

NRF2/BACH1 control of tyrosine catabolism links oxidative stress to hepatocellular carcinoma progression

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Abstract

Background

Downregulation of the main L-tyrosine (L-TYR) degradation pathway (*LTYM*) is a recurrent feature of liver disease and hepatocellular carcinoma (HCC) and correlates with poor prognosis, yet the underlying regulatory mechanisms remain unclear.

Methods

Here, we investigate this process using a novel murine model of hereditary tyrosinemia type I (FAH-G337S^{+/+}), which recapitulates subacute disease progression toward HCC.

Results

We show that L-TYR accumulation and the buildup of toxic intermediates, including fumarylacetoacetate and succinylacetone, are associated with robust downregulation of *LTYM* enzymes at both the protein and transcriptional levels. Mechanistically, we demonstrate that the transcription factor NRF2 directly binds to Maf recognition elements in the promoter regions of *LTYM* genes, leading to their repression. This regulation is likely mediated through competition with the transcriptional repressor BACH1, establishing a dynamic regulatory axis that reflects intracellular oxidative stress. Pharmacological perturbation of the NRF2 pathway with brusatol restores *LTYM* expression, supporting the functional relevance of this regulatory axis.

Conclusions

Our findings identify the *LTYM* pathway as a sensor of oxidative stress and reveal NRF2-mediated repression as a key driver of its dysregulation, providing a mechanistic link between altered tyrosine metabolism and poor clinical outcomes in HCC and other liver disorders.

INTRODUCTION

Tyrosine (L-TYR) is an amino acid with both ketogenic and glucogenic properties that plays an active role in tricarboxylic acid cycle anaplerosis, as well as in lipid and carbohydrate metabolism. In the liver, L-TYR serves as a precursor for a variety of metabolites and by-products (Fig. 1a). L-TYR primary pathway of degradation is its conversion into fumarate and acetoacetate (hereafter referred to as the *L-TYR* Main degradation pathway or *LTYM* pathway). In hepatocellular carcinoma (HCC), the enzymes of the *LTYM* pathway are frequently downregulated [1–3]. Specifically, several studies report reduced transcription of key genes in this pathway, including tyrosine aminotransferase (TAT) [4, 5], hydroxyphenylpyruvate dioxygenase (HPD) [6] and glutathione S-transferase zeta 1 (GSTZ1) [1]. Decreased expression of these

enzymes is associated with the accumulation of L-TYR in HCC[7] and MASLD [8], which in turn correlates with poor prognosis of the disease [1, 2]. In this context, targeting L-TYR catabolism is emerging as a promising therapeutic strategy in cancer [9, 10].

Metabolism of L-TYR has been extensively studied using models of hereditary tyrosinemia type I (HT1), a rare genetic disorder caused by a deficiency in fumaryl acetoacetate hydrolase (FAH), the last enzyme of the *LTYM* pathway [11, 12]. If left untreated, HT1 becomes life-threatening due to the development of HCC [13]. In HT1, and to a lower extent in HCC, accumulation of L-TYR can be linked to several metabolic disturbances, including the downstream build-up of toxic maleylacetoacetate (MAA) and fumarylacetoacetate (FAA). Both compounds are very electrophilic and can induce oxidative damage by reacting with sulfhydryl groups [14, 15]. In addition, FAA is mutagenic and is considered the main responsible for HCC proliferation in HT1 patients [11]. Finally, FAA and MAA can be reduced to succinylacetoacetate and further decarboxylated to succinylacetone (SA), with the latter severely damaging the kidney [16, 17]

It has been shown that FAA, MAA, and likely SA, can induce glutathione depletion [11] and chromosomal instability through activation of the Ras/ERK pathway [18] or the AKT survival pathway [19]. Importantly, in a model of impaired L-TYR degradation that progresses to HCC (*fah*^{-/-}), accumulated FAA has been reported to activate nuclear factor erythroid 2–related factor 2 (NRF2) [20]. This activation is likely a protective response to FAA-induced oxidative stress, as depletion of NRF2 in the same model (*fah/Nrf2*^{-/-}) triggers a strong inflammatory response and markedly reduces survival [20]. However, the precise mechanism underlying the interplay between FAA and NRF2 remains unclear.

NRF2 is a master regulator of cellular homeostasis, controlling redox balance and intracellular iron metabolism (among other processes), to promote cellular fitness and survival [21]. Under basal conditions, NRF2 is ubiquitously expressed but tightly regulated through its interaction with KEAP1, which retains NRF2 in the cytosol and acts as a substrate adaptor for an E3 ubiquitin ligase complex that targets NRF2 for proteasomal degradation [21]. Upon activation, NRF2 translocates to the nucleus, where it forms heterodimers with small Maf proteins and then bind to Maf recognition elements (MAREs) in specific promoter regions of its target genes. Notably, under stress conditions, NRF2 competes with the transcription factor BTB and CNC homology 1 (BACH1) for binding to MAREs [22]. In contrast to NRF2, BACH1 exerts opposing effects, promoting apoptosis and the generation of reactive oxygen species under stress absence [23, 24].

In certain cancer cells, somatic mutations in KEAP1 or related genes disrupt NRF2 ubiquitination [25, 26], resulting in constitutively elevated NRF2 levels. Alternatively, hyperactivation of the NRF2 pathway can occur in response to increased metabolic and oxidative stress, promoting metabolic reprogramming [27] and helping to maintain cellular homeostasis in high-demand environments [28]. Any case, high NRF2 activity has been associated with shorter progression-free survival and immunotherapy resistance in HCC [29].

Collectively, these findings suggest that NRF2 may directly or indirectly downregulate L-TYR degradation. We employed a novel mouse model of HT1 (FAH-G337S^{+/+}), designed to recapitulate a subacute HT1 phenotype, building on our previous characterization of pathogenic missense mutations [30]. We demonstrate here that FAH-G337S^{+/+} mice exhibit the characteristic HT1 phenotype including progression towards HCC. At the molecular level, FAH-G337S^{+/+} mice show L-TYR accumulation, downregulation of the *LTYM* pathway, and elevated NRF2 levels. We also show that NRF2 and BACH1 share MAREs in the promoter regions of the five enzymes of the *LTYM* pathway, acting as a sensor of the oxidative stress status. This mechanism explains the reduced expression of the L-TYR degradation enzymes in both HT1 and HCC: in HT1, elevated FAA (and likely SA) levels drive NRF2 activation, whereas in HCC, well-known mechanisms lead to NRF2 upregulation, ultimately converging on suppression of the *LTYM* pathway.

RESULTS

Obtention and general description of the recombinant murine model of HT1. To investigate L-TYR degradation, we developed a new mouse model that destabilizes FAH to produce HT1. The G337S mutation was inserted in the murine *fah* gene using the CRISPR/Cas9 technology (see Methods). The FAH-G337S^{+/-} heterozygous (HE) embryos were then implanted into surrogate C57BL/6J mice for gestation. The FAH-G337S^{+/+} homozygous mice (HO) were obtained by cross breeding HE mice. In all cases, the offspring were screened for the presence of the missense mutation, using postnatally DNA sequencing.

Figure 1b shows the liver expression of FAH for the WT, HE and HO mice. HO mice show impaired homeostasis in FAH because of the reduced kinetic stability induced by the mutation, as previously described [30]. FAH HO animals live longer than the previously reported models [31], with an average life expectancy of 35 days and with more than one fifth of them living more than 100 days in the absence of any treatment (Fig. 1c). The HO mice share with the HT1 patients the growth retardation, as shown by the animal weights (Fig. 1d) and size (Fig. 1c, inset). Still, the natural history of the disease is the evolution towards HCC, as evidenced by the relative weight of the liver (Figure S1) and the histological staining of the liver tissue: a double staining with α -fetoprotein (AFP, a marker of hepatocellular carcinoma, found elevated in HT1 [32] and Periodic acid-Schiff stain (PAS) for glycogen shows that liver integrity and functionality are significantly lost after two months (Fig. 1e-f). Additionally, Hematoxylin-Eosin (H/E) staining shows structural and functional loss in HO livers and the Ki67 marker also shows hepatic evolution towards HCC (Fig. 1e). Finally, the serological analysis of liver transaminases and other biochemical parameters also indicate liver pathology (Figure S1).

At the molecular level, the HO group shows all the metabolic abnormalities observed in HT1 (Fig. 1g), including hypoglycemia and the accumulation of L-TYR and SA among other metabolites. On the other hand, HE mice show normal phenotype, metabotype and biochemical parameters, consistent with the autosomal recessive character of the disease.

From a therapeutic perspective, L-TYR catabolism can be inhibited using nitisinone (2-[2-nitro-4-trifluoromethylbenzoyl]-1,3-cyclohexanedione; NTBC; trade name Orfadin). NTBC blocks the pathway upstream of FAH by potently inhibiting HPD [33]. Alternatively, circulating L-Tyr levels can be reduced by feeding animals a diet devoid of L-Tyr and low in L-Phe (LT diet). Both treatments normalized liver histology (Fig. 1d), indicating that they prevent progression toward HCC. Body weight was also restored to normal levels (Fig. 1b), and the metabolic profile was largely normalized (Fig. 1g), with the exception of L-TYR levels after NTBC treatment, which remained elevated, as expected upon HPD inhibition.

Downregulation of the L-TYR main degradation pathway in the liver. Figures 2a-c show the changes in protein levels in the enzymes of the *LTYM* pathway as measured by Western Blot (Figs. 2a-b and S2) and gene expression analysis (Figs. 2c-d and S3), in liver tissue. In general, there is a large enzyme dysregulation in the HO group (the HT1 phenotype), as compared to WT/HE samples. Consistent with the genetic alteration, the HO group mice deplete FAH, but the *LTYM* pathway is completely downregulated downstream of HPD in the liver, as already reported in HCC [3]. The apparent discordance between TAT mRNA and protein levels in HO mice is explained by post-translational stabilization of TAT under hypoglycemic conditions, as previously reported [34]. Consistent with this, HO mice exhibit marked hypoglycemia (Fig. 1g). This mechanism allows TAT protein to accumulate despite reduced transcription. In contrast, the remaining *LTYM* enzymes are transcriptionally downregulated, indicating that TAT regulation diverges from the other enzymes of the pathway.

The FAH-G337S^{+/+} mouse model progresses from a pure HT1 phenotype to one dominated by HCC. To assess this transition, we analyzed liver tissues from mice at different ages (Fig. 2e) and observed a progressive decrease in the mRNA expression levels of enzymes in the *LTYM* pathway over time. These findings indicate that this downregulation is maintained, and even reinforced, as the HCC phenotype becomes predominant.

To investigate the extent to which the observed downregulation is driven by metabolite accumulation in the HT1 model, NTBC treatment restores protein levels to near-normal values (Figs. 2b and S2), including TAT, likely reflecting the normalization of glycemic levels (Fig. 1g). This recovery is also evident at the transcriptional level (Fig. 2c). A comparable effect is observed when animals are fed with LT diet (Figs. 1g, 2a-d and S2). Collectively, these results indicate that downregulation of the *LTYM* pathway is sensitive to the concentrations of metabolites and/or intermediates in the FAH-G337S^{+/+} mouse model.

Several reports suggest that the *LTYM* pathway is also downregulated in liver disease and HCC [1–5]. To evaluate this, we analyzed the protein levels of *LTYM* pathway enzymes in liver tissues from multiple liver disease mouse models [35] (see Methods), including WT_WD1 a diet model that promotes obesity, insulin resistance, inflammation and metabolic dysfunction; OB_WD, a genetic/diet model that develops severe obesity, hyperphagia, insulin resistance, hyperglycemia and fatty liver; WT_WD2, a diet model that promotes obesity, insulin resistance, glucose intolerance, hepatic steatosis and low-grade inflammation; and GNMT, a genetic model that spontaneously develops steatosis and HCC. Our results show that, in most of the models and for most of the enzymes, their levels are downregulated as compared to their

relevant controls, often with multiple enzymes affected within the same model (Figs. 2f and S4). These findings are fully consistent with our observations in the HT1 model.

SA induces NRF2-mediated downregulation of the LTYM pathway. To further investigate the mechanism underlying *LTYM* pathway downregulation, we used a cellular model carrying the FAH-G337S genomic modification introduced via CRISPR/Cas 9 (HEK FAH-G337S cells) [36]. Under basal conditions, the protein levels of *LTYM* pathway enzymes are not downregulated in these cells, with the exception of FAH (Figs. 3a and S5), consistent with the absence of overt molecular and cellular phenotypes. However, upon exposure to SA, intracellular levels of toxic metabolites increase, leading to partial repression of *LTYM* pathway enzymes (Fig. 3a). Similar results are obtained when cells are exposed to homogentisic acid (Figure S5), the metabolic substrate of HGO. Taken together, these findings suggest that toxic *LTYM* intermediates, such as FAA, MAA, and/or SA, act as negative regulators of the pathway.

We therefore hypothesized that NRF2 may directly regulate the expression of genes within the *LTYM* pathway. Supporting this idea, NRF2 overexpression in HEK FAH-G337S (Figs. 3b, red lines, and S6) led to a significant, time-dependent (0-48h from NRF2 overexpression) reduction in the protein levels of all five *LTYM* pathway enzymes (Figs. 3c, black lines, and S6). Importantly, a similar effect is observed when the experiment is repeated in HEK WT cells (Figs. 3c and S6): a transient reduction in protein levels is detected at 24 h for all *LTYM* enzymes except HGO, which exhibits a comparable decrease at 48 h. This result indicates that regulation of the *LTYM* pathway by NRF2 does not require prior genetic alterations in the *LTYM* pathway, as occurs in HCC.

Finally, we examined the effect of NRF2 on alternative pathways of L-TYR degradation (Fig. 1a). In addition to the *LTYM* pathway, excess L-TYR can be metabolized through several secondary routes. For example, L-TYR can be acetylated in the liver to generate N-acetyl tyrosine. Alternatively, a recently described pathway converts 4-hydroxyphenylpyruvic acid into 4-hydroxymandelic acid and subsequently into the coenzyme Q10 [37]. We assessed the impact of NRF2 on these pathways in HEK G337S cells (Figs. 3d and S6). N-acetyltransferase 2 expression does not appear to be affected by *Nrf2* overexpression in HEK FAH-G337S cells. Similarly, no significant changes were observed for coenzyme Q3 methyltransferase, while 4-hydroxyphenylpyruvate dioxygenase-like protein showed only a partial downregulation. Overall, given that these represent secondary routes of L-TYR metabolism and exhibit only modest responses to NRF2, we did not pursue further analysis.

NRF2 and BACH1 bind to the same promoter regions of LTYM enzymes to regulate their expression. We then addressed the mechanism by which NRF2 regulates *LTYM* enzymes expression. NRF2 is overexpressed in HO mice liver, a trend that is exacerbated upon progression towards HCC phenotype (Fig. 2g). As characteristic for nuclear transcription factors, NRF2 enrichment in the nucleus is expected if it is directly involved in chromatin binding and transcriptional regulation. As shown in Fig. 4a (and Figure S7), NRF2 is not preferably located in the nucleus as compared to the cytosol for the HO mice, even though the elevated NRF2 expression (Fig. 2g) indicates that the nuclear concentration of NRF2 raises. Interestingly, BACH1 shifts from the nucleus to the cytosol in HO mice as compared to WT (or

NTBC treated) mice (Figs. 4a and S7). Taken together, these results indicate that BACH1 localization is modulated by the status of the L-TYR metabolism pathway.

We next investigated whether NRF2 and/or BACH1 regulate the transcription of *LTYM* enzymes through direct association with their promoters. Computational analysis of the promoter regions of genes encoding *LTYM* pathway enzymes, performed using the JASPAR CORE vertebrate database with a p-value cutoff of 0.001, identified multiple MARE sequences (Fig. 4b), consistent with previous reports [5]. Remarkably, the same analysis using the BACH1 motif yielded virtually identical putative binding sites, consistent with the shared DNA recognition sequences of these two transcription factors (Fig. 4c).

We selected a subset of these sites for Chromatin immunoprecipitation (ChIP)-qPCR analysis, prioritizing those located closest to the transcription start site (Fig. 4b, blue circles). These sites were analyzed independently for NRF2 and BACH1 occupancy using specific primers to the selected sites (Fig. 4c). ChIP-qPCR analysis showed that NRF2 preferentially binds to the promoter regions of *LTYM* enzymes in HO mice (Fig. 4d), with significant $\Delta\Delta C_t$ enhancements as compared to the control IgG. In contrast, BACH1 showed the opposite pattern, preferentially occupying MARE sites in WT mice compared with HO mice (Fig. 4e).

Notably, this mechanism appears to be shared across all enzymes of the *LTYM* pathway, with the selected sites in *Gstz1* and *Fah* showing the highest enrichment for NRF2 and BACH1, respectively. To further investigate heterogeneity in NRF2 binding, we analyzed three distinct MARE sequences computationally predicted in the promoter region of *Gstz1* (Fig. 4b). ChIP-qPCR revealed that two of the three analyzed sites displayed detectable NRF2 occupancy in HO mouse liver (Fig. 4f), indicating functional redundancy. Among these, the MARE element closest to the transcription start site consistently showed the strongest NRF2 signal, suggesting that promoter-proximal sites may be preferentially engaged under these conditions.

Finally, we examined the functional relevance of NRF2 binding to the promoter regions of *LTYM* pathway enzymes. Brusatol, a natural triterpenoid, is a potent inhibitor of NRF2 that enhances its ubiquitination in an independent way from KEAP1 levels, thereby impairing its activity as a transcription factor [38]. Mice treated with brusatol (see Materials and Methods) showed significant restoration of mRNA levels for all *LTYM* pathway enzymes, except TAT (Figs. 4e and S8), with corresponding recovery at the protein level (Figs. 4e and S9). These results indicate that direct binding of NRF2 to *LTYM* promoter regions is sufficient to modulate their expression.

DISCUSSION

Multiple studies indicate that downregulation of enzymes in the *LTYM* pathway is a hallmark of cancer and correlates with poor prognosis [1–3]. Here, we demonstrate in a bona fide murine model of HT1 that NRF2 binds to the promoter regions of *LTYM* pathway genes, leading to their transcriptional repression. This effect is also observed in aged mice, in which the phenotype is dominated by HCC, and is consistent with the downregulation of *LTYM* enzymes across several MASLD and HCC models. Together,

these findings suggest that this regulatory mechanism is also operative in HCC, providing a mechanistic basis for the observed association between reduced enzyme levels and poor prognosis.

Our findings indicate that NRF2 point towards a model where transcription factor binding to the *LTYM* promoter regions reflects a competitive model in which BACH1 occupies these sites under basal conditions, while upon NRF2 activation, BACH1 would be displaced and replaced by NRF2. Inhibition of this effect by brusatol further demonstrates that this interaction is functional and sufficient to drive downregulation of the pathway, with the notable exception of TAT, whose mRNA was not restored by brusatol (Fig. 4g), suggesting that TAT is primarily regulated at the post-translational level.

NRF2 and BACH1 act as opposing regulators of the cellular response to oxidative stress, with NRF2 promoting cytoprotective gene expression and BACH1 acting as a repressor of these same targets. Notably, we observed that NRF2 displaces BACH1 at multiple sites within the *LTYM* pathway, suggesting a coordinated regulatory mechanism that reflects the relative nuclear levels of these transcription factors and the nuclear export of BACH1. This supports the idea that expression of *LTYM* pathway enzymes may function as a sensor of oxidative stress.

Our dataset, including cellular experiments in WT HEK cells, is consistent with the notion that NRF2 binding to the *LTYM* pathway represents a general regulatory mechanism that can be activated under diverse conditions, likely due to the intrinsic capacity of this metabolic route to generate oxidative stress. In HT1, the inhibition of the *LTYM* pathway leads to increased levels of FAA and other toxic by-products, which we propose as the primary trigger for NRF2 activation, consistent with previous observations [20]. This may also explain why alternative L-TYR metabolic pathways, such as CoQ10 or NAT synthesis, are only minimally affected following NRF2 transfection in cells, as their resulting metabolites have not been associated with toxicity and may even contribute to reactive oxygen species detoxification [39]. The subsequent downregulation of the *LTYM* pathway may therefore confer a survival advantage to hepatocytes. Whether NRF2 inhibition with brusatol alters long-term disease progression remains to be determined.

In HCC, although L-TYR accumulation has also been reported to a certain extent, NRF2 activation is more likely driven by oxidative stress associated with metabolic reprogramming [27, 28] or by somatic mutations in KEAP1 [25]. Downregulation of the *LTYM* pathway may reduce fumarate production, potentially favoring alternative metabolic routes such as glutaminolysis [40, 41]. Overall, our data suggest that *LTYM* downregulation in HCC is closely associated with NRF2 activation and may represent a consequence of NRF2 signaling rather than a primary oncogenic driver.

This study has several limitations. First, all experiments were conducted in animal models, and extrapolation to human HCC relies on previously reported observations in the literature. Second, we did not account for potential time-dependent oscillations in transcription factor levels, as our focus was on long-term responses. Also, it is well-known that brusatol is not completely specific for NRF2. Finally, we did not explore other potential regulators of the *LTYM* pathway. However, the results obtained with brusatol underscore the strong biological relevance of NRF2-mediated repression of this pathway.

In summary, we demonstrate that tyrosine degradation pathway behaves as a redox-responsive metabolic sensor regulated by the NRF2/BACH1 axis: the main L-TYR degradation pathway is directly regulated by NRF2 through its binding to the promoter regions of the enzymes involved. This interaction likely competes with the complementary transcription factor BACH1, effectively positioning the pathway as a sensor of intracellular oxidative stress. Our findings expand our molecular knowledge of HT1 and provide a mechanistic explanation for the association between dysregulation of L-TYR degradation and poor prognosis in HCC, and they broaden the potential for pharmacological modulation of NRF2 in this disease.

MATERIALS AND METHODS

Animal experiments. Mice in the NTBC-treated group were put in separated cages with 7.5 mg/L NTBC in their drinking water *ad libitum* since pregnancy. The low L-TYR treatment (AIN93 AA L-TYR and L-PHE deficient supplemented with 0.3% of L-PHE, Tables S1-S2) was purchased from Safe Lab (www-safe-lab.com). NRF2 inhibition was performed by brusatol administration (Sigma-Aldrich, SML1868-25MG). Brusatol was dissolved in filtered PBS with DMSO 1% to reach a concentration of 2 mg/kg. Then, mice were injected intraperitoneally two times (0h and 24h). Finally, animals were sacrificed 5h after the last injection.

Animal models. A mouse model of HT1 with a pure C57BL/6 strain genetic background was developed by The Jackson Laboratory (Bar Harbor, ME USA) to introduce the *Fah* G337S missense mutation[42–46] (c.1009G > A, exon 12) with CRISPR/Cas9 technology in 1 of the 2 alleles of *Fah* gene to generate *Fah*^{G337S/WT} heterozygous mice. These mice were viable, and they were carried to the animal facility of CIC bioGUNE (Derio, Spain). *Fah*^{G337S/WT} heterozygous mice were crossed to generate an expected offspring of 25% *Fah*^{WT/WT} mice, 50% *Fah*^{G337S/WT} mice and 25% *Fah*^{G337S/G337S} homozygous mice, following Mendelian inheritance. Other animal models employed: WT_WD: C57BL/6N mice were fed Western diet for 42 weeks (high-fat, high-sugar, cholesterol-enriched). OB_WD: Ob/ob mice (leptin-deficient) were fed Western diet for 23 weeks. WT_HFD: C57BL/6J mice were fed high-fat diet for 28 weeks (45% kcal from fat). GNMT: C57BL/6J *gnmt* ^{-/-} mice.

Cell culture and transfection. Wild type and FAH-G337S HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% antibiotics, at 37° C, 5% CO₂. For NRF2 overexpression, 650,000 cells/well were transfected with an NRF2 expressing plasmid (Addgene plasmid #36971; <http://n2t.net/addgene:36971>; RRID:Addgene_36971) in a 6-well plate using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific, Waltham MA, USA).

To perform the transfection, 125 µL of Lipofectamine™ 3000 Reagent were mixed with 3.75 µL of Opti-MEM™ Medium (Thermo Fisher Scientific, Waltham MA, USA) for control wells or 7.5 µL for experimental wells to obtain diluted Lipofectamine™ 3000 Reagent. Then, master mix was prepared with 250 µL of Opti-MEM™ Medium, 2 µL/µg DNA of P3000™ Reagent (Thermo Fisher Scientific, Waltham MA, USA) and 5 µg of plasmidic DNA for each experimental well. 125 µL of master mix for control or experimental

wells were combined with 125 μ L of diluted Lipofectamine™ 3000 (1:1 ratio) and incubated for 15 minutes. The medium from all wells was changed and then 250 μ L of control lipid mixture or DNA-lipid mixture were added to each well. The final ratio of DNA and Lipofectamine™ 3000 Reagent was 2.5 μ g/7.5 μ L per well. After 24, 48 and 72h post-transfection, the medium from wells were aspirated and then plates were frozen in dry ice and stored at -80 °C until protein analysis. Control plates were frozen and stored together with 24h experimental plates.

Sample extraction from mice. Mice urine was collected by gently immobilizing each mouse to obtain fresh urine or obtained directly from the bladder after euthanasia (see expanded experimental section). Anesthesia was induced by placing each mouse in an inhalation chamber with 4% isoflurane (IsoFlo™, Abbot Laboratories) in 0.8-1% O₂. Blood was withdrawn from the retro-orbital plexus with a micro hematocrit capillary (BRAND™ micro hematocrit capillary BR749311) and collected in BD Microtainer™ serum separator gel tubes (365967, BD). Blood samples were allowed to clot for 5–15 min and were subsequently centrifuged (3500 rcf, 10 min, 4 °C). Serum was transferred to a non-autoclaved Eppendorf safe-lock tube and immediately stored at -80 °C. While anesthetized, mice were euthanized by cervical dislocation and organs were collected, frozen in liquid nitrogen and stored at -80 °C, unless otherwise indicated. Frozen brains were homogenized in liquid nitrogen, generating a powder that was subsequently aliquoted.

Determination of routine serum biomarkers. Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) serum activity, as well as albumin (ALB), cholesterol (COL), total protein (PT) and triglyceride (TG) levels were determined by using the Selectra Junior Spinlab 100 automated analyzer (Vital Scientific) according to the manufacturer's instructions. Standard controls were run before each determination, and the values obtained for the different biochemical parameters were always within the expected ranges.

Histological analysis. Paraffin Embedding: Mice organs were harvested, fixed in 10% neutral buffered formalin (Sigma Aldrich, Ref: HT501128-4L) for 24 h, dehydrated through graded alcohol concentrations, cleared in Histo-clear (AMS Ref: 64110-01), and embedded in paraffin at 60 °C. Blocks were cooled, sectioned at 5 μ m, and stored at room temperature until staining.

Hematoxylin and Eosin (H&E) staining: after dewaxed and hydrated, sections were immersed in Harris hematoxylin (Sigma-Aldrich Ref: MHS128-4L) for 5 min, rinsed in tap water for 5 min, and subjected to decolorization in 0.5% (v/v) HCl followed by a thorough rinse with tap water. Then the sections were counterstained with aqueous Eosin (Sigma-Aldrich Ref: HT1102128-4L) for 15 min and washed with tap water for 3 min. The sections were then dehydrated with graded ethanol solutions and mounted with permanent mounting media (DPX Sigma Aldrich Ref:06522-500ML).

PAS/AFP Double Staining: Sections were dewaxed, treated with periodic acid (Sigma Aldrich Ref: 395132-1L) for 10 min, and Schiff reagent (Sigma Aldrich Ref: 335206-500ML) for 20 min. Antigen retrieval was performed in pH 6 citrate buffer in a microwave for 20 min. Anti-AFP antibody (abcam Ref: ab46799,

1:200) was applied overnight at 4 °C, followed by anti-rabbit HRP-conjugated polymer incubation (Vector Ref: MP-7451) and DAB for color development (brown, Vector Ref: SK-4105). Counterstaining was performed with hematoxylin, and sections were dehydrated with grade ethanol solutions and mounted with DPX.

Ki67 staining: samples were pre-treated in PTLINK TE buffer pH 9 and blocked with 3% peroxidase for 10 minutes. Next, sections were incubated with the primary antibody (dakoM7248, DAKO) at a concentration of 1:500 overnight at 4°C, followed by secondary antibody (EnVision, DAKO) for 30 minutes at room temperature. Staining was developed with VectorVIP (VectorLabs) and counterstained with Mayer's hematoxylin.

Chromatin Immunoprecipitation assay (ChIP) for NRF2/BACH1. NRF2 and BACH1 binding sites to *LTBM* genes were studied through the iDeal ChIP-seq kit for transcription factors (Diagenode, reference number: C01010055). DNA was extracted from 200 mg of each WT and HO mice livers. It was cut into small pieces and mixed with fixation buffer (formaldehyde 1%) to be disaggregated in the Dounce Tissue Grinder (KIMBLE® KONTES®, reference number: 885301-0007). For each liver, disaggregation was completed with 20 strokes and 15 shearing movements of the pestle towards the glass mortar. The fixation was stopped with glycine, and the disaggregated tissue was sequentially centrifuged and combined with protease inhibitor cocktail and lysis buffer. The extracted chromatin was combined with shearing buffer and carried to the Bioruptor® Pico (Diagenode, reference number: B01080010) for sonication. Chromatin samples were separated into aliquots of 40 µl in 0.65 ml Bioruptor® Pico Microtubes (Diagenode, Reference number: C30010011). Sonication was performed in 33 cycles of 30" ON/30" OFF, and a DNA aliquot was used to check the correct sonication in a 1.5% agarose gel. ChIP reaction mix was prepared for the experimental IP containing NRF2 (Invitrogen, PA5-27882) and BACH1 (Proteintech, 14018-1-AP) antibodies respectively and negative control IP with IgG antibody (included in the kit). Then, sheared chromatin was combined with ChIP reaction mix and magnetic beads, samples were put on a magnetic rack and several washes and elutions were performed to discard the unspecific DNA. Chromatin was decross-linked, purified and resuspended in the appropriate buffer. The DNA concentration was estimated on an Agilent 2100 Bioanalyzer, using Agilent High Sensitivity DNA kit (Agilent Technologies, reference number:#5067 – 4626) in the Genome Analysis Platform in CIC bioGUNE.

The most probable binding regions for NRF2 and BACH1 were selected using the Eukaryotic Promoter Database (EPD, <https://epd.expasy.org/epd/>) for *Mus musculus*, combining the sequence retrieval with the search motif tool for NRF2 (Nfe2l2) or BACH1 (Bach1::Mafk), and JASPAR core. Regions around the position revealed for the motif were used to design the primers with the Primer-BLAST tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and were synthesized by Sigma-Aldrich.

Total RNA isolation. RNA from flash-frozen tissue and HEK 293T cells was extracted with TRIzol reagent (15596026, Invitrogen) as per manufacturer's instructions. RNA was heated to 65 °C for 5 min and

quickly chilled on ice. RNA concentration was assessed by measuring A_{260} using a Nanodrop spectrophotometer (ThermoFisher Scientific).

mRNA reverse transcription reaction. 1 μ g of RNA was treated with Amplification Grade DNase I (ThermoFisher) following the manufacturer's guidelines. cDNA was synthesized with M-MLV Reverse Transcriptase in the presence of Random Primers, dNTPs and RNaseOUT Recombinant Ribonuclease Inhibitor (all from Applied Biosystems), by using a Biometa™ Thermal Cycler (Analytik Jena). Mix was incubated at 25 °C for 10 min, then at 37 °C for 90 min and the reaction was inactivated by heating at 70 °C for 15 min. The resulting cDNA was diluted 10-fold in Nuclease-Free Water (Merck).

Quantitative PCR. An aliquot of 6.8 ng of cDNA was mixed with 1 μ M specific forward and reverse primers (Table S1) and SYBR Select Master Mix (ThermoFisher) constituting a final volume of 6.5 μ L, in MicroAmp Optical 384-Well Reaction Plates (ThermoFisher). Each reaction was performed in triplicate using the QuantStudio™ 5 Real-Time PCR System 384-well block (ThermoFisher). Ct values were extrapolated from the amplification curve and gene expression levels were normalized with GAPDH or β -actin housekeeping expression by implementing the $2^{-\Delta\Delta Ct}$ formula.

For isolated DNA from ChIP, 1 ng was mixed with 0.2 μ M designed forward and reverse primers (Table S2). The final volume per well was 6 μ L, in MicroAmp Optical 384-Well Reaction Plates (ThermoFisher). Each reaction was performed using the QuantStudio™ 5 Real-Time PCR System 384-well block (ThermoFisher). Relative genetic expression was calculated using the fold enrichment method, by resting the cycle threshold (Ct) of the experimental IP to the Ct of the negative control (IgG) and then using it in the formula of the fold enrichment $2^{-\Delta\Delta Ct}$.

Total protein extraction. Snap-frozen mouse liver slices were lysed in 1 mL of RIPA buffer supplemented with Protease and Phosphatase Inhibitor Cocktails (PIC) using a FastPrep-24 5G homogenizer (MP Biomedicals) in Precellys™ tubes (Bertin Technologies). HEK cells were harvested scrapping culture plates with PIC-supplemented RIPA buffer and lysed for 15 min on ice. Samples were centrifugated and supernatants were transferred to a fresh Eppendorf tube and total protein concentration was assessed by a Bradford protein assay (Bio-Rad) using BSA as a standard.

Cellular fractions separation. Cytosolic and nucleic fractions were separated using the ProteoExtract® Subcellular Proteome Extraction Kit (Sigma-Aldrich, Reference number: 539790), as it is described in its protocol. Fractions samples were stored at -20°C until its use.

Total protein concentration from each fraction was assessed by a Bradford protein assay (Bio-Rad), using BSA as a standard.

Immunoblotting. 15–20 μ g of total protein from mice organs or HEK 293T cells were loaded and separated by SDS-PAGE. Proteins were then transferred to a 0.2 μ m-pore size Amersham™ Protran™ nitrocellulose membrane (Cytiva) in 25 mM Tris, 186 mM glycine pH 8.3 and ethanol 20%. Blots were probed with antibodies (Table S6) diluted 1:1000 in 5% skim milk and 1:5000 diluted HRP-linked

secondary antibodies. In case of housekeeping proteins (GAPDH and β -actin), primary antibodies and the HRP-linked secondary antibodies were diluted 1:5000 in 5% skim milk. Chemiluminiscent bands were detected with Clarity™ Western ECL Substrate (Bio-Rad) and quantified by densitometric analysis using the open-source image processing programs Image J (<http://rsbweb.nih.gov/ij>) and IBright Analysis Software (Thermo Fisher Scientific, USA).

Metabolite extraction from mice tissue. 2-100 mg of frozen organs were placed into Precellys™ tubes with 1.4 mm zirconium oxide beads (Bertin Technologies). A mixture of 473.5 μ L of methanol and 300 μ L of chloroform was added to each tube and samples were homogenized in a FastPrep-24 5G homogenizer (MP Biomedicals). 253.7 μ L of chloroform and 300 μ L nuclease-free water (Merck) were then added and the mixture was vortexed before centrifugation at 12,000 rcf 10 min at 4 °C. The resulting upper aqueous phase was separated to a fresh Eppendorf™ tube, as well as the lipophilic down phase. Samples were then vacuum dried in a miVac Duo (GeneVac™) vacuum concentrator system and stored at -80 °C.

Quantification of metabolites by ^1H -NMR spectroscopy. ^1H NMR: 30 μ L of mice urine and serum were prepared as previously described [47, 48] and measured in a 600 MHz in vitro diagnostic research (IVDr) spectrometer (Bruker BioSpin GmbH, Germany), equipped with a double resonance broadband (BBI) probehead with a z gradient coil and BOSS-III shim. The system was calibrated daily for temperature, MeOD, and shim optimization, Sucrose. Serum samples were measured at 310 K and urine samples at 300 K.

Quantification of metabolites by LC/MS. Metabolite levels from approximately 25 mg of mice brain, liver, kidney and muscle were quantified using an Acquity UPLC system (Waters Associates, Milford, MA, USA) coupled with a Bruker Impact-II QTOF mass spectrometer (Bruker Daltonik GmbH, Germany), operating in both positive and negative ionization modes. Calibration was performed using sodium formate for mass accuracy. Sample analysis was randomized, with triplicate injections and extracted ion chromatograms used for metabolite detection. Internal standards and quality controls were included in all analyses, and calibration curves were generated from serial dilutions of metabolite standards.

Abbreviations

AFP

α -fetoprotein

BACH1

BTB and CNC homology 1

FAA

fumarylacetoacetate

FAH

fumaryl acetoacetate hydrolase

GSTZ1

glutathione S-transferase zeta 1

HCC
hepatocellular carcinoma
H/E
Hematoxylin-Eosin
HPD
hydroxyphenylpyruvate dioxygenase
HPDL
4-hydroxyphenylpyruvate dioxygenase-like protein
HT1
hereditary tyrosinemia type I
LT diet
low L-PHE and no L-TYR diet
LTYM
L-tyrosine (L-TYR) degradation pathway
MAA
maleylacetoacetate
MAREs
Maf recognition elements
NRF2
nuclear factor erythroid 2–related factor 2
NTBC
nitisinone
SA
succinylacetone
TAT
tyrosine aminotransferase.

Declarations

Ethics approval

All procedures involving animals were in accordance with the Spanish Guide for the Care and Use of Laboratory Animals and with International Animal Care and Use Committee Standards and they were approved by the CIC bioGUNE ethical review committee (Permit Number P-CBG-CBBA-0721 for animal breeding and P-CBG-CBBA-2321 and P-CBG-CBBA-0424 for animal characterization and interventions). CIC bioGUNE animal facility holds an AAALAC accreditation.

Consent for publication

Not applicable.

Competing interests

Not applicable.

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Author Contribution

JG-M, MG-G, VG, DF-R, and FL-O performed the mice and cell experiments. JG-M, MG-G, VG, MB, JL-B, AN-C, DF-R, and FL-O performed the experiments and collected and analyzed the data. OM designed the study, and JG-M, MG-G, NE, DF-R, FL-O, SL, AZ and JMM contributed to the conceptualization of the project. OM drafted the manuscript with assistance from all authors.

Acknowledgments

Not applicable.

Data Availability

All data and material during the current study are available from the corresponding author upon reasonable request.

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Figures

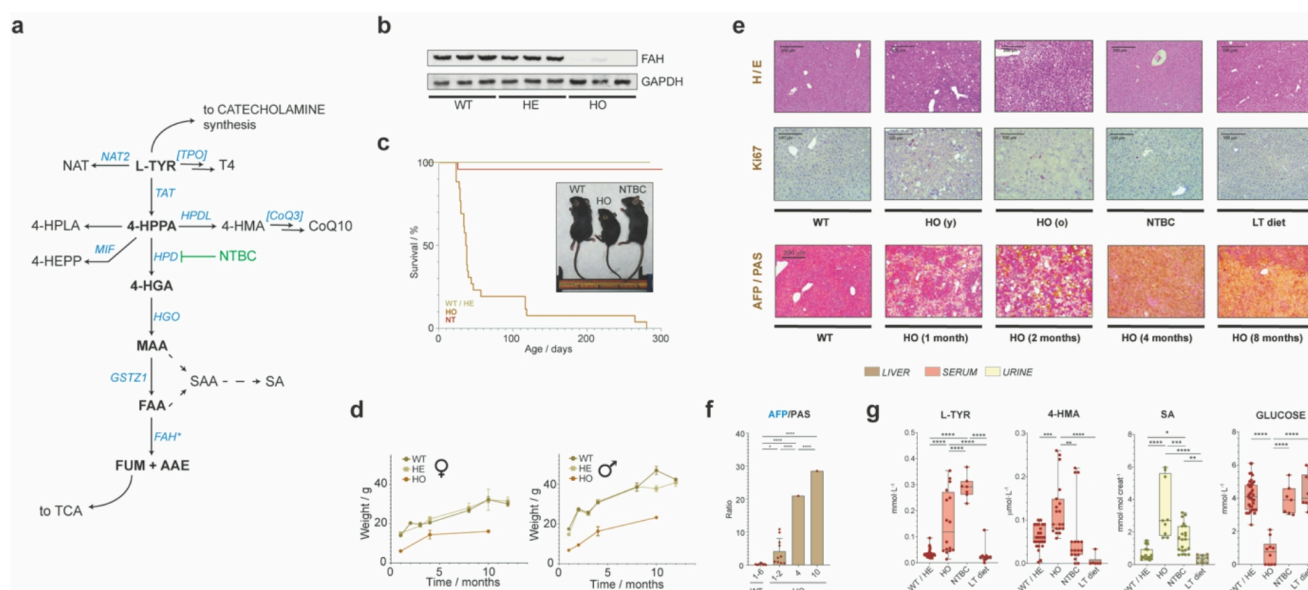


Figure 1

The *FAH-G337S^{+/+}* model of HT1. **a)** Scheme showing the main degradation pathways for L-TYR, including the main degradation path (*LTYM*, in bold) to produce FUM and acetoacetate. Toxic inhibitions are shown in red, while the NTBC therapeutic inhibition to HPD is green highlighted. Enzymes into brackets and stacked arrows indicate multiple reaction steps. **b)** Western blot analysis of FAH protein expression levels across genotypes (WT, HE, HO), illustrating the loss of protein in HO mice. **c)** Kaplan-Meier survival curves showing significant differences in lifespan between WT/HE and HO, and restoration of survival probability in NTBC-treated groups. Inset image size differences between WT, HO, and NTBC mice. **d)** Body weight measurements over time for both male and female mice in WT, HE, and HO groups. **e)** H/E: inflammation and immune cell infiltration staining and Ki-67 proliferation staining for the different groups, as labeled. Representative AFP and PAS co-stained liver sections showing progressive loss of hepatic function and structure and increase of HCC marker AFP in liver tissue of HO mice at different ages (1, 2, 4, and 8 months) compared to WT. **f)** Quantification of the AFP/PAS ratio across groups (WT, HE, HO), with a significant increase in HO, indicating HCC progression. **g)** Concentrations of urine (SA) and serum (SA, 4-HPAA) metabolites across groups. Legend for groups: WT/HE: wild type and heterozygous mice; HO: untreated homozygous mice; NTBC: nitisinone administered HO mice; LT diet: low L-TYR administered diet; (y) young; (o) old. Legend for p-values: * < 0.05; ** < 0.01; *** < 0.001; **** < 0.0001.

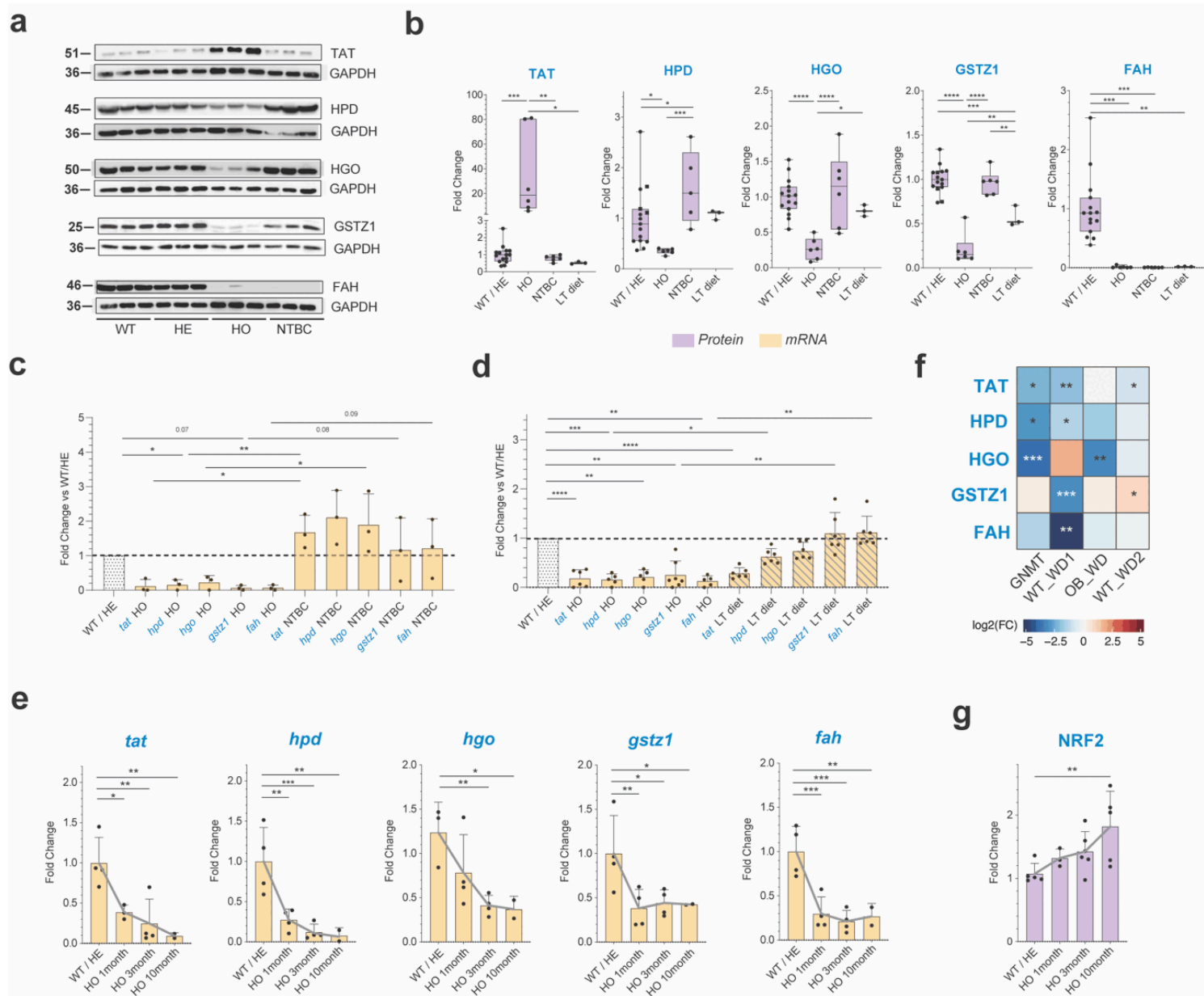


Figure 2

Downregulation of the L-TYR degradation pathway. a,b) Western blot analysis of representative *LTYM* pathway enzymes in the indicated mouse tissues and experimental conditions. Representative experimental data (a) Quantification of fold changes in protein expression for the indicated *LTYM* enzymes (b). *c,d*) Fold changes in mRNA levels of *LTYM* pathway enzymes following treatment with nitisinone (NTBC) (b) or a low L-TYR/L-PHE diet (LT diet) (c), relative to the HO group. *e*) Fold changes in mRNA levels of *LTYM* pathway enzymes as a function of age in HO mice. Lines connect mean values for each age group and are included to guide the eye. *f*) Heatmap showing protein expression levels of *LTYM* pathway enzymes across mouse models of MASLD and HCC, including GNMT, WT_WD, OB_WD and WT_HFD models. *g*) Protein expression levels of NRF2 as a function of age in HO mice. Lines connect mean values for each bar and are included to guide the eye.

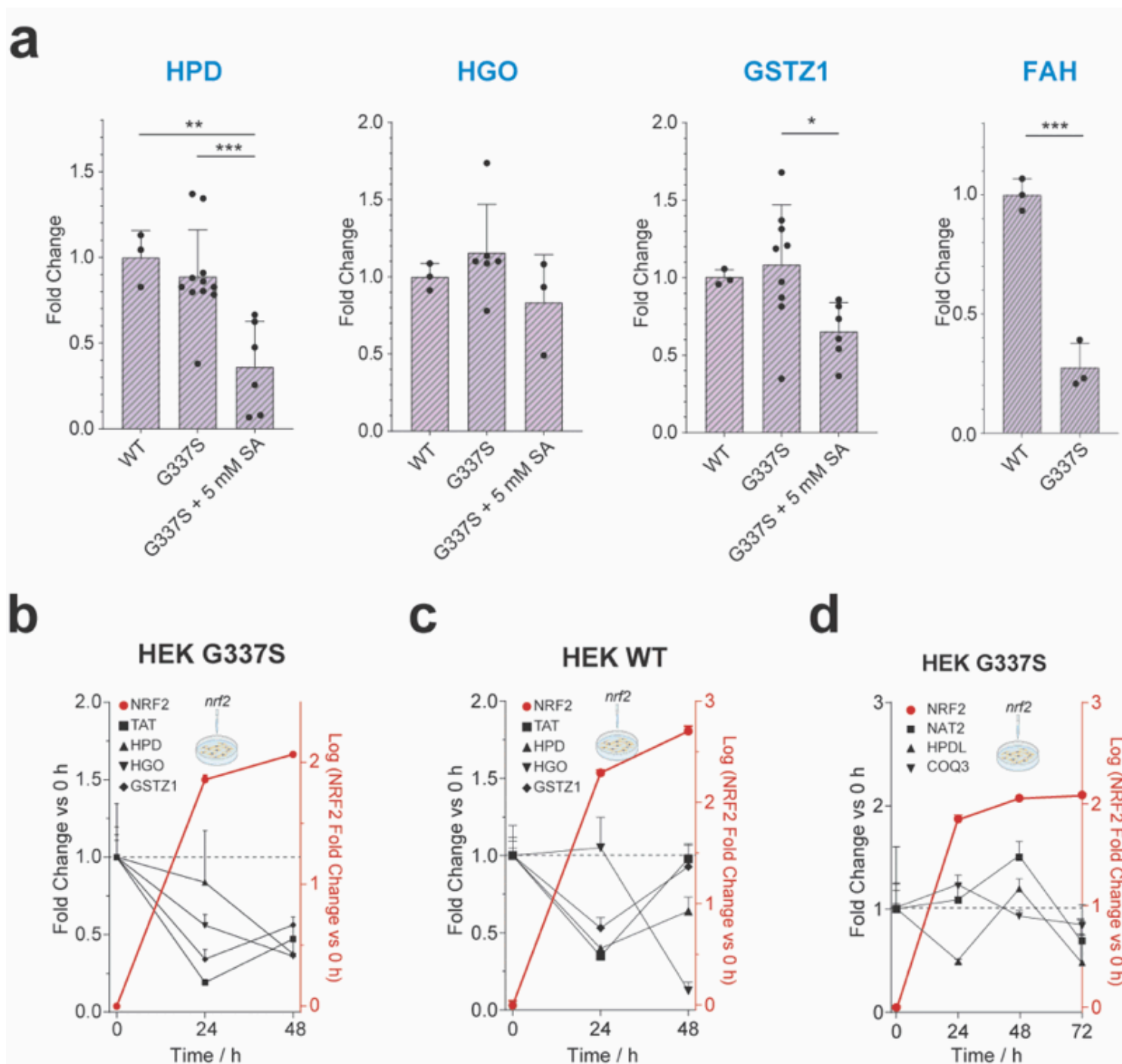


Figure 3

Cellular HEK FAH-G337S model. **a)** Western blot analysis of representative *LTYM* pathway enzymes in the indicated cellular extracts and experimental conditions. G337S denotes HEK FAH-G337S cells. Quantification of fold changes in protein expression for the indicated *LTYM* enzymes is shown. **b,c)** Protein expression levels of *LTYM* pathway enzymes (black lines; see legend) in HEK G337S cells (b) or WT HEK cells (c), determined by western blot as a function of time following NRF2 overexpression (red lines). **d)** Protein expression levels of additional pathways for L-TYR degradation intermediates (black lines; see legend) in HEK G337S cells, determined by western blot as a function of time following NRF2 overexpression (red lines).

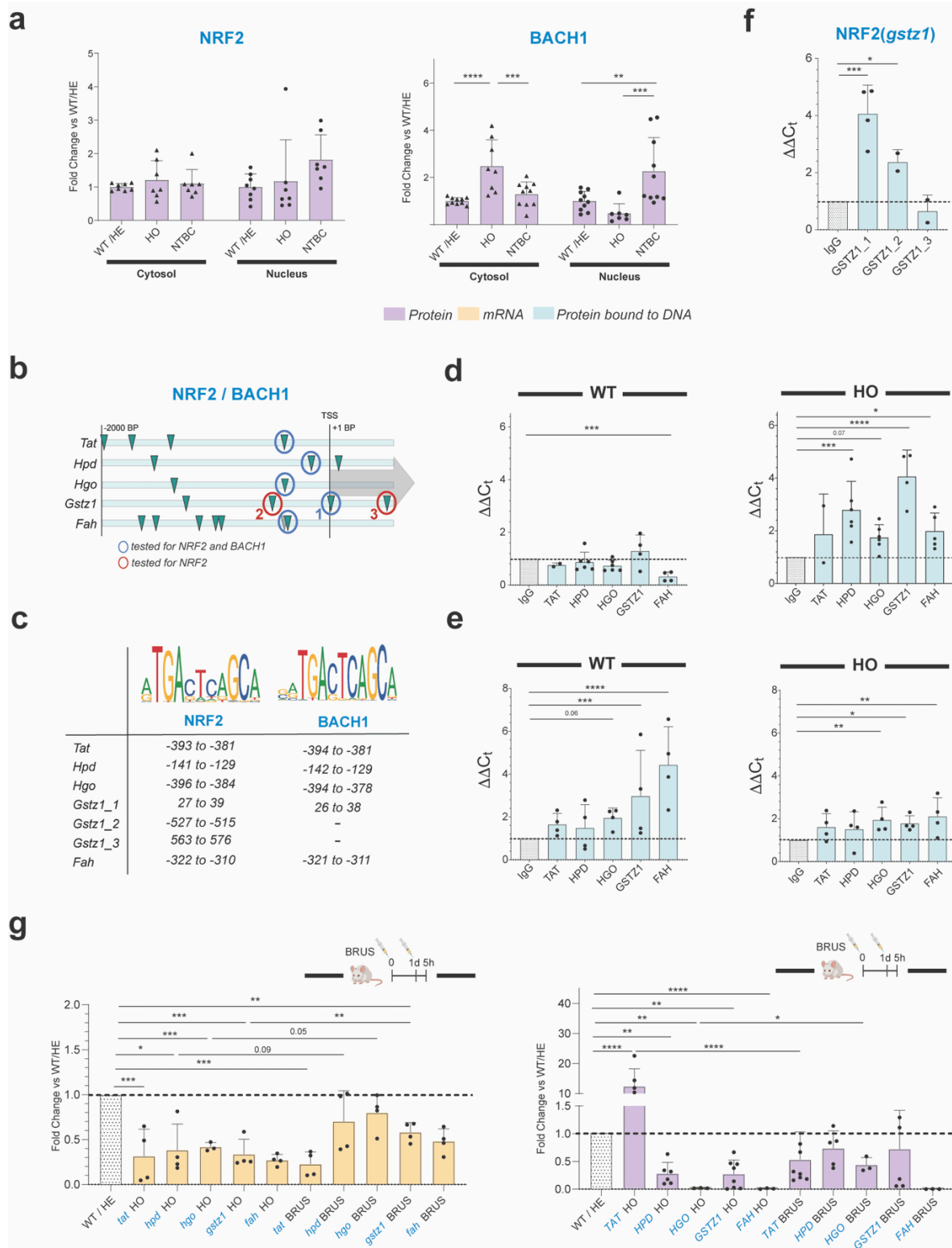


Figure 4

Role of NRF2 and BACH1 in regulation of the LTYM pathway. a) Relative protein expression levels of NRF2 (left) and BACH1 (right) in cytosolic and nuclear fractions from liver tissue extracts. **b)** Computationally predicted binding sites in the promoter region of the LTYM enzymes, indicated by vertical green wedges. Circled sites correspond to experimentally validated regions for NRF2 and BACH1 (blue circles) or NRF2 alone (red circles). **c)** Consensus DNA binding motifs for NRF2 and BACH1 as indicated. The table

shows the specific binding sites experimentally tested. **d,e)** Direct binding of NRF2 (b) and BACH1 (c) to promoter regions of *LTYP* pathway genes, as determined by ChIP-qPCR analysis. $\Delta\Delta C_t$ values represent differences in C_t values between samples combined with IgG (negative control) and samples combined with NRF2 or BACH1 antibodies. C_t values were derived from amplification curve analysis, and gene expression levels were normalized calculating the nonspecific adjustment (C_t IP – C_t of negative control) and then the fold enrichment ($2^{-\Delta\Delta C_t}$). **f)** ChIP-qPCR analysis of selected GSTZ1 promoter binding sites, as indicated. The relative positions of these sites within the promoter are shown in panel (b, red circles). **g)** Effect of brusatol administration on mRNA levels (left) and protein expression levels (right) of *LTYP* pathway enzymes.

Supplementary Files

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