	VectorBuilder	Cat# VB150925-10026
pAAV[Exp]-CMV > {mAgxt [NM_016702.3]}:IRES:EGFP	VectorBuilder	Cat# VB191228-2726vjg
pAAV[Exp]-TBG > {mAgxt [NM_016702.3]}:IRES:EGFP	VectorBuilder	Cat# VB200115-1168mpa
Biological samples		
Cryo Human hepatocytes, platable (Lot# HUM4108, HUM4229, HUM181761)	Lonza	HUCPI
Human hepatocytes	University of Regensburg, Charité University Medicine, and the Biobank of the Department of General, Visceral and Transplant Surgery in Ludwig-Maximilians-University (LMU) (https://www.htcr.de)	N/A
24-hour urine	Hudert et al., 2019	N/A
Liver biopsies	Hudert et al., 2019	N/A
Chemicals, peptides, and recombinant proteins		
MspI	NEB	Cat# R0106S
Klenow fragment (3' → 5' exo-)	NEB	Cat# M0212M
T4-ligase	NEB	Cat# M0202T
Hot Star Taq polymerase	QIAGEN	Cat# 203203
AMPure XP beads	Beckman Coulter	Cat# A63881
Hot FirePol DNA Polymerase	Solis BioDyne	Cat# 01-02-00500
Glucagon	Biomol	Cat# Cay24204
QIAzol Lysis-Reagent	QIAGEN	Cat# 79306
Collagen	Roche Diagnostics	Cat# 11171179001
William's Medium E	PAN Biotech	Cat# P04-29510
Gentamycin	PAN Biotech	Cat# P06-03001

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Stable L- Glutamine (2 mM)	PAN Biotech	Cat# P04-82100
Penicillin/Streptomycin (100 U/ml Pen; 100 U/ml Strep)	PAN Biotech	Cat# P06-07100
Insulin (ITS)	Sigma-Aldrich	Cat# I3146
Dexamethasone	Sigma-Aldrich	Cat# D4902-25MG
L-hydroxyproline	Applchem	Cat# A3742,0100
Sodium glycolate	Thermo Fisher Scientific	Cat# 351570250
Sodium glyoxylate monohydrate	Sigma-Aldrich	Cat# G4502
THFA (Prodh2 inhibitor)	Sigma-Aldrich	Cat# 341517
¹³ C ₂ oxalic acid	Cambridge Isotope Laboratories, Inc.	Cat# CLM-4449
¹³ C ₂ glycolic acid	Cambridge Isotope Laboratories, Inc.	Cat# CLM-2193
Picric acid	Merck	Cat# 623
Normal chow R/M-H 10-mm pellet	Ssniff, Soest, Germany	Cat#V1534
R/M-H 10-mm pellet with 1% hydroxyprolin	Ssniff, Soest, Germany	N/A
Western-type diet	Research Diet Inc	Cat#D09100301

Critical commercial assays

Ribo-Zero Gold Human/Mouse/Rat rRNA Removal Kit	Illumina	Cat# MRZG126
TruSeq Stranded totalRNA Sample Prep Kit	Illumina	Cat# 20020596
EZ-DNA methylation Gold kit	Zymo research	Cat# D5005
TruSeq ChIP Library Preparation kit	Illumina	Cat# IP-202-1012
Qubit HS-Kit	Thermo Fisher Scientific	Cat# Q32851
VECTASTAIN Elite ABC-Peroxidase Kit	Vector Laboratories	Cat# PK-6100, RRID:AB_2336819
Picrosirius red kit	Polyscience Europe GmbH	Cat#24901
Chromo-Map-DAB Kit	Roche	Cat# 05266645001
UltraMap anti-Rb HRP Kit	Roche	Cat# 05269717001
cDNA reverse transcription kit	Applied Biosystems	Cat# 4368813
BCA Protein Assay kit	Thermo Scientific	Cat# 23225
Triglyceride quantification kit	abcam	Cat# ab65336

Deposited data

Transcription profiling of primary mouse hepatocytes under different culture conditions	Godoy et al., 2016	E-MTAB-4444
Transcription profiling by array of H. sapiens liver cells to investigate global gene expression associated with the progression of NAFLD	Lake et al., 2011	E-MEXP-3291
Expression data for Nonalcoholic fatty liver disease patients	Moylan et al., 2014	GEO: GSE49541
Study of gene expression of human liver Hepatocellular carcinoma	Tung et al., 2011	GEO: GSE25097
Normalized RNA sequencing (RNaseq) data (analyzed on the Illumina HiSeq platform) were comparing hepatocellular carcinoma (HCC) (n = 163) to matched non-tumor liver tissue (n = 49)	TCGA	https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga
RNaseq, RRBS, ChIP-seq data	This manuscript	http://www.ebi.ac.uk/ena/browser/view/PRJEB32775

Experimental models: Organisms/Strains

Mouse: B6.V-Lep ob/ob JRj	Janvier Labs	N/A
Mouse: B6.V-Lep ob/+ JRj	Janvier Labs	N/A
Mouse: C57BL/6NRj	Janvier Labs	N/A

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Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse: C57BL/6Jrj	Janvier Labs	N/A
Mouse: Agxt–/–	Salido et al., 2006	N/A
Oligonucleotides		
Primers for targeted deep sequencing of mouse Agxt (1), Agxt (2), see Table S4	This paper	N/A
Primers for targeted deep sequencing of human AGXT upstream, AGXT (1), AGXT (2), see Table S4	This paper	N/A
Universal primer tags (fusion primer), see Table S4	This paper	N/A
Primer for targeted deep sequencing, 2 nd PCR, see Table S4	This paper	N/A
Taqman Assays, see Table S5	Thermo Fisher Scientific	N/A
Software and algorithms		
TrimGalore!	Babraham Institute, Simon Andrews	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/ ; RRID:SCR_011847
Gencode (version M2)	Harrow et al., 2012	https://www.gencodegenes.org/mouse/release_M2.html
grape-nf	CRG Barcelona, Roderic Guigó Serra, International Human Epigenome Consortium	https://github.com/guigolab/grape-nf/
DESeq2 (version 1.18.1)	Love et al., 2014	https://bioconductor.org/packages/release/bioc/html/DESeq2.html ; RRID:SCR_015687
Methylkit (1.3.8)	Akalin et al., 2012	https://bioconductor.org/packages/release/bioc/html/methylKit.html ; RRID:SCR_005177
BEDTools (v2.20.1)	Quinlan and Hall, 2010	https://github.com/arq5x/bedtools2 ; RRID:SCR_006646
DEEP pipelines GALv1 and DHSv3	Ebert et al., 2015	https://github.com/molgen.mpg.de/DEEP/comp-metadata
edgeR (version 3.8.6)	Robinson et al., 2010	https://bioconductor.org/packages/release/bioc/html/edgeR.html ; RRID:SCR_012802
deepTools	Ramírez et al., 2016	https://deeptools.readthedocs.io/en/develop/ ; RRID:SCR_016366
STRING (Version 10.5)	Snel et al., 2000	https://string-db.org/ ; RRID:SCR_005223
Integrative Genomics Viewer (IGV) browser	Robinson et al., 2011	https://www.broadinstitute.org/igv/ ; RRID:SCR_011793
BiQ Analyzer HT	Lutsik et al., 2011	https://biq-analyzer-ht.bioinf.mpi-inf.mpg.de/ ; RRID:SCR_008045

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Cristina Cadenas (Cadenas@ifado.de).

Materials availability

This study did not generate new unique reagents

Data and code availability

- The transcriptomic (RNaseq) and epigenomic (RRBS and DNase-seq) data generated during this study are deposited in the European Nucleotide Archive (ENA) at the EMBL-EBI under accession number PRJEB32775 (<http://www.ebi.ac.uk/ena/browser/view/PRJEB32775>) and are publicly available as of the date of publication.. The ENA accession numbers for each

individual sample are listed in [Data S2](#). Original western blot images and microscopy data reported in this paper will be shared by the lead contact upon request.

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Male ob/ob (Lepob/Lepob) and ob/+ (Leprob/+) as well as male C57Bl6N mice were purchased at the age of 8 weeks from Janvier labs. Female mice were excluded from the study to avoid sex-related differences reported in NAFLD mouse models ([Ganz et al., 2014](#)). All mice received food and water *ad libitum* and were kept under a 12 h light and 12 h dark cycle at a controlled ambient temperature of 25°C.

For dietary studies, 9 weeks old ob/ob and ob/+ mice were randomly separated into normal chow (R/M-H 10-mm pellet, Ssniff, Soest, Germany) or hydroxyproline-containing diet (1% hydroxyproline-containing R/M-H10-mm pellet, Ssniff) groups and fed for 10 days. For Western diet experiments, 9 weeks old C57Bl6N mice were randomly separated into normal chow or Western diet-fed groups. Mice were fed normal chow (Ssniff R/M-H 10-mm pellet standard diet, Ssniff) or a Western-type diet (Research Diet Inc., catalog number D09100301, containing 45% kcal fat, 20% kcal fructose and 2% weight cholesterol) for 6 weeks (short-term) or 30 weeks (long-term). Three days before the end of the indicated feeding schedules mice were transferred to single mouse metabolic cages (Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany) for 24-hour urine collections where they had access to water and to their corresponding diet. After 1-2 days acclimatization in the normal cages, mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg bw) and Rompun®/xylazin (10 mg/kg bw) for blood collection. Subsequently, mice were sacrificed by cervical dislocation for tissue collection.

A group of ob/+ and ob/ob mice (on a normal chow diet) received intraperitoneal injections of glucagon (3 mg/kg bw) or solvent (PBS). 3 h after injection mice were sacrificed and liver tissue was collected and snap frozen in liquid nitrogen for gene expression studies.

For the adeno-associated virus (AAV)-mediated delivery of Agxt, male ob/ob mice at the age of 9 weeks were used. A group ob/ob mice (on a normal chow diet) was randomly separated into two groups to either receive Agxt-AAV or EGFP-AAV (control). An additional group of age-matched male ob/+ mice was not injected and served as control. After two weeks, each group was again randomly separated into two. One received 1% hydroxyproline (1% Hyp) in the diet for a further two weeks while the other group continued receiving normal chow (NCD). For the collection of the 24-hour urine at the indicated time points, mice were transferred to metabolic cages as described above. At the end of the experiment mice were sacrificed for blood and tissue collection.

Additional male ob/ob and ob/+ mice as well as normal chow diet (NCD)-fed and Western diet (WD)-fed mice were subjected to liver perfusion to isolate hepatocytes for *in vitro* experiments at the age of 9-12 weeks.

Generation of Agxt deficient mice is described in detail elsewhere ([Salido et al., 2006](#)). Homozygous Agxt^{-/-} mice were kindly provided by E. Salido and C. Kurts. Age-matched C57Bl6J mice were used as wild-type controls. Confirmation of the genotype was carried out by PCR on genomic DNA extracted from ear punches, as described ([Salido et al., 2006](#)). Three Agxt^{-/-} male mice and three wild-type mice were subjected to liver perfusion at the age of 9-12 weeks to obtain primary hepatocytes for *in vitro* experiments. Additional male mice were placed in metabolic cages for three consecutive 24-hour urine collections and anesthetized for blood collection.

Animal handling was conducted according to the Principles of Laboratory Care and considering the recommendations of the Society of Laboratory Animal Science (Gesellschaft für Versuchstierkunde, GV-SOLAS, Germany) and all experiments were approved by the animal protection authorities of North Rhine-Westphalia (LANUV NRW, 84-02.04.2016.A149).

Primary human hepatocytes and human liver tissue from patients with liver resection

Primary human hepatocytes (PHH) were isolated from liver tissue samples obtained from healthy liver tissue that was resected from patients suffering primary or secondary liver tumors.

Informed consent of the patients for the use of tissue for research purposes was obtained according to the ethical guidelines of University of Regensburg, the Charité University Medicine, the University of Leipzig and the Biobank of the Department of General, Visceral and Transplant Surgery in Ludwig-Maximilians-University (LMU). The Biobank operates under the administration of the Human Tissue and Cell Research (HTCR) Foundation. The framework of HTCR Foundation ([Thasler et al., 2003](#)), which includes obtaining written informed consent from all donors, has been approved by the ethics commission of the Faculty of Medicine at the LMU (approval number 025-12) as well as the Bavarian State Medical Association (approval number 11142) in Germany.

Isolation of PHHs was performed using a two-step collagenase perfusion technique as described ([Godoy et al., 2013](#)) or with minor modifications ([Lee et al., 2013](#)). PHHs were processed for RNA isolation and subsequent qPCR, DNA methylation analysis and quantification of triglycerides. Remaining tissue was evaluated by a pathologist to determine the severity of liver disease. Stratification of liver tissue samples stained with hematoxylin and eosin was done according to histopathologic evaluation performed by a pathologist and the cut-off for steatosis was defined according to [Kleiner et al. \(2005\)](#) (> 5% steatotic hepatocytes). Quantification of the

triglyceride content of the corresponding isolated PHH was performed and compared to histopathology. Classification of the isolated primary human hepatocytes into control and steatotic cells was based on triglyceride quantification. The cut-off selected was $< 5,000$ RFU/mg protein. This value is in agreement with lack of visible lipid droplets in the corresponding tissue and it matches the mean value obtained in a group of control primary mouse hepatocytes of lean mice fed a normal chow.

Cryo-preserved replatable human hepatocytes (HUM4229, HUM4108, HUM181761, Lonza) were used for *in vitro* experiments with glyoxylate precursors.

Pediatric NAFLD cohort

24-hour urine samples and liver biopsies were available from overweight or obese children and adolescents, aged 10 to 17 years, with biopsy-proven NAFLD. Patients were recruited from the pediatric obesity outpatient clinic and pediatric gastroenterology outpatient clinic of Charité between June 2014 and October 2017 as previously described (Hudert et al., 2019). The study conformed to the guidelines of the Declaration of Helsinki and was approved by the local institutional review board at Charité University Medicine Berlin. Informed consent was obtained from all parents or guardians.

The liver biopsies were evaluated and scored by experienced pathologists, using the histological scoring system for non-alcoholic fatty liver disease (NAFLD) by the NASH Clinical Research Network (NASH CRN). This included the scoring of steatosis (0 = $< 5\%$ of hepatocytes; 1 = 5%–33%; 2 = 34%–66%; 3 = $\geq 67\%$), lobular inflammation (0 = 0 foci/200x field; 1 = < 2 foci/200x field; 2 = 2–4 foci/200x field; 3 = > 4 foci/200x field), and hepatocellular ballooning (0 = none; 1 = few; 2 = many/prominent). To obtain the NAFLD activity score (NAS), which ranges from 0 to 8, the scores obtained for each of the three features were totaled. Portal inflammation was scored as 0 = none; 1 = mild; or 2 = more severe than mild. Fibrosis was classified in four stages: 0 = no fibrosis; 1 = portal/periportal or zone 3; 2 = portal/periportal and zone 3; 3 = bridging fibrosis; 4 = cirrhosis.

24-hour urine samples (acidified) were obtained from 31 participants. One sample was excluded due to likely under-collection (generally defined as 24-hour urine volume < 500 mL and creatinine excretion < 4 mmol/24 h), thus yielding $N = 30$. As recommended for the interpretation of urine oxalate results in children and adolescents (Clifford-Mobley et al., 2015), we considered the age-related reference ranges (normal value of oxalate excretion in 24-hour urine sample: < 0.50 mmol/1.73 m²/day for all ages; normal values of oxalate/creatinine molar ratio: < 70 –82 mmol/mol for 5–14 years and < 40 mmol/mol for > 16 years) (Hoppe, 2012) as well as body surface area information. Body surface area (BSA) was calculated as $BSA = \text{SQR} [Bw \text{ (kg)} \times Ht \text{ (cm)} / 3600]$.

METHOD DETAILS

Sample collection

Collection of 24-hour urine for quantification of oxalate was done in metabolic cages. The urine collection tubes contained 35 μ L 6 N HCl for acidification of the collected urine. The water and food intake were recorded daily by weighting the water bottle and the feeding drawer and carefully inspecting the cage for water or food spilling. Once the urine volumes were determined, the acidified urine samples were centrifuged at 1,000 rpm for 10 min and the supernatant was stored in aliquots at -80°C until further analysis.

Blood was collected in syringes previously loaded with EDTA from three anatomical positions of the mouse: the portal vein (representing the liver inflow), the hepatic vein (representing the liver outflow) and the mixed heart blood (representing the systemic circulation), as previously described (Ghallab et al., 2019). The blood samples were centrifuged at 13,000 rpm for 10 min and the separated plasma was stored at -80°C until further analysis.

Liver tissue samples were collected from defined anatomical positions as previously described (Ghallab et al., 2016): For histological stainings and immunohistochemistry, the left liver lobe was fixed in 4% PFA for 48 h, washed in PBS and paraffin-embedded. For RNA and protein expression, DNA methylation and quantification of triglyceride content, tissue specimens were collected from the remaining lobes, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

Isolation and cultivation of primary mouse hepatocytes

The isolation of primary hepatocytes from mouse liver was based on the two-step collagenase perfusion technique, as described in Godoy et al. (2013). Mice were weighed and anesthetized with an intraperitoneal injection of Rompun® (10 mg/kg bw) and ketamine (100 mg/kg bw). After perfusion, the obtained cell suspension was passed through a 100 μ m sieve to remove undigested tissue and centrifuged (5 min at 50 $\times$ g at 4°C). Subsequently, the hepatocyte pellet was further purified by two additional steps of washing and re-centrifugation. The viability ($> 95\%$) was determined by trypan blue dye exclusion and the purity of the hepatocyte enriched fraction was examined under the microscope. If necessary, an additional centrifugation step was performed.

For genomic DNA isolation (for DNA methylation analyses), aliquots of 5×10^6 hepatocytes were pelleted, snap-frozen in liquid nitrogen and stored at -80°C until further processing. For isolation of RNA (for RNAseq analysis), aliquots of 5×10^6 hepatocytes were homogenized in 1 mL TRIzol, snap frozen and stored at -80°C until further processing. Furthermore, aliquots of 1×10^7 hepatocytes were used for nuclei isolation and subsequent DNase I digestion for DNase I-seq analysis.

For *in vitro* experiments, primary mouse hepatocytes were cultured under sterile conditions at 37°C and 5% CO₂ within aqueous saturated vapor in collagen-coated 6-well plates with a layer of collagen on top (referred to as “sandwich on top,” SOT), as previously described (Godoy et al., 2013) at a cell density of 0.85×10^6 cells/well in William’s E medium (PAN Biotech) supplemented with dexamethasone (100 nM), insulin (2 ng/mL) (both from Sigma Aldrich), stable L-glutamine (2 mM) penicillin/streptomycin

(100 U/ml), gentamycin (10 µg/ml) (all from PAN Biotech). For treatments with glucagon (10 nM, Sigma Aldrich) insulin-free medium was used. Incubations with L-hydroxyproline (AppliChem), glycolate (as sodium glycolate, Thermo Fisher Scientific) and glyoxylate (as sodium glyoxylate monohydrate, Sigma Aldrich) were performed at the indicated concentrations for 48 h. Where indicated, the Prodh2 inhibitor THFA (10 mM) was given 1 h prior hydroxyproline exposure. After 48 h cell supernatants were collected for quantification of metabolites and cell lysates were prepared for normalization of metabolites to protein content and analysis of gene expression.

Gene expression data from *in vitro* cultivated primary mouse hepatocytes were obtained from ArrayExpress, (<http://www.ebi.ac.uk/arrayexpress>) under the accession number E-MTAB-4444 (Godoy et al., 2016).

Vector production, virus packaging and transduction of primary hepatocytes

The vectors pAAV-CMV-EGFP (control vector ID: VB150925-10026; complete name: pAAV[Exp]-CMV > EGFP) and pAAV-CMV-mAgxt (vector ID: VB191228-2726vjg, complete name: pAAV[Exp]-CMV > {mAgxt[NM_016702.3]}:IRES:EGFP) were produced and packaged into adeno-associated virus DJ (AAV-DJ) by Vector Builder for expression under the control of the ubiquitous cytomegalovirus (CMV) promoter. Transduction of PMHs with viral particles was performed using a multiplicity of infection of 5×10^5 gene copies/cell according to the manufacturer's instructions. Hepatocytes were plated on collagen-coated 6-well plates with a collagen layer on top (SOT) as described above. The following day, transduction took place, and after 24 h hepatocyte culture medium containing different concentrations of hydroxyproline was added, and cells were cultivated for further 72 h. In this set of experiments, hepatocytes were cultivated for a longer period of time to ensure protein translation of the delivered *Agxt* gene. Cells and supernatants were collected as described above.

Gene transfer in mice

For delivery of *Agxt* in mice, the vector pAAV-TBG-mAgxt (vector ID: VB200115-1168mpa, complete name: pAAV[Exp]-TBG > {mAgxt[NM_016702.3]}:IRES:EGFP), designed for the specific expression of *Agxt* in the liver under the control of the hepatic thyroxine-binding globulin (TBG) promoter, was produced and packaged into adeno-associated virus DJ (AAV-DJ) by Vector Builder. Transduction of the mouse liver was performed by i.v. injection of 4.5×10^{12} gene copies per mouse in PBS with a 27Gauge needle.

RNA sequencing (RNaseq) and identification of differentially expressed genes (DEGs)

RNA was isolated using the TRIzol method. Briefly, 1 mL TRIzol was added to 5×10^6 cells followed by vortexing, a 5-min incubation at room temperature, and addition of 200 µL chloroform. After mixing, further incubation at room temperature for 2–3 min and centrifugation (12,000 g) at 4°C for 5 min, the clear supernatant was mixed with 500 µL isopropanol and incubated at room temperature for 10 min. After centrifugation (12,000 g) at 4°C for 10 min, the supernatant was discarded and the pellet washed with 1 mL cold 75% ethanol followed by vortexing and centrifugation (7,500 g, 4°C, 5 min). The pellet was dried and dissolved in RNase-free water. For preparation of the libraries, rRNA was removed with the Ribo-Zero Human/Mouse/Rat rRNA Removal Kit from Biozym Scientific GmbH. The RNA library was prepared using the TruSeq Stranded totalRNA Sample Prep Kit from Illumina (San Diego, CA) according to the manufacturer's instructions. All samples were sequenced using an Illumina HiSeq 2500 sequencer v4 (Illumina, San Diego, CA) with 10 transcriptome samples per lane and an average of 25 million paired-end reads (2×125 bp) at IKMB NGS core facilities.

Reads were trimmed for adaptor and low-quality tails ($Q < 20$) with TrimGalore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and subsequently aligned to the mm10 reference genome with gene models from Gencode (version M2) (Harrow et al., 2012) by the IHEC supported pipeline grape-nf (<https://github.com/guigolab/grape-nf>) -grape-nf wraps STAR (version 2.4.0j) (Dobin et al., 2013) and RSEM (version 1.2.21) (Li and Dewey, 2011).

DESeq2 (Version 1.18.1) (Love et al., 2014) was used to detect differentially expressed genes (DEGs) with minimal absolute FC of 1.5 and maximal adjusted P value of 0.001. Genes for which less than four samples had an FPM (fragments per million mapped reads) above 0.1 were discarded.

Reduced Representation Bisulfite Sequencing (RRBS) and identification of differentially methylated regions (DMRs)

RRBS was performed as previously described (Luetke-Eversloh et al., 2014), with small modifications: Briefly, 500 ng of genomic DNA were digested overnight at 37°C using 50–100 U of MspI enzyme (NEB, Ipswich, USA) followed by end repair and A-tailing reaction with Klenow fragment ($3' \rightarrow 5'$ exo-) (NEB). After ligation of sample-specific sequencing adaptors using T4-ligase (NEB), bisulfite conversion with the EZ-DNA methylation Gold kit (Zymo research, Irvine, USA) and 12–18 cycles of PCR amplification using Hot Star Taq polymerase (QIAGEN, Hilden, Germany) were performed. Libraries were then purified with Agencourt AMPure XP beads (Beckman Coulter, Brea, USA), went through quality checks (qPCR and Agilent Bioanalyzer) and were sequenced on a Illumina HiSeq2500 (TruSeq SBS Kit v3 – HS chemistry) in a single read run with 100 bp read length. Trimming, alignment to the mm10 reference genome and DNA methylation value calling were performed as previously described (Brosch et al., 2018).

Differentially methylated regions (DMRs) were detected as described before (Brosch et al., 2018) using MethylKit (1.3.8) (Akalin et al., 2012) with minor modifications. DMRs were defined as 750 bp tiles (step size: 650bp) with at least 5 CpGs, a maximum q-value of 0.001 and minimal methylation difference of 5%. Mitochondria, X and Y chromosomes were excluded. The resulting DMRs were then annotated to Gencode gene models (version M2) (Harrow et al., 2012) with the help of BEDTools (v2.20.1) (Quinlan and Hall, 2010).

DNase I sequencing and identification of differential open regions

DNase I sequencing (DNase-seq) was performed as previously described (Schmidt et al., 2017). Briefly, nuclei were isolated from 1 × 10⁷ cells by using buffer A (60 mM KCl, 15 mM Tris-HCl (pH 8.0), 15 mM NaCl, 1 mM EDTA (pH 8.0), 0.5 mM EGTA (pH 8.0), 0.5 mM spermidine free base) supplemented with IGEPAL (0.1% final concentration) and incubation on ice for 15 min. Nuclei were treated with DNase I (0, 40, 50, 60, 80U/ml) for 3 min at 37°C and the reaction was stopped at 55°C for 1 h with stop buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.1% SDS, 100 mM EDTA (pH 8.0), 1 mM spermidine and 0.3 mM spermine) supplemented with proteinase K (50 µg/ml). DNA was then purified using phenol chloroform extraction and double-hit fragments of 100–500 bp were selected by sequential purifications with Agencourt AMPure XP Beads (Beckman Coulter, Brea, USA). Sequencing libraries were prepared from 6.5–8 ng of purified DNA (from 80 U/ml-digest) using the TruSeq ChIP Library Preparation kit (Illumina, San Diego, USA) according to the manufacturer's protocol and sequenced on an Illumina HiSeq2500 (v3 paired-end flow cell).

Raw reads were processed with the DEEP pipelines GALv1 and DHSv3 (Ebert et al., 2015) (<https://github.molgen.mpg.de/DEEP/comp-metadata>). Differential open regions (DORs) were detected by contrasting the number of 5' read ends that mapped to the union of MACS2 peaks (as generated with bedtools), for each sample. The comparison was done between control and steatotic samples, with edgeR (version 3.8.6) (Robinson et al., 2010), DORs were filtered for a minimal absolute FC of 1.3 and maximal adjusted p value of 0.05. For Figure 1C, DORs and flanking ± 1kb regions were ordered by mean signal and plotted as a heatmap using deepTools (Ramírez et al., 2016).

Integrative analysis and visualization of genome-wide data

For the integration of expression, methylation and open chromatin, DMR and DORs, or DMR-DOR pairs with at least 1bp overlap of the corresponding DMR and DOR, assigned to the closest gene, were overlapped with DEGs based on Ensembl ID. For visualization of DEGs associated with DMRs and DORs, z-scores were calculated for each dataset (Figure 1D).

Gene set enrichment analysis of DEGs with associated DMRs and DORs was performed using STRING (Version 10.5) (Snel et al., 2000).

Genomic tracks of expression, methylation and chromatin accessibility data (bigwig) were visualized in the Integrative Genomics Viewer (IGV) browser (replicates overlaid) (Robinson et al., 2011).

Targeted deep sequencing

DMRs were validated with targeted deep sequencing. 300 ng of genomic DNA were bisulfite treated using EZ-DNA Methylation Gold kit (Zymo Research). Bisulfite PCRs were carried out in 30 µl mixes, including 3 µl of 10x HotFirePol Buffer BD (Solis BioDyne, Tartu, Estonia), 2.4 µl of 10 mM dNTPs (Fisher Scientific, Pittsburgh, USA), 3 µl of 25 mM MgCl₂ (Solis BioDyne, Tartu, Estonia), 0.5 µl of amplicon specific forward and reverse primer (10 µM each), 0.5 µl of Hot FirePol DNA Polymerase (5 U/µl; Solis BioDyne), 2 µl of bisulfite converted DNA and 18.1 µl of double distilled water, with the following conditions: 15 minutes denaturation at 95°C, followed by 42 cycles of 1 min at 95°C, 2 min at 54–58°C and 1 min at 72°C, and a final 5-minute extension step at 72°C. Amplicons were generated using fusion primers consisting of a 3' sequence-specific part and a universal 5'-part containing the nucleotide sequences for Illumina sequencing. Primer sequences and conditions are listed in Table S4.

PCR products were visualized on 1.2% agarose gels and purified using Ampure XP beads (Beckman Coulter) according to manufacturer's recommendations. Products were then measured using the Qubit Fluorometer (Qubit HS-Kit, Thermo Fisher Scientific, Carlsbad, USA) and diluted to 4 nM. After finalizing the NGS tags by a second PCR (5 cycles) and a final clean-up step (AMPure XP Beads), samples were diluted to 10 nM, pooled and sequenced on an Illumina MiSeq (Illumina v3 chemistry, 2 × 300 bp paired end).

The raw data were quality checked using FastQC, trimmed for adaptors or low-quality bases with cutadapt and Trim Galore! ("Babraham Bioinformatics - Trim Galore!" 2017) (Martin, 2011) and paired reads were joined using the FLASH tool. Reads were then sorted according to NGS barcode adaptor and initial 15bp sequence to assign Sample ID and amplicon ID respectively. Finally, reads were evaluated with BiQ Analyzer HT (Lutsik et al., 2011) and R including alignment, filtering out low quality reads ('minimal sequence identity' of 0.9, 'minimal conversion rate' of 0.95, 'maximal fraction of missing sites' of 0.3) and analysis of methylation levels and patterns.

Analysis of publicly available human liver disease datasets

For investigation of gene expression changes in human liver disease microarray datasets from the public data repositories ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>, accession number E-MEXP-3291), Gene Expression Omnibus (GEO: GSE49541, GEO: GSE25097) and The Cancer Genome Atlas (TCGA) (<https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) were retrieved.

E-MEXP-3291 (Lake et al., 2011) was analyzed on Affymetrix GeneChip Human Gene 1.0 ST arrays and was used to compare non-alcoholic steatohepatitis (NASH) (n = 16) to healthy liver tissue (n = 19). GSE49541 (Moylan et al., 2014) was analyzed on Affymetrix GeneChip Human Genome U133 Plus 2.0 arrays and used to compare severe NAFLD (NASH fibrosis stage 3–4, n = 32) with mild NAFLD (fibrosis stage 0–1, n = 40). GSE25097 (Tung et al., 2011) was analyzed on Human RSTA Affymetrix 1.0 Custom CDF microarrays and was used to compare cirrhotic liver (n = 40) to non-tumor liver tissue (n = 243). In addition, normalized RNA sequencing (RNAseq) data (analyzed on the Illumina HiSeq platform) were retrieved from TCGA comparing hepatocellular carcinoma (HCC) (n = 163) to matched non-tumor liver tissue (n = 49).

Microarray gene expression data were processed and quantile normalized using the Piano R package (Våremo et al., 2013), as previously described (Grinberg et al., 2014). Differential expression analysis was also carried out using the Piano package, and p values were corrected for multiple testing by the method of Benjamini and Hochberg (Benjamini and Hochberg, 1995). For differential expression analysis of RNaseq data, the R package DESeq (Anders and Huber, 2010) was used. Genes that showed a |fold change (FC)| of at least 1.3 and a false discovery rate (FDR) adjusted p value ≤ 0.05 in the pairwise comparison of healthy liver versus NASH (E-MEXP-3291), mild NAFLD versus severe NAFLD (GSE49541), and non-tumor tissue to diseased/tumor tissue (GSE25097 and TCGA) were considered differentially expressed. To enable a direct comparison of differentially expressed genes analyzed on different platforms, the probe sets included on the Affymetrix chips (annotated to Ensembl Gene IDs) and the genes included in the TCGA dataset (recognized by Entrez Gene IDs) were annotated to their official gene symbols, as provided by HUGO gene nomenclature Committee (HGNC). Comparison to differentially expressed genes in the mouse models used in this study was also based on official gene symbols.

Tissue fixation, histopathology and immunohistochemistry

4 μ m-thick formalin-fixed paraffin-embedded liver tissue sections were used for tissue staining and immunohistochemistry. Hematoxylin and eosin (H&E) staining was performed with a standard protocol, as previously described (Ghallab et al., 2016). Liver fibrosis was visualized with picrosirius red using a commercially available kit (Polyscience Europe GmbH, Eppelheim, Germany) according to the manufacturer's instructions.

For immunohistochemistry of GS and F4/80, the paraffin sections were deparaffinised in Roti®-Histol three times à 10 min and rehydrated through a decreasing ethanol gradient. For antigen retrieval, samples were immersed in 10 mM citrate buffer, pH 6, and heated in a microwave twice for 7 min. Samples were treated with 3% H₂O₂ in PBS to block endogenous peroxidase and blocked with 3% BSA/3% Tween 80 in PBS in a humidified chamber for 60 min at room temperature to mask unspecific binding sites. Primary antibodies were diluted appropriately (anti-GS: Cat# G2781, Sigma Aldrich, diluted 1:2000; anti-F4/80: Cat#MCA497R, BioRad, diluted 1:50) in 0.3% BSA/3% Tween 80 and the tissue samples were incubated over night at 4°C in a humidified chamber. After extensive washing, incubation with horseradish peroxidase-conjugated secondary antibodies (VECTASTAIN Elite ABC kit, Vector Laboratories, UK) was performed for 30 min in a humid chamber at room temperature. Antibody binding was visualized by staining with 3,3'-diaminobenzidine solution (Vector Laboratories) for 2-5 minutes, and counterstained with Mayer's hematoxylin to visualize nuclei. Finally, the stained sections were dehydrated, by passing in an ascending ethanol series, and preserved by mounting in entellan. For imaging, the optical microscope (Olympus BX41) was used.

Immunohistochemistry of mouse Agxt (with ab261910, Abcam, diluted 1:250-1:1000) and human AGXT (with HPA035370, Sigma-Aldrich, diluted 1:2500) was performed in an autostainer (VENTANA DISCOVERY ULTRA, Roche). For antigen retrieval, samples were treated in EDTA buffer for 24 min at 96°C. The Chromo-Map-DAB Kit and the Ultra-Map-HRP Kit (Roche) were used for detection according to the manufacturers instructions.

Quantitative real-time PCR

Total RNA was extracted from liver tissue, as well as from cultivated and freshly isolated hepatocytes using Qiazol (QIAGEN, Hilden, Germany) according to the manufacture's protocol. RNA (1-2 μ g) was transcribed to cDNA with the high capacity cDNA reverse transcription kit (Applied Biosystems). Real-time quantitative PCR was performed with the Applied Biosystems ABI 7500 Fast Real-Time PCR System with the Taqman technique. Eif2 or Gapdh were used as housekeeping genes in mouse samples and GAPDH or UBC were used as housekeeping genes in human samples. Control, non-steatotic liver tissue or hepatocytes were taken as a reference. The relative gene expression was calculated following the 2- $\Delta\Delta$ Ct method (Livak and Schmittgen, 2001). A list of Taqman assays is provided in Table S5.

Western Blot analysis

Whole cell lysates were collected in RIPA lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (v/v), Nonidet P-40 substitute 0.5% (w/v) sodium deoxycholate, 0.5% (w/v) SDS) containing protease and phosphatase inhibitors (Sigma). For protein quantification the BCA Protein Assay kit (Thermo Scientific) was used. Equal amounts of protein from the samples of interest were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis SDS-PAGE and transferred to a polyvinylidene difluoride PVDF membrane (Perkin Elmer). Membranes were incubated over night at 4°C with the primary antibodies, diluted as follows: Agxt: 1:1000; Hao1: 1:1000; Grhpr: 1:1000; Prodh2: 1:1000; Ldha: 1:13000; Gapdh: 1:5000; β -Actin: 1:5000. After removal of the primary antibody, membranes were incubated with secondary antibodies conjugated to horse radish peroxidase (anti-mouse and anti-rabbit IgG HRP-linked antibody from Cell Signaling) for 30-60 min at room temperature. Protein bands were visualized using chemiluminescence on a Vilber Fusion Fx7 imager (Vilber Lourmat). Densitometric quantification was performed with the ImageJ Software Version 1.52a.

Quantification of triglycerides

Triglyceride levels were quantified using a commercially available kit (ab65336, Abcam) according to manufacturer's instructions. This assay is based on the conversion of triglycerides as well as mono- and diglycerides to fatty acids and free glycerol. Glycerol is further oxidized and reacts with a probe to generate a fluorescent product (Ex/Em = 535/587 nm). The relative fluorescence signal

was measured using a Tecan Infinite 200PRO plate reader (Tecan Group AG) and was normalized to the protein amount of the extract quantified the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions.

Quantification of metabolites in biological samples and normalization

The oxalate and glycolate concentrations in urine samples (acidified), in plasma or in cell supernatants (in serum-free medium) were quantified by LC-MS/MS as previously described (Schriewer et al., 2017) using a QTrap5500 mass spectrometer (AB Sciex) coupled to a Shimadzu UFLC system. Minor modifications from the described method included the injection of a lower sample volume (2 μ L), the use of formic acid instead of trifluoroacetic acid as the mobile phase additive to improve ionization efficiency, and the use of the 61.0 and 62.0 product ions as quantifiers for oxalic acid and $^{13}\text{C}_2$ oxalic acid respectively, as well as the use of the 47.0 and 48.0 ion products as quantifiers for glycolic acid and $^{13}\text{C}_2$ glycolic acid. Urine samples were diluted appropriately; cell culture supernatants remained undiluted. Plasma samples were first subjected to precipitation to remove plasma protein. The analytes were quantified using uniformly ^{13}C -labeled internal standards (Cambridge Isotope Laboratories, Inc.) with the support of the Analyst Software Version 1.6.2 and the MultiQuantTM Software Version 2.1.1 (AB SCIEX).

Urinary creatinine was determined using the reaction with picric acid according to Jaffe (1886). Briefly, urine was diluted in distilled water and mixed with basic picric acid solution. After 45 min at room temperature in the dark, the absorbance at 492 nm was measured in a microplate reader (Tecan Infinite 200PRO plate reader) and a calibration curve was used for quantification.

The amount of oxalate and glycolate in cell supernatants was normalized to the protein content of the corresponding cell lysate to yield nmol oxalate or glycolate per mg protein. The amount of oxalate in urine samples was normalized to creatinine to yield μg oxalate per mg creatinine or mmol oxalate per mol creatinine. In urine from human participants, body surface area was considered and excreted oxalate was calculated as mmol oxalate per 1.73 m^2 per 24-hour. In the 1% hydroxyproline feeding experiments in ob/+ and ob/ob mice, the total amount of urinary oxalate was additionally normalized to the total amount of ingested food (μmol oxalate per g ingested food) to correct for the hyperphagic behavior of ob/ob mice.

In experiments involving inhibitors or viral transduction, the metabolite quantification results were presented as fold change (i.e., increase in oxalate/glycolate after addition of different concentrations of substrate compared to non-exposed (0 mM)). This was also the case when the absolute values of metabolite in steady state (0mM) differed among the donors, or between the experiments.

QUANTIFICATION AND STATISTICAL ANALYSIS

In the animal studies, comparisons between groups were performed using parametric methods. N values represent the number of animals. P values were calculated with an unpaired, two-sided t test. A p value of < 0.05 was considered statistically significant. All data are shown as mean $\pm$ SD, except for plasma/urine where data are shown as mean $\pm$ SEM.

To study correlations among the obese pediatric patients and in the patients with liver resection, Spearman's rank correlation coefficient was used. Comparisons between two groups were performed using nonparametric statistics because the data did not pass the normality test (Kolmogorov Smirnov test). N values represent the number of patients/participants. P values were calculated by Mann-Whitney U test, two-tailed. A p value of < 0.05 was considered statistically significant. All data are shown as mean $\pm$ SD.

All statistical details can be found in the figure legends. The calculations were performed using GraphPad PRISM 7.0.