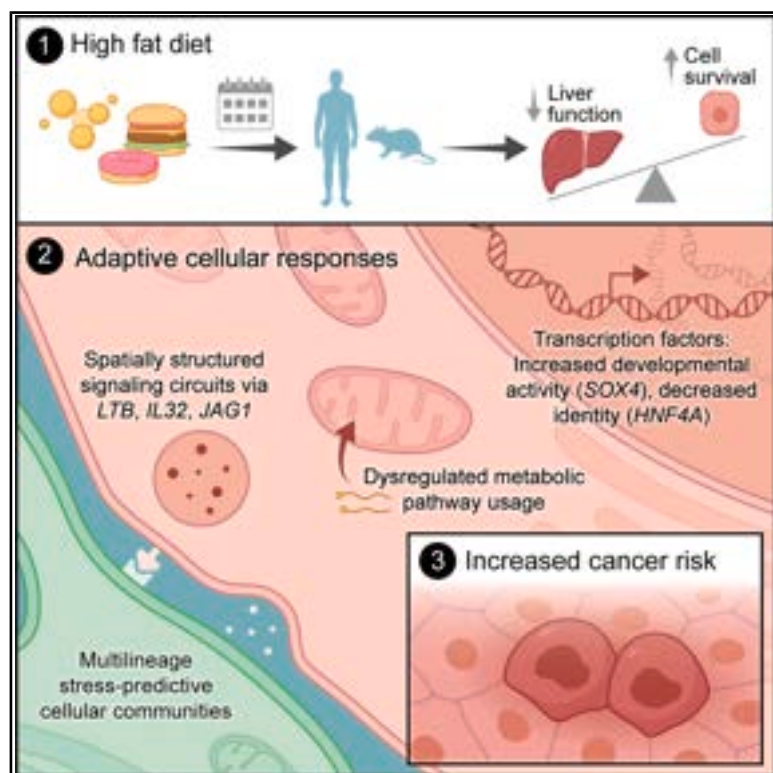


Hepatic adaptation to chronic metabolic stress primes tumorigenesis

Graphical abstract



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In brief

Environmental stresses, such as a high-fat diet, disrupt liver cell balance by promoting pro-survival, developmental, and regenerative programs while impairing key tissue functions. These stress responses—driven by metabolism, transcription factors, and intercellular signaling—increase cancer risk months to years in the future.

Highlights

- High-fat diet (HFD) reduces hepatocyte differentiation and physiological output
- Non-transformed hepatocytes activate stress and regenerative programs under HFD
- These responses prime tumor formation months in the future and stratify survival
- We identify regulatory roles for metabolism and intra- and intercellular signaling



Article

Hepatic adaptation to chronic metabolic stress primes tumorigenesis

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SUMMARY

During chronic stress, cells must support both tissue function and their own survival. Hepatocytes perform metabolic, synthetic, and detoxification roles, but chronic nutrient imbalances can induce hepatocyte death and precipitate metabolic dysfunction-associated steatohepatitis (MASH, formerly NASH). Despite prior work identifying stress-induced drivers of hepatocyte death, chronic stress' functional impact on surviving cells remains unclear. Through cross-species longitudinal single-cell multi-omics, we show that ongoing stress drives prognostic developmental and cancer-associated programs in non-transformed hepatocytes while reducing their mature functional identity. Creating integrative computational methods, we identify and then experimentally validate master regulators perturbing hepatocyte functional balance, increasing proliferation under stress, and directly priming future tumorigenesis. Through geographic regression on human tissue microarray spatial transcriptomics, we uncover spatially structured multicellular communities and signaling interactions shaping stress responses. Our work reveals how cells' early solutions to chronic stress can prime future tumorigenesis and outcomes, unifying diverse modes of cellular dysfunction around core actionable mechanisms.

INTRODUCTION

During chronic stress, cells must buffer environmental perturbations to ensure their own survival while contributing to tissue

functions necessary for organismal health.¹ Hepatocytes perform wide-ranging functions, including nutrient metabolism, protein secretion, and chemical detoxification.^{2,3} Moreover, they possess substantial regenerative capacity, able to restore



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normal liver mass and function after acute injury as dramatic as surgically removing two-thirds of the liver.⁴ However, this intrinsic regenerative ability can prove insufficient during chronic stress, resulting in progressive tissue damage.^{5,6} For instance, persistent western or obesogenic diets can drive metabolic dysfunction-associated steatotic liver disease (MASLD, formerly NAFLD/NASH), affecting >33% of people globally.^{7,8} The progressive form of MASLD, termed metabolic dysfunction-associated steatohepatitis (MASH), manifests as hepatic inflammation, fibrosis, cirrhosis, organ failure, and hepatocellular carcinoma (HCC), the second-leading cause of years of life lost to cancer.^{9–15}

Epidemiological studies indicate that each successive MASH fibrosis stage portends an increasing risk of HCC.¹⁶ However, among pre-cancer liver disease patients, only patients with cirrhosis, but not earlier fibrosis stages, exhibit significant increases in somatic mutational burden.^{17–19} Likewise, among a MASH-predominant cohort, somatic mutations were enriched in metabolic enzymes but not HCC drivers.²⁰ Intriguingly, work in other organs has described environmental stressors driving non-mutational priming of longer-term dysfunction and tumorigenesis, including transcriptional and epigenetic adaptations in response to inflammation in the pancreatic and skin epithelia and high-fat diets (HFDs) in intestinal stem cells.^{21–26} Likewise, environmental stressors can non-mutationally select for pre-existing transcriptional heterogeneity.^{27,28} Accordingly, we hypothesized that hepatocytes may also respond to chronic stress by developing progressively dysfunctional cell states that, while not solely genetically defined, are poised for tumorigenesis.

Prior work in MASLD has largely focused on tissue-level histology and organ-level function in the context of specific gene knockouts or immune subset depletions or examined broad contributors to cell death, such as reactive oxygen species, unfolded protein response, or lipotoxicity.^{9–11,29–32} Comparatively little is known about phenotypic changes in surviving cells and their dynamics during chronic stress. Outstanding questions include (1) which pathways and functional rebalancing are induced with progressive exposure to environmental stressors? (2) How do early responses connect to long-term consequences like tumor outcomes? And (3) which decision-making circuits causally mediate cellular (mal)adaptations? A deeper understanding of longitudinal stress responses (and their accompanying disease

repercussions) could help elucidate how the liver coordinates homeostatic functions while buffering stressors and the deleterious effects of ongoing injury.^{33,34} Furthermore, discovery of causal drivers could lead to novel therapeutic strategies and improved patient stratification in MASLD and HCC.

Here, we demonstrate that non-transformed hepatocyte responses to chronic metabolic stress prime tumorigenesis by perturbing cellular identity and homeostatic functions. We conduct longitudinal single-cell multi-omics analyses of a diet-only mouse model of chronic stress via metabolic overload, spanning early steatosis to spontaneous tumorigenesis. With these data and via extensions to human MASLD/HCC cohorts, we define progressive rebalancing of hepatocyte activity: this includes upregulation of early developmental markers and functions promoting the survival of individual cells concomitant with decreases in core identity and professional functions, such as reduced expression of lineage-determining transcription factors (TFs), rate-limiting enzymes, and immunomodulatory secreted proteins. By creating a computational framework to discover putative regulators of disease-associated gene programs and then testing them via *in vitro* and *in vivo* perturbations, we experimentally validate transcriptional and metabolic master regulators that drive dysregulation during chronic metabolic stress, increase hepatocyte proliferative capacity, and directly prime future tumorigenesis. Leveraging spatial transcriptomics data on human liver tissue microarrays, we demonstrate spatial multicellular hubs and signaling interactions linked to extreme manifestations of hepatocyte stress responses. Our results identify principles of longitudinal cellular responses to chronic stress and connect them to cancer formation and outcomes.

RESULTS

Metabolic stress alters pro-survival and core functional setpoints among hepatocytes

As an exemplar of chronic stress, we implemented an HFD liver injury model that does not involve exogenous genetic or chemical insult.^{35–38} HFD C57BL/6 mice developed systemic, histological, and functional comorbidities seen in MASH patients: progressive simple steatosis, inflammation, fibrosis, and spontaneous HCC tumorigenesis (Figures 1A–1D and S1; STAR Methods).^{39,40} Our mouse model additionally formed

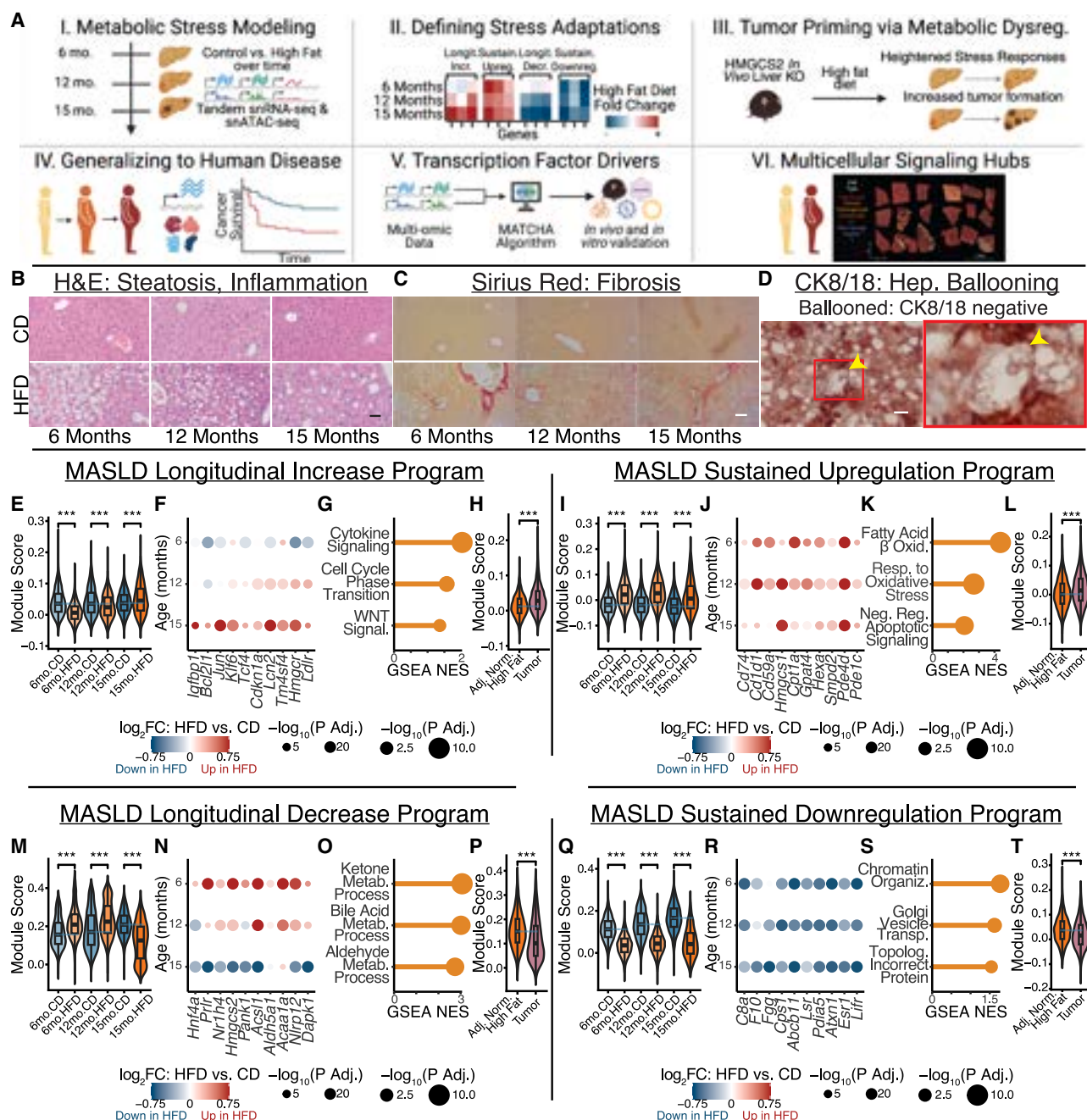


Figure 1. Dynamic responses of hepatocytes undergoing chronic metabolic stress

(A) Study schematic.

(B–D) Histology of tissue morphology (B; scale bar, 50 μ m), fibrosis (C; scale bar, 50 μ m), and hepatocyte ballooning (D; CK8/18 staining; scale bar, 15 μ m).

(E–H) Longitudinal Increase program expression in chronic metabolic stress mouse hepatocytes (snRNA-seq), with aggregate expression (E), expression changes of representative genes (F), enriched GO:BP gene sets (G), and held-out mouse snRNA-seq tumor cells and adjacent normal hepatocytes not used in program derivation (H).

(I–L) Sustained Upregulation program, following (E)–(H).

(M–P) Longitudinal Decrease program, following (E)–(H).

(Q–T) Sustained Downregulation program, following (E)–(H).

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spontaneous liver tumors that similarly exhibited histologic and transcriptional states characteristic of HCC, including HCC diagnostic biomarkers and established liver cancer marker gene sets (Figure S1; Table S1).^{41–47} To define dynamic cellular responses to chronic metabolic stress, we conducted longitudinal single-cell transcriptional and epigenetic profiling for HFD and control diet (CD) mice, recovering all major parenchymal, stromal, and immune subsets (see Table S2 for cell-type marker genes; see key resources table for interactive visualizations and data access through the Alexandria Project/Single Cell Portal: SCP3352, SCP3354, and SCP3356).

We initially focused our analyses on progressive stress responses among hepatocytes, given their central role in executing homeostatic tissue functions impacted in MASLD. Reasoning that genes with temporally coordinated expression trajectories might capture linked biological processes or cellular responses, we defined four expression programs: (1) “Longitudinal Increase” and (2) “Sustained Upregulation” programs, which describe genes progressively or consistently elevated with long-term metabolic stress, respectively, and (3) “Longitudinal Decrease” and (4) “Sustained Downregulation” programs, whose genes are progressively or consistently decreased, respectively (Table S3; STAR Methods).

Examining the Longitudinal Increase and Sustained Upregulation programs, we found that long-term metabolic stress increased expression of genes maintaining cellular survival and mitigating apoptosis (e.g., anti-cell death *Bcl2l1*, *Cd1d1*, and *Cd59a*; regeneration-linked *Cdkn1a* and *Tcf4*) (Figures 1E, 1F, 1I, and 1J).^{41,48–58} Specific cholesterol synthesis (*Hmgcr*, rate-limiting; *Hmgcs1*) and β -oxidation enzymes (*Cpt1a*, rate-limiting) were also upregulated,^{59,60} as were metabolic regulators and enzymes generating signal transduction metabolites.^{61–65} Supporting these interpretations, gene set enrichment analyses (GSEA) indicated enrichment of externally defined Gene Ontology: Biological Process (GO:BP) gene sets related to cell cycle regulation, negative regulation of apoptotic signaling, WNT signaling, and β -oxidation (Figures 1G and 1K; Table S4). Intriguingly, although the programs were derived from non-transformed hepatocytes’ progressive responses to chronic metabolic stress, we found that both the Longitudinal Increase program and the Sustained Upregulation program were significantly further elevated in diet-driven spontaneous mouse liver tumors (Figures 1H and 1L).

By contrast, the Longitudinal Decrease and Sustained Downregulation programs contained genes core to hepatocyte functional identity and homeostatic responsibilities, including the master lineage regulator *Hnf4a*,⁶⁶ diverse metabolic enzymes (e.g., *Hmgcs2*, rate-limiting for ketogenesis; *Cps1*, rate-limiting for the urea cycle), secreted proteins (e.g., complement and coagulation factors), and tumor suppressors (Figures 1M–1T; Table S4).^{67–81} Decreases in *Hmgcs2* were especially notable given that (1) ketogenesis occurs downstream of lipid oxidation,

(2) both ketogenesis and cholesterol synthesis incorporate lipid oxidation byproduct acetoacetyl-coenzyme A (CoA),⁶⁷ and (3) many cholesterol synthesis enzymes (including rate-limiting *Hmgcr*) increased with chronic metabolic stress. Coherently, both of these programs were significantly further downregulated in matched murine spontaneous tumors (Figures 1P and 1T).

Multimodal validation of hepatocyte longitudinal stress responses and dysfunction

Consistent with transcriptomic changes, we observed decreases in the protein-level abundances of hepatocyte Longitudinal Decrease and Sustained Downregulation stress response genes: (1) the master lineage-determining transcription factor HNF4A, (2) additional functional markers, and (3) secreted proteins produced by hepatocytes, including the clinical biomarker ALB, further confirmed in circulating plasma (Figures 2A–2J and S2A). Meanwhile, we detected increases in the protein-level abundance and proportion of hepatocytes positive for the WNT target GLUL (Figures 2C–2E). When culturing human liver HepG2 cells in lipid-rich media *in vitro*, we independently observed transcriptional changes, lipid accumulation, and protein synthesis shifts concordant with our *in vivo* stress responses (Figures S2B–S2H; STAR Methods).^{82,83}

Further, shotgun lipidomics demonstrated extensive remodeling of liver lipids. Triglycerides were significantly enriched with HFD at 6 and 12 months, aligning with histologic quantification (Figures 2K and S1B). Progressive increases in triglyceride total carbons aligned with upregulation of the elongation enzyme *Hacd1* (Figures S2I and S2J) and increased carbon lengths during liver regeneration.⁸⁴ At 15 months, cholesterol- and sphingolipid-related lipid species exhibited the strongest increases with HFD (Figure 2L). Increased liver abundances of cholesterol and cholesterol-related lipids aligned with upregulation of *Hmgcr* (cholesterol synthesis rate-limiting enzyme), *Hmgcs1*, and *Ldlr*, as well as downregulation of *Hmgcs2* (ketogenesis rate-limiting enzyme) (Figures 1F, 1J, and 1N). These observations were supported by plasma cholesterol measurements (Figure S2K). Upregulation of sphingolipid-related enzymes *Smpd2* and *Hexa* further linked liver transcriptional and metabolic states: sphingolipids mediate MASH progression⁸⁵ and tumor immune evasion,⁸⁶ and pre-tumorigenesis increases in sphingolipids suggest metabolic shifts that may complement non-transformed hepatocytes’ transcriptional responses related to immune evasion (e.g., *Cd59a* and *Cd1d1*).

Metabolic pathway rebalancing exacerbates stress responses and primes future liver tumorigenesis

To examine the direct functional consequences of metabolic stress (i.e., as opposed to aging), we further explored whether diet-driven metabolic changes were sufficient to exacerbate hepatocyte stress responses and tumor outcomes. We focused on decreases in HMGCS2 (3-hydroxy-3-methylglutaryl-CoA

In (E)–(G), (I)–(K), (M)–(O), and (Q)–(S), snRNA-seq of $n = 13$ mice, 51,065 hepatocytes. In (H), (L), (P), and (T), snRNA-seq of $n = 6$ adjacent normal or tumor samples, 10,707 hepatocytes or tumor cells. In (E), (I), (M), and (Q), the blue dashed line indicates the CD median. In (H), (L), (P), and (T), the blue dashed line indicates the adjacent normal HFD median. GSEA through the fgsea package; Mann-Whitney U test for all others; Benjamini-Hochberg multiple-testing correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Figure S1 and Tables S1, S2, S3, and S4.

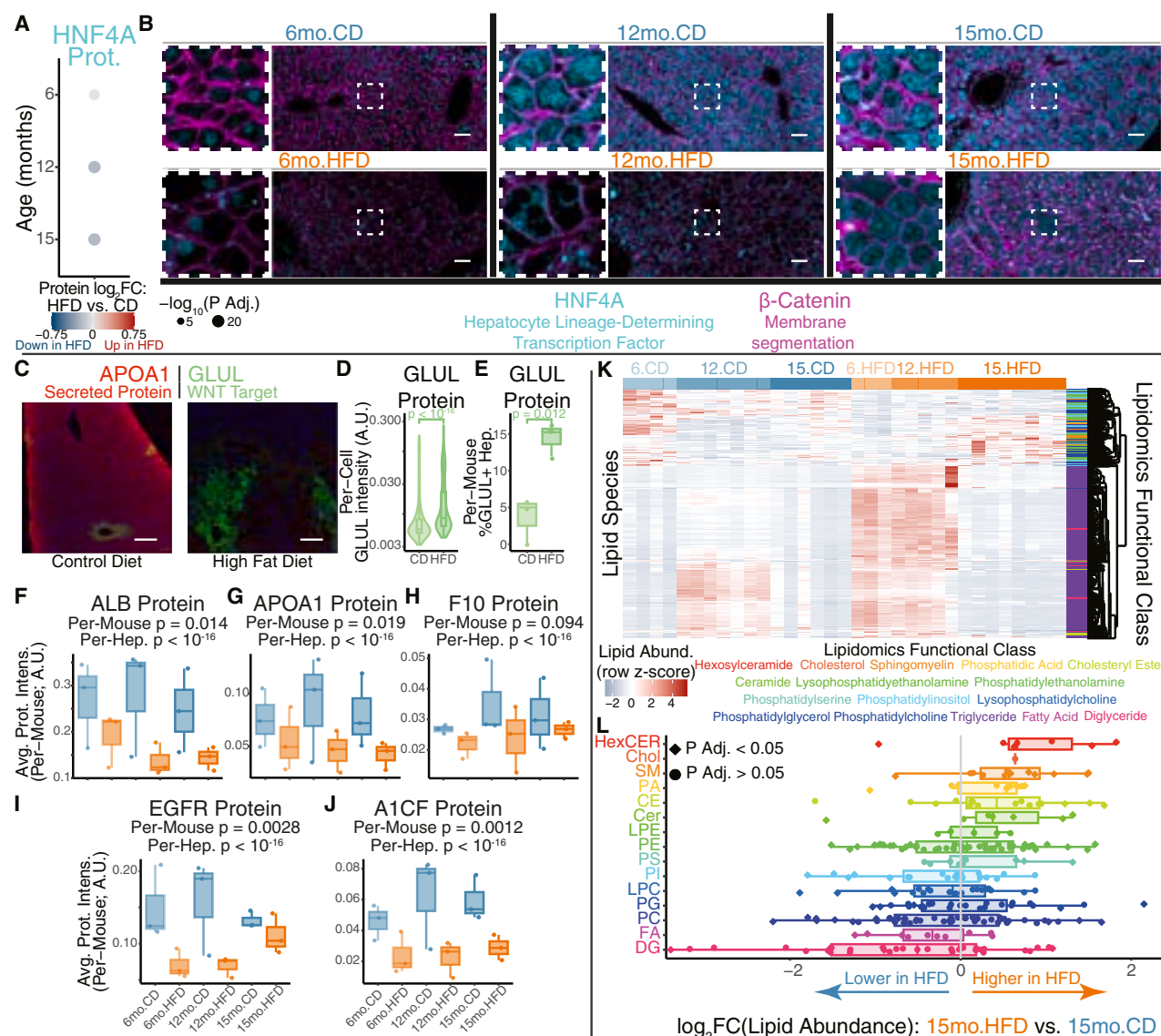


Figure 2. Multimodal validation of hepatocyte metabolic stress responses

(A) Log₂(high-fat vs. control diet fold-change) in multiplexed immunofluorescence HNF4A protein nuclear abundance ($n = 18$ mice, 350,842 nuclei).

(B) Representative HNF4A images (scale bar, 50 μ m).

(C) GLUL and APOA1 multiplexed immunofluorescence in control (left) or high-fat diet (right) ($n = 6$ mice).

(D) Average GLUL protein intensity ($n = 6$ mice).

(E) Per-mouse percent GLUL+ hepatocytes ($n = 6$ mice).

(F–J) Multiplexed immunofluorescence intensity for (F) ALB, (G) APOA1, (H) F10, (I) EGFR, and (J) A1CF ($n = 18$ mice, 2,378,590 cells).

(K) Longitudinal lipid abundances with long-term high-fat diet, measured via shotgun lipidomics ($n = 33$ mice).

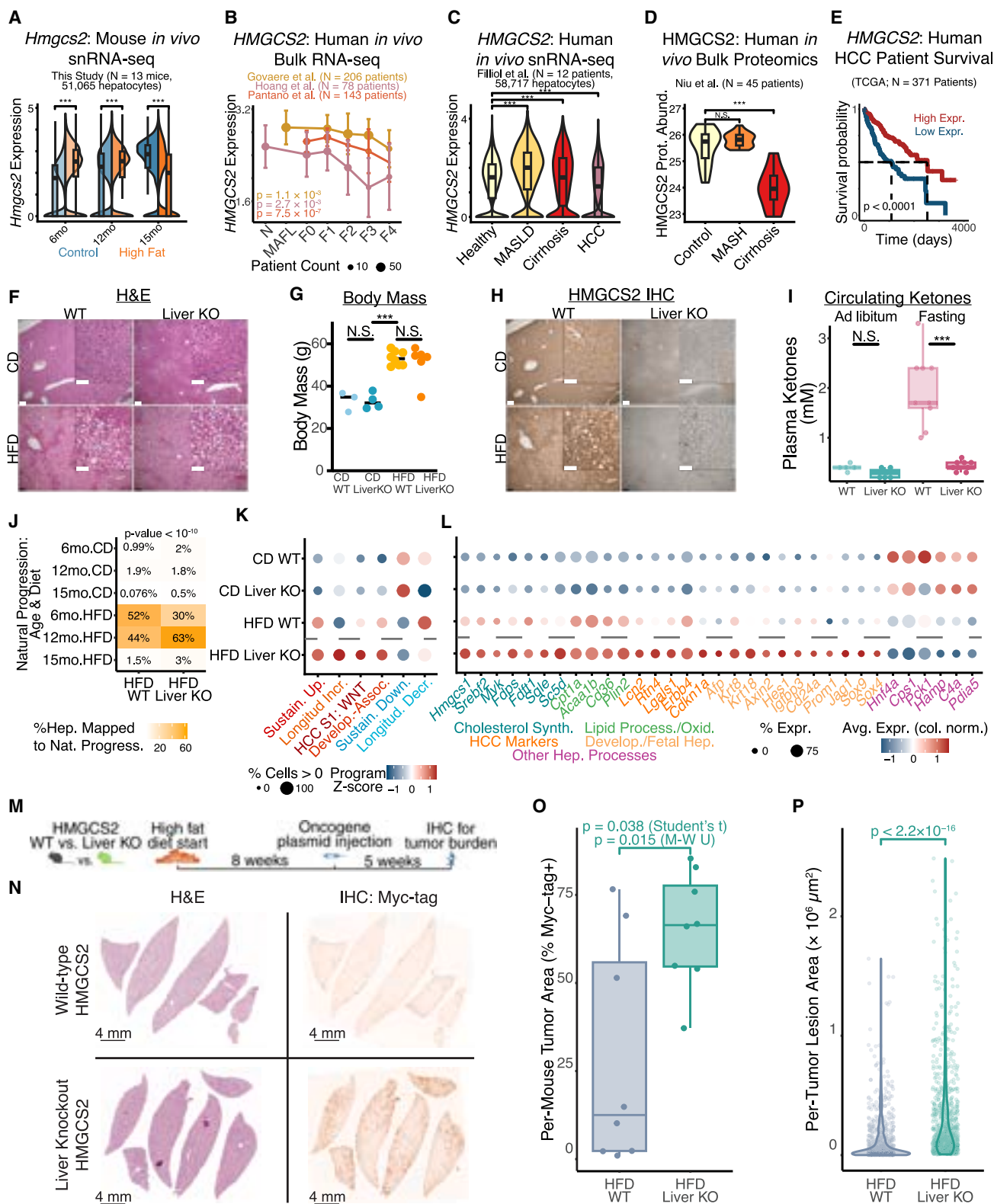
(L) Lipid abundance changes after 15 months of high-fat vs. control diet ($n = 14$ mice).

p values through the Mann-Whitney U test with Benjamini-Hochberg correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

See also Figure S2.

synthase 2), the rate-limiting enzyme for ketogenesis and part of the Longitudinal Decrease program. HMGCS2 showed robust decreases at the transcriptional and protein levels as part of metabolic stress-driven responses, and lower HMGCS2 expression predicted worsened human HCC survival outcomes (Figures 1N and 3A–3E). Ketone body production from lipid oxidation byproducts is also known to be impaired in human steatohepatitis.^{87–90}

To model metabolic stress-driven *Hmgcs2* expression decreases, we generated a liver-specific knockout mouse model (*Hmgcs2*^{fl/fl}; *Alb-Cre*). HFD drove steatosis across genotypes, but weight gains and circulating glucose remained unaffected by genotype, as expected (Figures 3F, 3G, and S2L). However, liver damage and circulating cholesterol increased with the combination of HFD and HMGCS2 LiverKO (Figures S2M and S2N).



Anti-HMGCS2 immunohistochemistry and reduced post-fast circulating ketones validated the protein-level and functional efficacy of the knockout, respectively (Figures 3H and 3I). We proceeded to conduct single-nucleus RNA sequencing (snRNA-seq) across *Hmgcs2* genotypes and diet conditions to examine whether decreases in HMGCS2 were sufficient to exacerbate and accelerate hepatocytes' progressive stress responses (snRNA-seq; $n = 8$ mice, 27,119 cells) (Table S2; Key resources table, Alexandria Project/Single Cell Portal: SCP3355).

Through reference mapping of snRNA-seq expression profiles, we found that HMGCS2 loss accelerated hepatocyte stress responses. Approximately equal proportions of HFD.WT hepatocytes mapped to the natural progression 12-month (44%) and 6-month (52%) conditions. However, with HFD.LiverKO, over twice as many hepatocytes mapped to the 12-month HFD condition (63%) as to the 6-month HFD condition (30%) (Figure 3J). HFD.LiverKO similarly drove extreme expression of hepatocyte stress response programs, even relative to diet-induced shifts in wild-type mice (Figure 3K). Diverse cholesterol synthesis-related genes were upregulated with the combination of HMGCS2 LiverKO and HFD (e.g., *Hmgcs1*, *Srebf2*, and *Mvk*), in line with the metabolic branch point between ketogenesis and cholesterol synthesis (Figure 3L). Additionally, combined HFD and HMGCS2 LiverKO induced dysfunctional cell states involving development-associated markers and HCC-linked intercellular signaling proteins, as well as reduced functional and identity markers.

Extending HMGCS2's stress-driven natural history and gene regulatory effects, we sought to directly demonstrate HMGCS2's causal role in priming future tumorigenesis. We modeled HCC tumorigenesis through hepatocyte delivery of plasmids encoding hMET, constitutively active S45Y-CTNNB1, and Sleeping Beauty transposase via hydrodynamic tail vein injection to HFD mice^{54,91–96} (Figure 3M). HMGCS2 LiverKO causally increased tumorigenesis in the context of chronic metabolic stress, as evidenced by increased area of tumor outgrowth on a per-mouse level (Figures 3N and 3O), as well as increased sizes

of individual tumor nodules (Figure 3P). Additionally, the length of HFD exposure influenced the degree of tumor priming, as 2 weeks of HFD prior to oncogene introduction was also sufficient to drive increased tumorigenesis in an independent cohort of HMGCS2 LiverKO mice, but to a lesser degree (Figure S2O). Congruently, across three human cohorts, we additionally found that reduced HMGCS2 expression in non-transformed human liver tissue predicted increased HCC risk up to 15 years in the future⁹⁷ (Figure S2P). These results support the cumulative nature of hepatocyte stress responses in establishing a fertile soil for future tumorigenesis.

Pre-transformation stress responses extend to human disease and predict cancer survival

Given the physiological fidelity of our HFD mouse model to human MASLD/HCC (Figure S1; STAR Methods) and the functional consequences of metabolic rewiring for murine HCC, we next examined connections between our cellular chronic stress responses and publicly available human MASLD/HCC data (Figure 4A). In human patients, the Longitudinal Increase program was further elevated with progressive MASLD severity across cohorts using bulk RNA-seq and snRNA-seq readouts (Figures 4B and 4C).^{82,98–101} Similarly, Longitudinal Increase protein-level abundances increased across MASLD progression in human liver tissue proteomics (Figure 4D).¹⁰² Moreover, the Longitudinal Increase program was further augmented within human HCC and prognostically stratified HCC patient survival,¹⁰³ with high expression predicting worsened outcomes (Figures 4C and 4E).

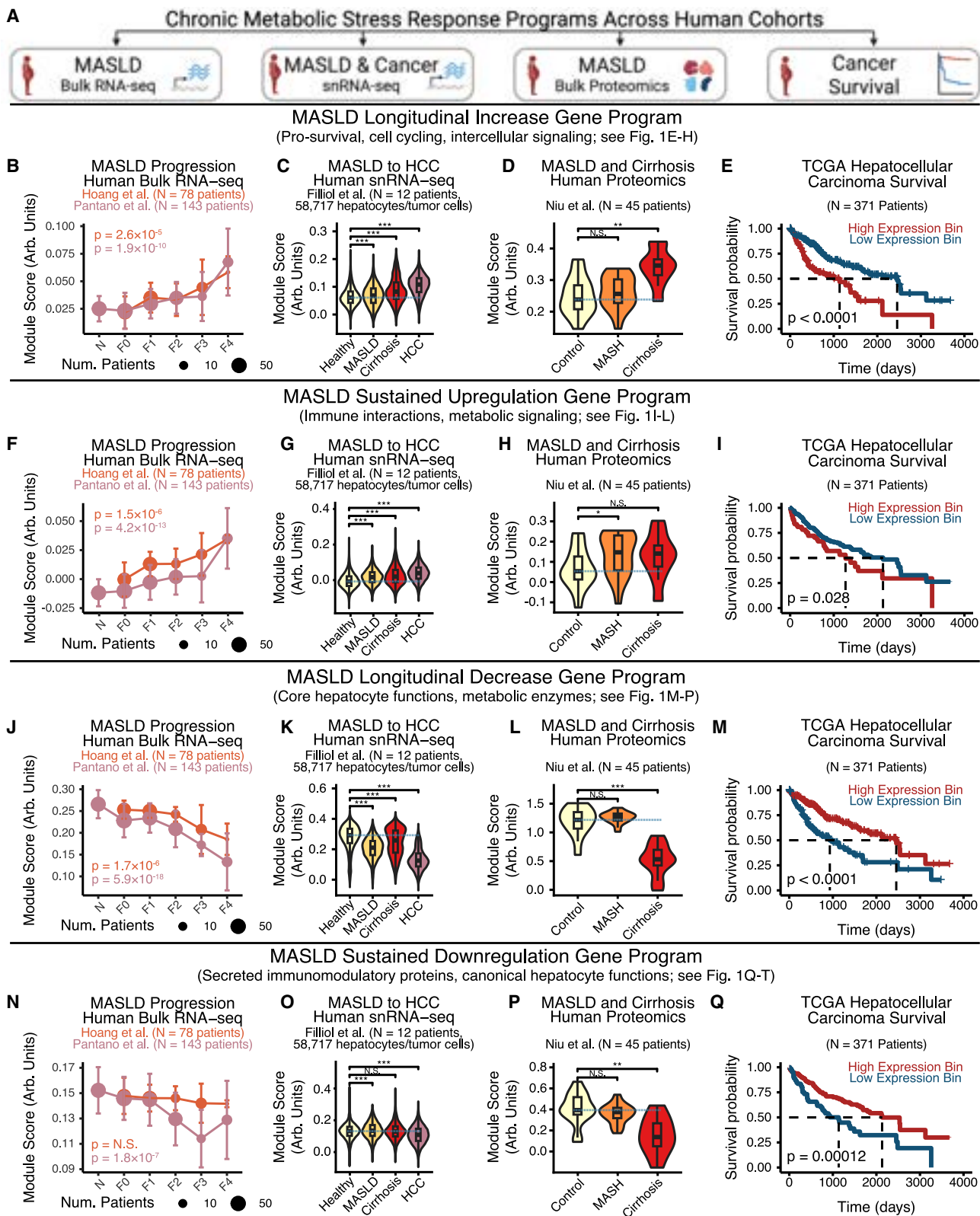
The Sustained Upregulation, Longitudinal Decrease, and Sustained Downregulation programs also significantly associated with disease severity, tumor phenotypes, and patient survival outcomes across mice and humans (Figures 4F–4Q). As anticipated, the Longitudinal Increase and Sustained Upregulation programs behaved opposite to the Longitudinal Decrease and Sustained Downregulation programs. Across studies, measurement modalities, and species, aggregate scores were driven by similar underlying genes (Figures S3A–S3F).

Figure 3. In vivo validation of HMGCS2 as a regulator of hepatocyte stress responses and tumor priming

- (A) Mouse snRNA-seq hepatocyte *Hmgcs2* expression with natural metabolic stress progression ($n = 13$ mice, 51,065 hepatocytes).
(B) Human bulk RNA-seq *HMGCS2* expression with MASLD progression ($n = 427$ patients across 3 independent cohorts).
(C) Human snRNA-seq hepatocyte or tumor cell *HMGCS2* expression with MASLD or HCC ($n = 12$ patients, 58,717 cells).
(D) Human bulk proteomics HMGCS2 protein abundance with MASLD progression ($n = 45$ patients).
(E) HMGCS2 stratification of human HCC survival outcomes ($n = 371$ patients).
(F) H&E staining of wild-type (WT) or *Hmgcs2*^{fl/fl}; *Alb-Cre* (HMGCS2 LiverKO) mice on CD or HFD (main image scale bar, 200 μ m; inset scale bar, 100 μ m).
(G) Mouse body mass ($n = 21$ mice).
(H) HMGCS2 immunohistochemistry after 6 months on control or high-fat diet.
(I) Circulating ketone body concentrations after 24-h fast ($n = 30$ mice).
(J) Hepatocyte reference mapping from the HMGCS2 LiverKO cohort ($n = 8$ mice, 25,420 hepatocytes) to natural stress response progression ($n = 13$ mice, 51,065 hepatocytes).
(K) Gene program expression with diet and HMGCS2 genotype.
(L) Gene expression across diet and HMGCS2 genotype.
(M) Tumorigenesis priming experimental schematic.
(N) H&E and Myc-tag immunohistochemistry (IHC) of tumor burden with HMGCS2 genotype.
(O) Tumor area per mouse ($n = 14$ mice).
(P) Distribution of individual lesion sizes.

p values in (B) were calculated with Student's t test on the linear regression slope coefficient, p value in (E) was calculated with log-rank test, p value in (J) was calculated using Fisher's exact test, and all other p values were calculated using a Mann-Whitney U test with Benjamini-Hochberg correction.

See also Figure S2 and Table S2.



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In view of spontaneous tumorigenesis in our HFD mice, we sought to further connect our murine observations with human cancer incidence. We similarly investigated how HCC-associated signatures manifest in early MASLD before overt tumorigenesis. Here, we found multi-omic and cross-species activation even at early MASLD stages of (1) a liver developmental signature characteristic of liver epithelial progenitor cells and (2) HCC human patients' S1 signature, characteristic of tumors with elevated WNT pathway activation and early tumor recurrence (Figures S3G–S3P).^{47,104–112} Intriguingly, we also found that our chronic metabolic stress response programs were consistently and coherently dysregulated across HCC tumors linked to metabolic disorders (i.e., alcohol-related liver disease, MASLD), viral infection (i.e., hepatitis B and hepatitis C virus), or no known risk factors (Figure S3Q). They were also invoked in acute regeneration (acetaminophen overdose) (Figure S3R).¹¹³ This cross-disease and cross-injury conservation could suggest that our programs represent more generalized liver responses to diverse environmental stresses.

Epigenetic dysregulation and WNT pathway priming under chronic metabolic stress

We next sought to identify the broader cell-intrinsic regulatory networks that may initiate, drive, and sustain our hepatocyte stress response programs. Incorporating matched epigenetics data via murine HFD snATAC-seq, we found concordance between transcriptional expression and epigenetic accessibility (Figure S4A; Table S2; Key resources table, Alexandria Project/Single Cell Portal: SCP3356). Examining genome-wide accessibility of TF binding motifs revealed progressive increases in AP-1 complex motif accessibility, which mediates long-lasting epigenetic rewiring and tissue memory of inflammation in other organs (Figure 5A).^{25,114–116} Likewise, HFD increased motif accessibility of TFs involved in liver regeneration and development (SOX4, SOX9, TEAD, TCF7, LEF1).^{117–120}

We further investigated temporally varying chromatin states through pseudotemporal analyses (Figures 5B and S4B; STAR Methods). Reinforcing our prior multi-omic, cross-species analyses, members of the Longitudinal Decrease program exhibited maximal chromatin accessibility early in the HFD pseudotime progression, whereas members of the Longitudinal Increase program peaked toward the pseudotime terminus. Direct examination of longitudinal accessibility changes at gene loci supported pseudotemporal ordering (Figures 5B and 5C), with decreased epigenetic accessibility at the *Hmgcs2* locus supporting our findings on its transcriptional, proteomic, and functional dysregulation with chronic metabolic stress and HCC (Figures 1N, 3, and S3).

We additionally sought to identify indications of epigenetic priming, leveraging co-accessibility between intergenic chromatin peaks and promoters or gene bodies to capture distal enhancer-gene regulatory interactions.^{121,122} For WNT/ β -catenin target *Axin2*, for example, we identified several distal chromatin regions co-accessible with *Axin2*'s promoter/gene body and elevated in accessibility with HFD across time points (Figure 5D). More broadly, WNT-associated and HCC-linked genes exhibited increased epigenetic accessibility but only modest changes in transcription at our earliest time point (6 months) (Figures 5D and 5E). However, these genes were strongly upregulated months later after tumorigenesis (15 months) (Figure 5F). Genes with the opposite directionality (i.e., early decreases in chromatin accessibility preceding transcriptional downregulation with longer-term metabolic stress) included (1) metabolic enzymes and secreted proteins, (2) suppressors of HCC and fetal hepatocyte-associated phenotypes, and (3) *Esr1* as a receptor for MASLD-protective estrogen signaling (Figures 5E and 5F).^{123,124} Paralleling hepatocyte transcriptomic and proteomic stress responses, discovery of early WNT- and HCC-linked chromatin changes that foreshadow later transcriptional shifts suggests that even early exposure to metabolic stress may establish a latent permissive chromatin landscape that contributes to subsequent activation of regeneration-, development-, and cancer-associated pathways.

To further examine how dysregulated WNT pathway signaling related to our HFD hepatocyte stress programs, we collated publicly available data across multiple WNT perturbation approaches spanning *in vivo* and *in vitro* model systems.^{125–128} Neither WNT pathway activation nor decreases were sufficient to drive holistic hepatocyte stress response programs (Figures S4C–S4Q). Instead, overlapping genes between WNT perturbations and stress response programs were enriched for fatty acid, cholesterol, and bile acid metabolism Hallmark gene sets (Figures S4E, S4H, S4K, S4N, and S4Q). Likewise, across multiple model systems and signatures, lipid treatment alone was not sufficient to increase activation of WNT-associated pericentral signatures (Figures S4R and S4S).^{129–131} This suggests our stress response programs may be partially influenced by WNT signaling but are likely mediated by additional molecular drivers.

Prioritizing causal TFs shaping chronic stress responses via MATCHA

To discover causal factors that might be more holistically regulating our hepatocyte stress responses, we developed MATCHA (Multi-omic Ascertainment of Transcriptional Causality

Figure 4. Chronic stress responses extend to human cohorts and connect to cancer phenotypes and outcomes

(A) Analysis schematic.

(B–E) Longitudinal Increase program in human bulk liver RNA-seq (B; $n = 221$ patients across 2 independent cohorts), human snRNA-seq hepatocytes and tumor cells (C; $n = 12$ patients, 58,717 cells), human bulk liver proteomics (D; $n = 45$ patients), or human HCC survival outcomes (E; $n = 371$ patients).

(F–I) Sustained Upregulation program, following (B)–(E).

(J–M) Longitudinal Decrease program, following (B)–(E).

(N–Q) Sustained Downregulation program, following (B)–(E).

p values in (B), (F), (J), and (N) were calculated with Student's t test on the linear regression slope coefficient. Survival outcome p values were calculated with the log-rank test. All others were calculated with Mann-Whitney U test with Benjamini-Hochberg correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

See also Figure S3.

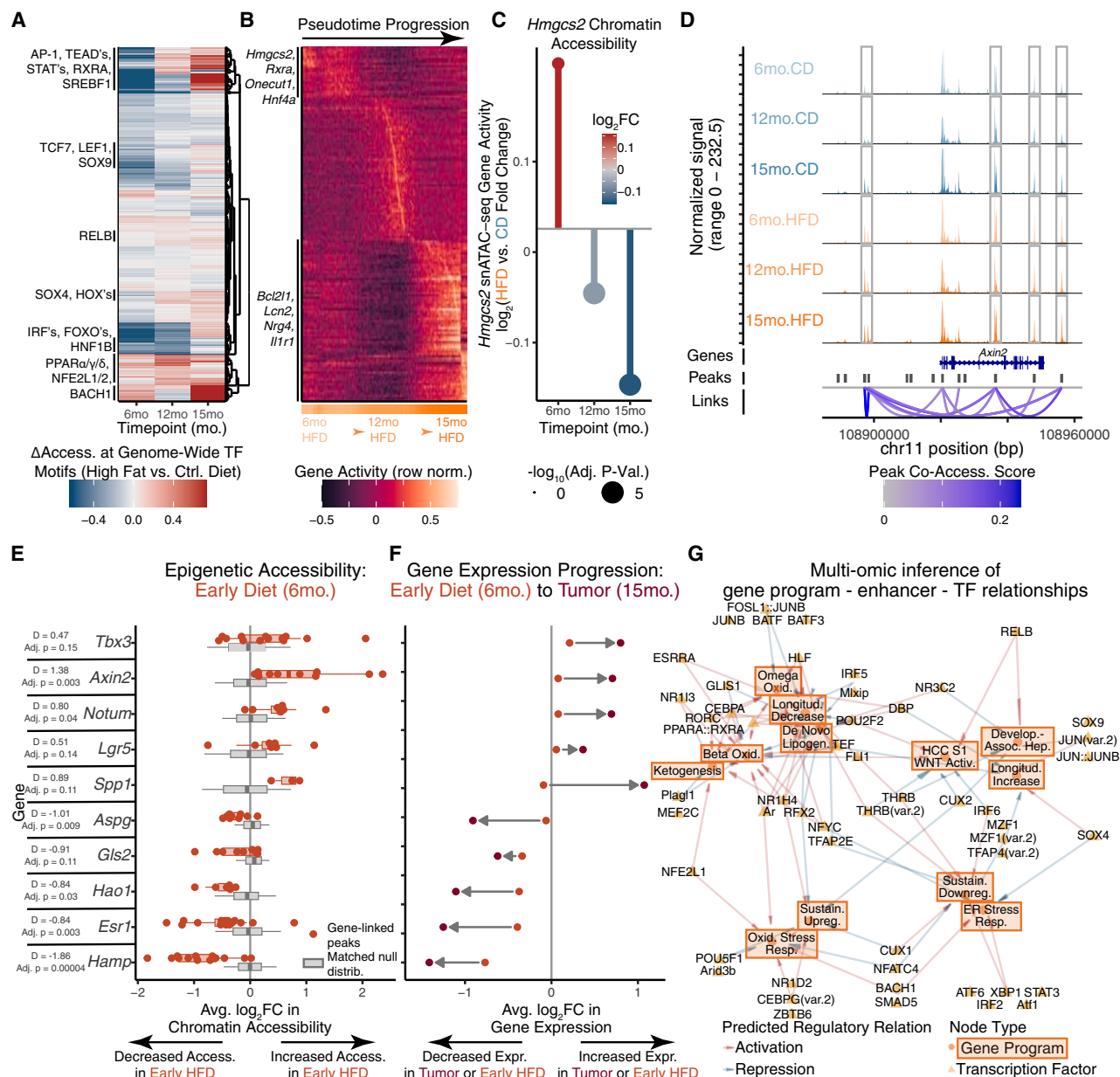


Figure 5. Chronic metabolic stress drives altered epigenetic trajectories and WNT pathway priming

(A) Chromatin accessibility deviation of TF motifs across age and diet ($n = 13$ mice, 48,010 hepatocytes).

(B) Epigenetic gene activity trajectories of HFD hepatocytes ordered by pseudotime progression.

(C) Chromatin accessibility gene activity at the *Hmgcs2* genomic locus $\log_2$ (high-fat vs. CD fold-change).

(D) Coverage plot of epigenetic accessibility at the *Axin2* gene locus across age and diet.

(E) Accessibility changes of gene-linked peaks at the 6-month time point, between actual observed HFD-vs.-CD fold-changes (red) and GC-and-background-matched null distribution (gray).

(F) Expression changes of genes at 6-month HFD-vs.-CD (red) or 15-month tumor-vs.-adjacent-normal (brown) comparisons.

(G) MATCHA-inferred network of TFs predicted to regulate MASLD-relevant gene programs.

All p values were calculated using a Mann-Whitney U test with Benjamini-Hochberg correction, and effect size was quantified through Cohen's D.

See also [Figures S4](#) and [S5](#) and [Table S2](#).

via Hierarchical Association). Harnessing user-specified gene programs and multi-omic datasets (optionally spanning species), MATCHA nominates core regulatory TFs of diverse pro-

grams via context-dependent modeling of cell-type- and tissue-specific gene regulatory landscapes ([STAR Methods](#); [Resource availability](#)).



As a proof-of-concept, MATCHA recovered ground-truth causal TFs for metabolic stress-relevant signatures defined externally through GO:BP.^{47,104,105} The top TFs prioritized for GO:BP's endoplasmic reticulum (ER) stress response signature were XBP1 and ATF6, well-established master regulatory TFs (Figures S5A–S5G).¹³² Top TFs for GO:BP's β -oxidation signature included FXR/NR1H4 and PPARA (both with agonists reaching phase 3 clinical trials for MASH fibrosis)^{133,134} and NR1I2, CEBPA, and NR1I3 (all with preclinical evidence for liver metabolic regulation) (Figures S5H–S5N).^{135,136}

To identify core TFs driving hepatocytes' wide-ranging stress responses, we applied MATCHA to construct a bipartite regulatory network between gene programs and TFs, supported by epigenetic and transcriptional evidence across mice and humans (Figure 5G). Among identified TFs was THRB, whose agonist resmetirom recently became the first U.S. Food and Drug Administration-approved therapy for MASH with moderate-to-advanced fibrosis.^{137,138} MATCHA inferred regulatory links between (1) THRB motifs, (2) activation of Longitudinal Decrease and Sustained Downregulation programs, and (3) repression of Development-Associated and HCC S1 WNT programs (Figure 5G). We chose 15 TFs (20 isoforms) to prioritize for experimental validation (Figure S5O).

Validating cell-intrinsic drivers of stress responses that also increase proliferative capacity *in vivo*

To validate hepatocyte-intrinsic transcriptional drivers of metabolic stress responses, we conducted human *in vitro* TF overexpression in HepG2 cells in lipid-rich media,¹³⁹ with scRNA-seq, live-cell functional imaging, and immunofluorescence readouts (Figure 6A; Tables S2 and S5; Key resources table, Alexandria Project/Single Cell Portal: SCP3357). Negative control non-transduced or blue fluorescent protein-transduced cells clustered by media condition (i.e., lipid vs. control media) but not transduction status, supporting specificity of measured lipid-induced metabolic stress responses (Figure 6B). As a positive control, we confirmed that PPARA overexpression in lipid-rich media upregulated known targets (Table S5).¹⁴⁰ Likewise, RELB, JUN, and RORC overexpression drove metabolic processing genes and signaling regulators, consistent with these TFs as effectors of nuclear factor κ B (NF- κ B), AP-1, and metabolic sensing.^{141–144} SOX4, CUX2, and HLF decreased protein secretion genes and increased fetal-associated HCC markers, aligning with roles in liver organogenesis and development.^{117,145–148} We focused on SOX4 and RELB given their strong effects on metabolic stress-associated transcriptional and functional states.

SOX4 and RELB overexpression each increased the Longitudinal Increase, Sustained Upregulation, and HCC S1 signature programs, with simultaneous decreases in Longitudinal Decrease and Sustained Downregulation programs (Figures 6C, 6D, S6A, and S6B). Functionally, SOX4 and RELB decreased lipid accumulation but increased reactive oxygen species (ROS) accumulation and protein-level nuclear p53 accumulation. SOX4 additionally increased cell cycling (nuclear Ki67), as elevated proliferative capacity despite cellular stress (Figures S6C–S6G; STAR Methods).

Toward human *in vivo* relevance, SOX4 and RELB each increased with MASLD severity and predicted worsened HCC patient survival (Figures 6E–6H). Analogous to a “natural genetic perturbation experiment” in human HCC patients, copy-number variations (CNVs) at the RELB genomic locus drove significant changes in RELB expression itself but also downstream stress response programs with mutually concordant directionalities (Figures S6H–S6P; STAR Methods). Leveraging cohorts linking initial liver gene expression to long-term cancer incidence, elevated expression of SOX4 and RELB in non-transformed human liver tissue conferred elevated future HCC risk and rate, connecting SOX4 and RELB regulation of non-transformed hepatocyte stress responses to future human HCC tumorigenesis (Figures S6Q and S6R).

Given SOX4's *in vitro* transcriptional, functional, and proliferative effects, we pursued *in vivo* validation through AAV-mediated SOX4 overexpression in HFD mouse livers (Figures 6I and S6S–S6X; STAR Methods; Key resources table, Alexandria Project/Single Cell Portal: SCP3358). Through snRNA-seq reference mapping, a significantly increased proportion of SOX4-overexpressing hepatocytes mapped to the 15-month HFD time point of hepatocytes' natural metabolic stress progression (Figure 6J), indicating SOX4 drives and accelerates hepatocyte transcriptional reprogramming *in vivo*. SOX4 overexpression altered hepatocyte stress response programs with directionalities concordant with the months-to-years progression of chronic stress responses (e.g., upregulated developmental, pro-survival, and regeneration-linked cell cycle regulators; decreased metabolic enzymes and secreted proteins) (Figures 6K and 6L). Toward protein-level and functional effects *in vivo*, SOX4 overexpression directly increased the proportion of actively cycling Ki67+ hepatocytes, as well as increased protein abundance of cyclin D1, known to increase with proliferation (Figures 6M–6O). Finally, SOX4 overexpression increased the protein-level abundance of WNT effector β -catenin (Figure 6O). Collectively, our data suggest that hepatocyte-intrinsic factors can be sufficient to drive our transcriptional stress response programs and potentiate hepatocyte proliferation within a diet-stressed liver microenvironment.

(I) Mouse *in vivo* genetic perturbation experimental schematic.

(J) Reference mapping of transcriptional profiles from hepatocyte SOX4 vs. EGFP overexpression snRNA-seq data ($n = 4$ mice, 1,424 hepatocytes) to natural progression of responses to chronic metabolic stress ($n = 13$ mice, 51,065 hepatocytes).

(K) Hepatocyte differential expression with SOX4 overexpression.

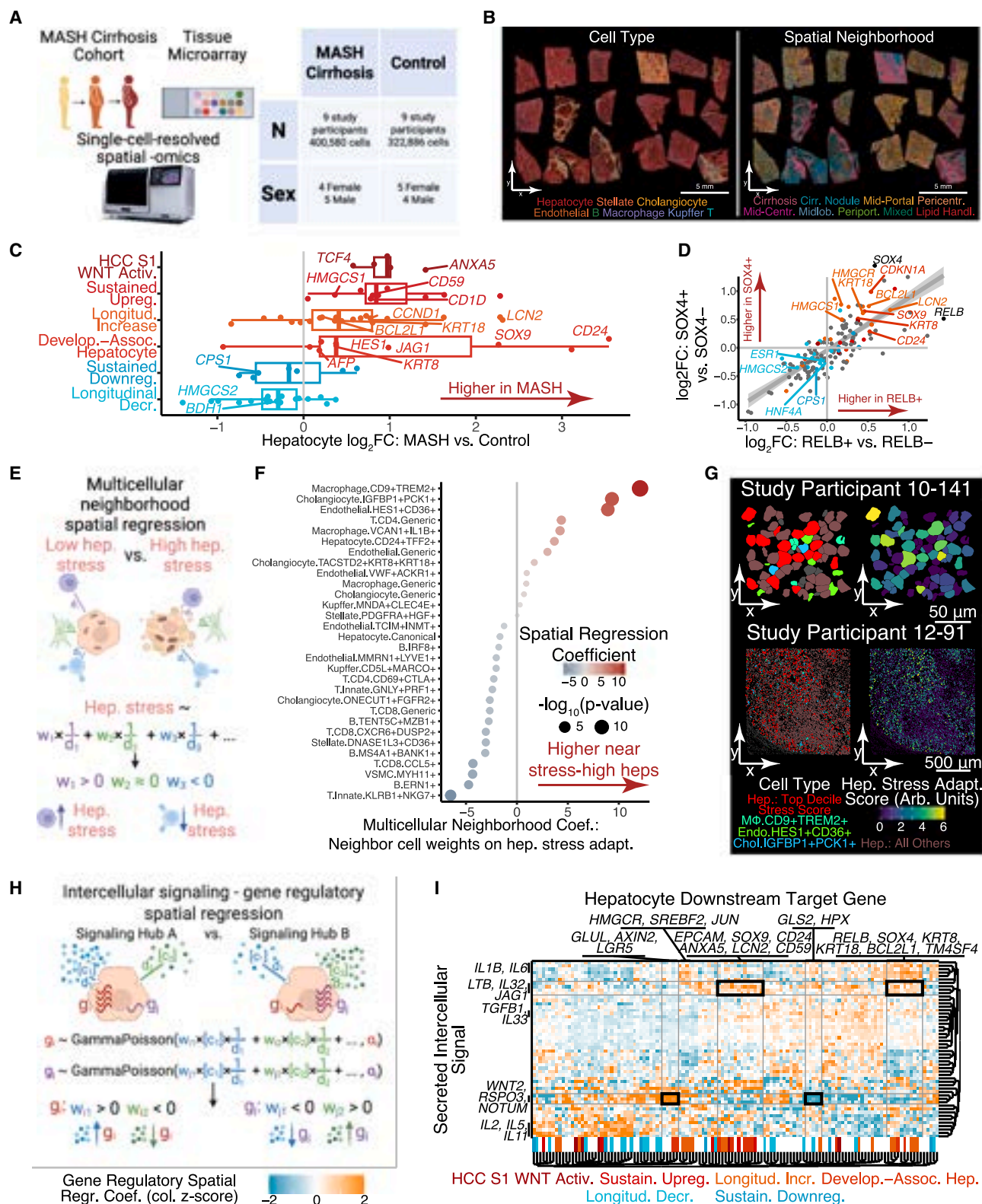
(L) Distribution of stress response program differential expression with SOX4 overexpression.

(M and N) Mouse liver anti-Ki67 immunohistochemistry representative images (M, scale bar, 100 μ m) and quantification (N) with SOX4 overexpression.

(O) Mouse liver western blotting with SOX4 overexpression.

p values in (E) and (G) were calculated with Student's t test on the linear regression slope coefficient. Survival outcome p values were calculated with the log-rank test, and all other p values were calculated with a Mann-Whitney U test with Benjamini-Hochberg correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

See also Figure S6 and Tables S2 and S5.



(legend on next page)

Human spatial transcriptomics identifies spatially structured signaling drivers of hepatocyte stress responses

Finally, we sought to uncover potential environmental signals and spatially structured intercellular interactions that might participate in our hepatocyte stress response programs. To extend our stress response findings to the tissue context of MASH cirrhosis, we assembled a sex-balanced human MASH cirrhosis liver tissue microarray and conducted single-cell-resolved spatial transcriptomics ($n = 18$ study participants, 723,466 cells) (Figures 7A, 7B, and S7; Table S2; Key resources table, Alexander Project/Single Cell Portal: SCP3359).

Corroborating our prior cross-species multi-omics analyses, hepatocyte stress response programs changed with predicted directionalities in human MASH cirrhosis: increases in the Human HCC S1 WNT activation, Sustained Upregulation, Longitudinal Increase, and Development-Associated hepatocyte gene programs and decreases in the Sustained Downregulation and Longitudinal Decrease gene programs (including strong downregulation of *HMGCS2*) (Figure 7C; complementing human transcriptional and proteomic analyses in Figures 3, 4, 6, S2, S3, and S6). Supporting MATCHA computational inference and genetic perturbation validations, SOX4+ and RELB+ hepatocytes exhibited higher expression of stress-increased gene expression programs and lower expression of stress-decreased gene program members (including *HMGCS2*), relative to SOX4- and RELB- hepatocytes (Figure 7D).

Toward potential contributions of adipose-liver signaling, we found that hepatocyte spatial stress responses remained robust in human study participants with lean MASH vs. MASH with elevated BMI (BMI 26.6 ± 0.81 vs. 35.7 ± 0.80 , corresponding to weight differences of ~ 50 pounds) (Figure S7E; STAR Methods).¹⁴⁹ Through meta-analyses of mouse models disentangling obesity and liver pathology, as well as human liver organoids treated with lipids or the cytokine TGFB1, we likewise found neither obesity alone nor lipid alone was sufficient to drive advanced metabolic stress responses (Figures S7F–S7H).^{131,150–152}

Next, to identify disease-associated intercellular interactions and multicellular hubs, we implemented geographic regression on hepatocyte stress responses as a function of local neighborhood cellular compositions (Figure 7E; STAR Methods). Most strongly co-localized with extreme hepatocyte stress responses were *CD9+TREM2+* scar-associated macrophages, implicated in increasing stellate cell collagen transcription in cirrhosis (Figure 7F).¹⁵³ Concordantly, in our mouse HFD scRNA-seq

and multiplexed immunofluorescence, we found enrichment of these scar-associated macrophages. We note that these cells were enriched in our mouse model even at early disease stages (Figures S7I–S7N).^{153–155} The second- and third-most enriched cell types in our human MASH cirrhosis microarray spatial-omics (*IGFBP1+PCK1+* cholangiocytes and *HES1+CD36+* endothelial cells) have each been linked to liver disease responses,^{156,157} and these cell types' spatial co-localization suggests signaling interactions promoting hepatocytes' stress responses. Spatial hubs of these three cell types colocalizing with hepatocyte stress responses could be readily seen at the microscale and mesoscale (Figure 7G).

To reveal spatially structured signaling regulatory networks, we implemented geographic regression on each individual hepatocyte gene as a function of secreted signaling protein transcripts in hepatocytes' local neighborhoods (negative binomial/Gamma-Poisson generalized linear modeling) (Figure 7H; STAR Methods). Reassuringly, our approach recapitulated spatially structured periportal-pericentral WNT gene regulatory circuits, where pericentrally abundant WNT ligands were (1) the strongest positive signals driving known pericentral markers and well-established WNT targets and (2) the strongest negative, suppressive signals for periportal markers (Figure 7I). Our analysis prioritized *LTB*, *JAG1*, and *IL32* as key signals positively co-regulating members of the Sustained Upregulation and Longitudinal Increase gene programs, including dedifferentiation markers, cell death-protective genes, and damage-associated downstream signaling. *LTB* activates non-canonical NF- κ B signaling via RELB.¹⁵⁸ Likewise, *JAG1*-mediated Notch signaling promotes liver fibrosis,¹⁵⁹ and circulating interleukin (IL)-32 is a MASLD biomarker.¹⁶⁰ *RELB* and *SOX4* themselves co-clustered and exhibited similar upstream signaling regulation as members of the Sustained Upregulation and Longitudinal Increase gene programs, further implicating these TFs with hepatocyte stress responses.

DISCUSSION

During chronic environmental stress, cells must rebalance their activities to ensure survival while contributing to tissue functions essential for organism-level physiology. We show that hepatocyte responses to chronic metabolic stress include activating regeneration-linked and development-associated states while suppressing canonical tissue functions, priming tumorigenesis. The cellular programs we identify are active—and in some instances exacerbated—from the early stages of chronic stress

Figure 7. Spatial transcriptomics atlas of human MASH reveals multicellular signaling context of hepatocyte stress responses

- (A) Experimental schematic and study participant table.
 (B) Tissue microarray colored by cell type (left) and multicellular neighborhood membership (right) ($n = 18$ patients, 723,466 cells).
 (C) Hepatocyte expression changes between MASH vs. control.
 (D) Hepatocyte expression changes with RELB and SOX4.
 (E) Schematic of multicellular neighborhood spatial regression.
 (F) Multicellular neighborhood spatial regression coefficients, linking fine-level cell states to stress responses in surrounding hepatocytes.
 (G) Representative fields-of-view of multicellular neighborhoods and stress responses in neighboring hepatocytes, at microscale (top) and mesoscale (bottom).
 (H) Schematic of intercellular signaling gene regulatory spatial regression.
 (I) Gene regulatory spatial regression coefficients, linking intercellular signals to gene expression in surrounding hepatocytes.
 p values in (F) calculated with Student's t test on the linear regression slope coefficient. See also Figure S7 and Table S2.

to late-stage alterations associated with cancer and predict cancer survival. Moreover, they generalize across species and assays, ranging from bulk, single-cell, and spatial transcriptomics to proteomics, with corresponding lipidomic readouts. Dissecting cell-intrinsic regulatory networks, we demonstrate that epigenetic dysregulation precedes and presages these transcriptional and proteomic shifts, suggestive of epigenetic priming. We computationally identify and experimentally validate causal hepatocyte-intrinsic regulators sufficient to drive stress response programs and functional outcomes *in vivo*. Through spatial -omics, we further uncover multicellular niches and signaling interactions that may extrinsically shape hepatocyte stress responses and their regulatory mechanisms. Our work supports a model in which hepatocytes' initial lipid responses—mediated, in part, by SOX4 activation and HMGCS2 repression—trigger multicellular circuits (e.g., cell-intrinsic epigenetic rewiring and cell-extrinsic spatially structured intercellular signaling) that converge back on hepatocytes to activate generalizable stress responses, priming tumorigenesis.

Our diet-only mouse model exhibits functional, histologic, and cellular phenotypes paralleling human MASH without genetic manipulation or exogenous chemical insults. Although our HFD mice do not develop significant bridging fibrosis (possibly due to mouse-vs.-human lifespan differences), we showed our murine findings extend to clinical human cirrhosis samples, as well as multiple external cohorts. Collectively, our datasets provide rich resources on the intersection of aging and metabolic stress: longitudinal mouse single-cell multi-omics, human spatial transcriptomics, and harmonized human MASLD/HCC transcriptomic and proteomic cohorts. When coupled with the work of others, they suggest our stress response programs may have broad relevance to acute regeneration, development, and wide-ranging HCC risk factors (alcohol, viral infections, and MASLD), indicative of conservation and convergence across diverse classes of insult.

Progressive decreases in lineage-determining HNF4A and canonical hepatocyte functional genes, alongside increases in developmental markers, raise the question of how stress responses relate to dedifferentiation. Partial hepatocyte dedifferentiation occurs following acute stressors like hepatectomy and acetaminophen overdose, where subsets of hepatocytes downregulate canonical functions and activate fetal-like states to restore liver mass.^{4–6,113,161} Genetically induced dedifferentiation priming improves hepatocyte survival during subsequent acute stressors,¹⁰⁷ but long-term dedifferentiated states predispose to liver failure and worsened survival in HCC.^{162–164} Dedifferentiation is a cancer hallmark, where cancer cells unlock fetal, plastic cell states associated with elevated proliferation and tumor progression.^{165,166} Our data suggest environmental stress may drive long-term maintenance of partially dedifferentiated states in non-transformed hepatocytes, thereby causing (1) improved survival of individual cells by activating the liver's regenerative capacity but (2) maladaptive reductions in the liver's tissue-level functions and homeostatic setpoints, ultimately incurring (3) early induction and priming of transcriptional and epigenetic programs that support cancer formation.

Our work reveals that HMGCS2 reductions during chronic metabolic stress can prime future tumorigenesis by (1) acceler-

ating extreme stress response transcriptional states and then (2) increasing tumor formation after oncogenic mutation introduction. As context, fatty acid oxidation produces acetyl-CoA that can be condensed to form acetoacetyl-CoA, and ketogenesis can then transform acetoacetyl-CoA into ketone bodies. Acetyl-CoA accumulation has been proposed to increase liver tumorigenesis risk via histone acetylation that facilitates broadly accessible, permissive chromatin landscapes.¹⁶⁷ By contrast, hepatocytes primarily synthesize ketone bodies and export them from the liver but largely do not catabolize them.^{168–172} Our work shows that HMGCS2 decreases are a sufficient mechanistic driver to heighten hepatocyte stress response programs and subsequently support tumorigenesis, elevating altered metabolic pathway usage as a unifying cause of progressive transcriptional dysregulation and ultimate cancer formation alike. It remains to be seen if pharmacologic augmentation of HMGCS2 expression (potentially through pan-PPAR agonists such as those currently in phase 3 clinical trials, NCT04849728) can successfully reverse these effects. Likewise, future work will be needed to uncover potential upstream drivers of HMGCS2 loss: our human liver tissue spatial transcriptomics support links between increased SOX4 abundance and decreased HMGCS2 expression (Figure 7D). Likewise, insulin signaling, LXR α agonists, or fibroblast growth factor 21 (FGF21) knockout can each decrease HMGCS2 abundance and/or ketogenesis pathway usage.^{173–176}

We show that hepatocytes' natural responses to chronic metabolic stress epigenetically prime WNT members and HCC markers for transcriptional alterations months later, extending prior experimental perturbation studies of WNT regulators like RNF43/ZNRF3.^{127,130} In other organs and diseases, AP-1 synergizes with context-specific TFs to maintain poised epigenetic landscapes and transcriptional heterogeneity that serve as memory of past inflammation and can be selective in response to subsequent environmental pressures.^{25,27,28} For instance, in the skin and pancreas, inflammatory bouts lasting only a handful of days are sufficient to drive effects months later (e.g., improved wound healing and increased cancer risk),^{21,22,26,177} suggesting even short stressors can instill persistent tissue memory and altered tissue function. In nasal epithelium, cytokine modulation can partially revert dysfunctional progenitor memory of allergic inflammation toward therapeutic restoration of homeostatic baselines.^{116,178} Hepatocytes' primed accessibility at AP-1 binding motifs and WNT-related loci occurred at relatively early disease stages preceding when many patients are diagnosed.⁹ Future investigations into the timescales of stress response initiation, its persistence, and its functional manifestations *in situ* could provide not only fundamental biological understanding but also potential prognostic and therapeutic value before overt disease. Moreover, given evidence that weight loss and newer pharmacological agents can stabilize or even reverse histologic features of MASLD/MASH (e.g., GLP-1 receptor agonists), a key question will be to what extent this memory of chronic stress can be erased by lifestyle or therapeutic interventions and the potential implications for HCC risk even after histologic evidence of disease improvement.^{179–182}

To uncover core TFs that causally unify user-specified gene programs, like our hepatocyte stress responses, we developed

MATCHA. MATCHA infers gene programs—co-accessible enhancer—causal TF triads. One key strength is MATCHA's emphasis on incorporating tissue and cell-type-specific data, capturing the context dependence of chromatin regulatory landscapes and TF activity. Toward uncovering “central hub” TFs with strong co-regulatory connections across the multiple stress response programs derived here, MATCHA recovered ground truths and targets of phase 3 clinical trials but also discovered comparatively less-explored TFs that we experimentally validated *in vitro* and *in vivo*. Thus, MATCHA advances the discovery of individual regulatory nodes sufficient to co-regulate and couple phenotypes with complex functional associations and temporal trajectories.

Validating MATCHA predictions, RELB and SOX4 regulated hepatocyte stress response transcriptional programs and functional metabolic phenotypes. RELB constitutes the transcriptional effector of the non-canonical NF- κ B signaling pathway,¹⁸³ which has received much less attention in liver (patho)physiology than canonical NF- κ B signaling (with the two pathways having different activating ligands, intracellular signaling cascades, and output phenotypic effects).^{30,183–192} Likewise, SOX4 is active during fetal liver epithelial progenitor fate specification toward cholangiocytes,^{108,117} and ectopic SOX4 expression *in vivo* drives lineage reprogramming in bile duct injury models and hepatoblastoma (rare pediatric liver cancer, 80%–90% of patients with WNT-activating *CTNNB1* mutations).^{193–196} Our work demonstrates that natural hepatocyte responses to chronic metabolic overload are critically mediated by SOX4 and, for the first time to our knowledge in the context of highly prevalent environmental stressors, reveals SOX4's direct gene regulatory and functional effects: downregulating canonical hepatocyte functions, activating regenerative and cell survival programs, and increasing proliferative capacity in stressful tissue environments that collectively align with the posited de-differentiation interpretation of hepatocyte stress response programs.

Complementing our discovery and validation of cell-intrinsic drivers, we defined multicellular signaling hubs and upstream signals poised to promote hepatocyte stress responses through spatial transcriptomics atlases of human MASH cirrhosis patients. The discovery of recurrent stress-enriched cellular communities suggests multi-lineage crosstalk, where immune, epithelial, and structural cell states mutually support and shape progressive hepatocyte responses to chronic metabolic stress. Signals nominated through our analyses represent a rich springboard for future investigations into cell-extrinsic causal drivers of maladaptive epithelial alterations and future tumorigenesis. One open question centers on the relative therapeutic merits of targeting hepatocytes' cell-intrinsic stress response regulators directly (i.e., as immediate drivers sufficient to drive stress responses programs and tumor outcomes) vs. modulating cell-extrinsic drivers (i.e., to rebalance the tissue-scale multicellular coordination shaping hepatocytes' states and fates). Likewise, future work will be necessary to determine how distinct coresident cell types and signals inform the content and durability of hepatocytes' stress memory and how these may vary across different stressors.

One final important question is whether stress response programs uncovered in this work are necessarily co-regulated or can be disentangled for selective modulation. Pareto analyses

have investigated core archetypes of cellular contributions to collective tissue-scale tasks, such as division of labor along the liver's periportal-pericentral axis.^{197–199} However, these analyses focused on steady-state healthy hepatocytes and did not consider dynamics or disease progression. Thus, disease contexts present additional complexity (but also opportunities) for understanding how cells and tissues navigate the state space of potential phenotypes and maintain essential functions despite external stressors. Our work revealed transcriptional and metabolic central hub regulators of wide-ranging dysfunction and tumor priming, unifying diverse stress program temporal trajectories. Future work could attempt to decouple these programs, therapeutically activating only beneficial features while mitigating deleterious effects. Engineered transcriptional activators may support both cellular survival and tissue function without priming worsened cancer outcomes. Alternatively, targeting key enzymes to tune relative metabolic fluxes could provide greater control of tissue homeostatic setpoints, thereby buffering against environmental stressors. Similarly, modulation of select hepatocyte-extrinsic factors may potentiate preferred hepatocyte and tissue-level responses, while mitigating others.

Together, our work demonstrates an underappreciated role for how cells' longitudinal responses to environmental stresses seed future cancer risk and worsen patient survival. Our data suggest that early aberrant activation of regeneration-linked and development-associated programs occurs concomitantly with blunting of canonical tissue functions, creating a fertile transcriptional and epigenetic milieu for tumorigenesis upon eventual mutational accumulation, months to years later. Ultimately, our work provides a foundation for revealing the principles behind cellular and tissue decision-making during stress, translating complex descriptions of disease dysfunction into unifying core mechanisms, and deriving fundamental connections on how even early stress can precipitate cellular responses and activity rebalancing, which prime long-term tumorigenesis.

Limitations of the study

As one limitation, our mouse *in vivo* HMGCS2 experiments leveraged the widely used, liver-specific Alb-Cre knockout, which causes gene deletion in both hepatocytes and cholangiocytes during embryogenesis. To control for potential epigenetic or developmental compensation, our experiments included all combinations of diet and genotype conditions, helping support specificity of findings to the combination of HMGCS2 loss and chronic metabolic stress. Future work could include extensions to HMGCS2 perturbations at different stages of development or stress progression. Likewise, within the liver epithelial compartment targeted by the constitutive Alb-Cre promoter, hepatocytes comprise ~97%–99% of liver epithelial cells, whereas cholangiocytes comprise ~1%–3%.²⁰⁰ Furthermore, individual hepatocytes have ~3-fold higher expression of *Hmgcs2* than do individual cholangiocytes, and hepatocytes are canonically considered to be the predominant site of ketogenesis.^{169,201} As another limitation, a remaining open question includes how ketogenesis decreases result in intermediate epigenetic alterations en route to transcriptional stress alterations and functional tumorigenesis priming, in line with metabolite-epigenetic cross-talk as a research frontier in cancer metabolism generally.²⁰² We

previously showed that ketone bodies regulate intestinal stem cell regenerative capacity via histone deacetylase inhibition.^{203,204} Future work could further unveil interactions by which ketogenesis and associated metabolites regulate the hepatocyte stress responses. Finally, we emphasize that our use of the term “chronic metabolic stress” acknowledges potential contributions from diet-only modeling to diverse intermediates (e.g., direct nutrient overload and imbalance, insulin resistance, gut-liver crosstalk, adipose-liver signaling, microbiome-liver interactions, liver innervation, etc.) that collectively converge on phenotypic alterations and sufficient causal drivers discovered here.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Alex Shalek (shalek@mit.edu).

Materials availability

This study did not generate new, unique reagents.

Data and code availability

All code used for single-cell and spatial analyses are accessible at Zenodo under <https://doi.org/10.5281/zenodo.17387657>. MATCHA is available on GitHub for download and use at <https://github.com/ctzouanas/MATCHA>. For data generated in this study, scRNA-seq, snRNA-seq, snATAC-seq, and spatial transcriptomics digital gene expression matrices, metadata, and interactive visualization tools can be found through the Alexandria Project, a Bill & Melinda Gates Foundation-funded portal (part of the Single Cell Portal hosted by the Broad Institute of MIT and Harvard), and specific study IDs are listed in the [key resources table](#). This paper analyzes existing, publicly available data, accessible at accession numbers listed in the [key resources table](#). Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

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STAR★METHODS

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

- **KEY RESOURCES TABLE**
- **EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS**
 - Mouse Husbandry and High Fat Diet
 - Human Banked Samples for Tissue Microarray
- **METHOD DETAILS**
 - Mouse Functional Testing and Histology
 - Multiplexed Immunofluorescence
 - Immunoblotting
 - Lipidomics
 - Live-Tissue Dissociation for scRNA-seq
 - Nuclei Isolation for snRNA-seq + snATAC-seq
 - Lentiviral Production
 - Lentiviral Transduction
 - Liver Cell Lipid Culture
 - Liver Cell scRNA-seq and Functional Imaging
 - Human Liver Tissue Microarray Construction
 - Human Liver Xenium Spatial Transcriptomics
- **QUANTIFICATION AND STATISTICAL ANALYSES**
 - sc/snRNA-seq QC, Filtering, and Annotation
 - snATAC-seq QC, Filtering, and Annotation
 - Gene Program Derivation
 - Cancer Signature Induction in Stress Response
 - Longitudinal Epigenetic Alterations & Priming
 - MATCHA Transcription Factor Inference
 - Control Analyses of RELB CNVs on Programs
 - Xenium Spatial Transcriptomics

SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit monoclonal anti-HMGCS2	Abcam	Cat#ab137043; RRID: AB_2749817
Rabbit polyclonal anti-Ki67	Abcam	Cat#ab15580; RRID: AB_443209
Rabbit monoclonal anti-Myc tag	Cell Signaling Technology	Cat#2278; RRID: AB_490778
Donkey anti-rabbit IgG (H+L)	Jackson ImmunoResearch	Cat#711-067-003; RRID: AB_2340595
Rabbit polyclonal anti-A1CF	ThermoFisher	Cat#PA5-60608; RRID: AB_2637598
Chicken polyclonal anti-ALB	Abcam	Cat#ab106582; RRID: AB_10888110
Chicken polyclonal anti-APOA1	ThermoFisher	Cat#PA5-20721; RRID: AB_11153391
Rabbit monoclonal anti-EGFR	Abcam	Cat#ab52894; RRID: AB_869579
Rabbit monoclonal anti-F10	Abcam	Cat#ab196023; RRID: AB_3717528
Mouse monoclonal anti-GLUL	BD	Cat#BDB610518; RRID: AB_397880
Guinea pig polyclonal anti-keratin 8/18	Progen	Cat#GP11; RRID: AB_2904125
Rabbit monoclonal anti-HNF4A	Abcam	Cat#ab201460; RRID: AB_2927380
Rabbit polyclonal anti-CXCR6	ThermoFisher	Cat#PA5-79117; RRID: AB_2746233
Rat monoclonal anti-TOX	Abcam	Cat#ab237009; RRID: AB_2883984
Rabbit monoclonal anti-CD4	Abcam	Cat#ab183685; RRID: AB_2686917
Rabbit monoclonal anti-CD8A	Abcam	Cat#ab217344; RRID: AB_2890649
Mouse monoclonal anti-CTNNB1	BD Biosciences	Cat#610154; RRID: AB_397555
Rabbit monoclonal anti-CCND1	Cell Signaling Technology	Cat#2978; RRID: AB_2259616
Rabbit monoclonal anti-VCL	Cell Signaling Technology	Cat#13901; RRID: AB_2728768
Horse anti-mouse IgG; HRP-linked	Cell Signaling Technology	Cat#7076; RRID: AB_330924
Goat anti-rabbit IgG; HRP-linked	Cell Signaling Technology	Cat#7074; RRID: AB_2099233
Rat monoclonal anti-Ki67	ThermoFisher	Cat#14-5698-82; RRID: AB_10854564
Rabbit monoclonal anti-p53	Cell Signaling Technology	Cat#2527; RRID: AB_10695803
Bacterial and virus strains		
AAV8-TBG-SOX4:WPPE	VectorBuilder	N/A
AAV8-TBG-EGFP:WPPE	VectorBuilder	N/A
Chemicals, peptides, and recombinant proteins		
High-fat diet for mice	Research Diets	Cat#D12492
Control diet for mice	Research Diets	Cat#D12450J
Critical commercial assays		
Vectastain Elite ABC immunoperoxidase detection kit	Vector Laboratories	Cat#PK6100
Signalstain DAB substrate kit	Cell Signaling Technology	Cat#8049
ImmPACT AMEC Red	Vector Laboratories	Cat#SK-4285
Chromium Next GEM Single Cell ATAC v1.1	10x Genomics	Cat#1000175
10x Xenium (Human Multi-Tissue and Cancer)	10x Genomics	Cat#1000626
Illumina Sequencing: NextSeq 500/550, 2000, 6000	Illumina	N/A
Deposited data		
Longitudinal mouse <i>in vivo</i> high-fat vs. control diet liver scRNA-seq (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3354

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Longitudinal mouse <i>in vivo</i> high-fat vs. control diet liver snRNA-seq (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3352
Mouse <i>in vivo</i> [HMGCS2 liver knockout vs. wild-type] × [high-fat vs. control diet] liver snRNA-seq (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3355
Longitudinal mouse <i>in vivo</i> high-fat vs. control diet liver snATAC-seq (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3356
Human <i>in vitro</i> transcription factor overexpression HepG2 scRNA-seq (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3357
Mouse <i>in vivo</i> SOX4 overexpression vs. EGFP control liver snRNA-seq (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3358
Human <i>in vivo</i> liver tissue microarray spatial transcriptomics (generated in this study)	This study	Alexandria Project: https://singlecell.broadinstitute.org/single_cell/study/SCP3359
Human <i>in vivo</i> liver bulk RNA-seq of MASLD disease progression (publicly available)	Govaere et al. ⁸²	GEO: GSE135251
Human <i>in vivo</i> liver bulk RNA-seq of MASLD disease progression (publicly available)	Pantano et al. ¹⁰¹	GEO: GSE162694
Human <i>in vivo</i> liver bulk RNA-seq of MASLD disease progression (publicly available)	Hoang et al. ¹⁰⁰	GEO: GSE130970
Human <i>in vivo</i> liver snRNA-seq of MASLD disease progression and HCC (publicly available)	Filliol et al. ⁹⁸	DOI: 10.1038/s41586-022-05289-6
Human <i>in vivo</i> liver bulk proteomics of MASLD and cirrhosis (publicly available)	Niu et al. ¹⁰²	PXD027722
Human <i>in vivo</i> liver HCC bulk RNA-seq, genomic copy number variations, and survival outcomes (publicly available)	TCGA Liver Cancer (LIHC) Cohort ¹⁰³	UCSC Xena (tcga.xenahubs.net)
Mouse <i>in vivo</i> liver scRNA-seq acute regeneration (publicly available)	Walesky et al. ¹¹³	GEO: GSE136679
Human <i>in vivo</i> bulk RNA-seq on non-transformed liver tissue and future cancer risk (publicly available)	Fujiwara et al. ⁹⁷	GEO: GSE193084
Mouse <i>in vivo</i> liver WNT activation via RNF43/ZNRF3 KO bulk RNA-seq (publicly available)	Belenguer et al. ¹²⁷	GEO: GSE133213
Mouse <i>in vivo</i> liver WNT activation via RSPO3 overexpression bulk RNA-seq (publicly available)	Uno et al. ¹²⁸	GEO: GSE282890
Human <i>in vitro</i> hepatocyte organoid WNT activation via WNT media bulk RNA-seq (publicly available)	Dowbaj et al. ¹²⁶	GEO: GSE274971
Mouse <i>in vivo</i> liver WNT decrease via endothelial or stellate cells RSPO3 KO bulk RNA-seq (publicly available)	Sugimoto et al. ¹²⁵	GEO: GSE256377
Human <i>in vitro</i> liver organoid lipid or TGFB1 treatment scRNA-seq (publicly available)	Hess et al. ¹³¹	GEO: GSE207889
Pericentral, RSPO-inducible gene signature	Sun et al. ¹³⁰	DOI: 10.1016/j.stem.2021.05.013
Pericentral gene signature	Ben-Moshe et al. ¹²⁹	DOI: 10.1038/s42255-019-0109-9
Mouse <i>in vivo</i> db/db vs. control liver bulk RNA-seq	Matsushita et al. ¹⁵⁰	GEO: GSE188344

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Mouse <i>in vivo</i> methionine-choline-deficient vs. control diet liver bulk RNA-seq	Paquette et al. ¹⁵¹	GEO: GSE156918
Mouse <i>in vivo</i> methionine-choline-deficient vs. control diet liver bulk RNA-seq	Yue et al. ¹⁵²	GEO: GSE205974
Experimental models: Cell lines		
HepG2	MIT Koch Institute Cell Line Repository	idCELL#310
Experimental models: Organisms/strains		
Wild-type C57BL/6 mice	Jackson Laboratory	Strain #000664
<i>Alb-Cre</i> C57BL/6 mice	Maintained in lab (originally from Jackson Laboratory)	Strain #003574
<i>Hmgcs2^{fl/fl}</i> C57BL/6 mice	Maintained in lab (available from Jackson Laboratory)	Strain #068091
<i>Hmgcs2^{fl/fl}; Alb-Cre</i> C57BL/6 mice	Crossed in lab	N/A
Recombinant DNA		
pT3-EF5a-hMet-V5	Monga Lab	N/A
pT3-EF5a-S45Y-CTNNB1-Myc	Monga Lab	N/A
pCMV/Sleeping Beauty	Monga Lab	N/A
Plasmids to produce lentivirus overexpressing human transcription factors	Joung et al. ¹³⁹	AddGene: MORF Collection
Software and algorithms		
MATCHA	This study	https://github.com/ctzouanas/MATCHA
DropSeqTools	Macosko et al. ²⁰⁵	https://github.com/broadinstitute/Drop-seq
STARsolo	Kaminow et al. ²⁰⁶	https://github.com/alexdobin/STAR
Cumulus	Li et al. ²⁰⁷	https://github.com/lilab-bcb/cumulus
CellRanger	10x Genomics	https://www.10xgenomics.com/software
QuPath	Bankhead et al. ²⁰⁸	https://qupath.github.io/
CellPose	Stringer et al. ²⁰⁹	https://github.com/mouseland/cellpose

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mouse Husbandry and High Fat Diet

C57BL/6 mice in the MIT cohort were housed and cared for in accordance with the American Association for the Accreditation of Laboratory Animal Care and approved by MIT's Committee on Animal Care. A long-term high-fat diet containing 60% kcal from fats (Research Diets D12492) was provided ad libitum to male mice starting at the age of 8-12 weeks for 6-15 months. Sex- and age-matched control mice were provided a purified Control diet containing 10% kcal fats and matched sucrose (Research Diets D12450J). Our HFD liver injury recapitulated many of the cardiometabolic comorbidities seen in patients with MASLD including obesity (higher body weight), dyslipidemia (elevated serum cholesterol), systemic insulin resistance (impaired glucose tolerance), and hepatocellular damage (increased alanine aminotransferase levels) (Figures 1A–1D and S1).³⁹ Histologically, HFD livers exhibited (initially pericentral) steatosis, inflammation, ductular reaction, pericellular fibrosis, and hepatocellular ballooning (Figures 1C, 1D, and S1). Spontaneous HCC developed without manipulation after 15 months on HFD in two independent cohorts at different institutions (Figure S1). We additionally sought to contextualize phenotypes observed in the diet-only mouse model used in this work to those observed in human patients with the progression of MASLD and HCC. H&E and trichrome stains were blindly scored by a clinical liver pathologist,²¹⁰ revealing that HFD mice typically demonstrate simple steatosis at 6 months and MASH by 12 months (Figure S1). HFD livers developed classic ballooning degeneration by 12 months, often associated with human patients' progression to more severe MASH (Figure 1D).²¹¹ Hepatocyte ballooning was confirmed by a second clinical liver pathologist and further validated by negative staining of CK8/18^{212,213} (Figure 1D). These temporal patterns of steatosis and fibrosis align with the well-known "burnt-out NASH" and "cryptogenic cirrhosis" phenotypes where hepatic fat is lost in human patients with end-stage liver disease.^{11,214,215} As further contextualization of the scope of the model used in this work and resultant experimental datasets, a meta-analysis of 3,920 MASLD models found that ~75% of studies have durations shorter than our first timepoint,³⁹ significantly shorter than the full MASH progression and absent readouts as rich as parallel scRNA-seq, snRNA-seq, and snATAC-seq. As

part of their meta-analysis, Im et al. developed a 0-14 point scoring system for evaluating mouse models' metabolic stress phenotypes: 1 point each for obesity, insulin resistance, dyslipidemia, raised aminotransferases, lobular inflammation, portal inflammation, hepatocellular ballooning, NASH, fibrosis (presence/absence), maximum fibrosis stage (from 1-4), and hepatocellular carcinoma formation. Applying this scoring system to our study, the mouse model presented in this work captured 12 of the 14 points (obesity, insulin resistance, dyslipidemia, raised aminotransferases, lobular inflammation, portal inflammation, hepatocellular ballooning, NASH, presence of fibrosis, maximum fibrosis stage 2, and hepatocellular carcinoma formation). Contextualized against the broader landscape of mouse modeling efforts, comparisons to Im et al.'s meta-analyses indicate that the mouse model implemented in this work captures and mimics a variety of phenotypes important and associated with liver responses to metabolic overload. We also highlight that other models' acceleration of progression through genotoxic chemicals or genetic manipulation increases differences from human disease pathogenesis and progression, especially when considering analyses targeted at understanding the natural dynamics of cellular trajectories during chronic stress.

C57BL/6 mice in the second mouse cohort at Brigham and Women's Hospital were housed and cared for in accordance with the American Association for the Accreditation of Laboratory Animal Care and approved by Brigham and Women's Hospital Committee on Animal Care, with diets started at the age of 4 weeks in these mice (Research Diets D12492 and D12450J). Spontaneous HCC developed in both mice cohorts after 15 months of HFD.

Alb-Cre mice (#003574) were purchased from Jackson Laboratory and described previously.²¹⁶ *Hmgcs2^{fl/fl}* were generated in-house and described previously.²⁰³ The following mice were bred in-house: *Hmgcs2^{fl/fl}*; *Alb-Cre*. Studies involving liver-specific loss of HMGCS2 were performed using littermates, whereby Cre-negative mice served as controls. For snRNA-seq experiments, mice were maintained on high-fat or control diets for 6 months. Live animal imaging was arranged through the Koch Institute at MIT Animal Imaging and Preclinical Testing Core and performed using a Varian 7T MRI imaging system. For hydrodynamic tail vein injection (HTVI) experiments, HMGCS2 WT and LiverKO mice were placed on a HFD for approximately 8 weeks (primary cohort) or 2 weeks (supplemental cohort). Hydrodynamic tail vein injection was performed according to Tao et al.⁹¹ Briefly, 10 μ g/mL of pT3-EF5 α -hMet-V5, 10 μ g/mL of pT3-EF5 α -S45Y-CTNNB1-Myc, and 0.8 μ g/mL of pCMV/Sleeping Beauty transposase (all plasmids a gift of Dr. Monga) were co-injected in a volume of 3mL (primary cohort) or 2.5mL (supplemental cohort) into the tail vein. Mice were humanely euthanized after 4 weeks in accordance with the American Association for the Accreditation of Laboratory Animal Care and approved by MIT's Committee on Animal Care.

For adeno-associated virus experiments, 10¹² GC virus (AAV8-TBG-SOX4:WPPE or AAV8-TBG-EGFP:WPPE, VectorBuilder) was diluted in 100 μ L PBS and injected retro-orbitally into C57BL/6 mice with dose and timing based on Katsuda et al.¹⁹³

Human Banked Samples for Tissue Microarray

A human liver tissue microarray was constructed from FFPE samples that were banked and stored for research purposes at Mass General Brigham (Mass General Brigham IRB number I00000363, Federalwide Assurance number FWA00000142, MGB IRB protocol #1999P004983). Complete cohort metadata with respect to MASH cirrhosis status, biological sex, age, and race/ethnicity can be found in metadata tables at Alexandria Project/Single Cell Portal SCP3359. Participants were chosen based on review of medical records for a history of MASLD/MASH, as well as liver histology annotated by expert pathologist review as MASH cirrhosis. Study participants were additionally filtered for: 1) negative for other potential liver diseases (e.g., hepatitis B, hepatitis C, alcoholic liver disease); 2) balanced representation of biological sex in MASH cirrhosis and control arms (i.e., overall cohort: 9 female and 9 male study participants; MASH cirrhosis: 4 female and 5 male study participants; control: 5 female and 4 male study participants); and, 3) representation of lean MASH (BMI mean=26.6, standard deviation=0.81) and MASH with elevated BMI (BMI mean=35.7, standard deviation=0.80) within our MASH cirrhosis arm. Study participants had a mean age of 53.9 years, standard deviation 15.8 years.

METHOD DETAILS

Mouse Functional Testing and Histology

Cholesterol, ALT, Albumin

For cholesterol, ALT, and albumin measurements, blood was collected through cheek bleed or cardiac puncture in Microvette 200 Z-Gel containers (Sarstedt 20.1291). Briefly, samples were centrifuged immediately after collection, and serum was frozen at -80°C before being submitted for IDEXX lab testing as coordinated by the Division of Comparative Medicine at MIT.

HMGCS2 IHC

Tissue was fixed in 10% normal buffered formalin prior to paraffin-embedding. 4-5 micron sections underwent deparaffinization and rehydration prior to antigen retrieval using Borg Decloaker RTU solution (Biocare Medical, BD1000G1) and a pressurized Decloaking Chamber (Biocare Medical, NxGen). Antibodies and respective dilutions used for immunohistochemistry are as follows: rabbit monoclonal anti-HMGCS2 (1:2000, Abcam, ab137043), rabbit polyclonal anti-Ki67 (1:1000, Abcam, ab15580), rabbit monoclonal anti-Myc tag (1:100, Cell Signaling Technology, #2278), and rabbit polyclonal anti-glutamine synthetase (1:1500, Sigma, #G2781) with dilutions performed in Signalstain Antibody Diluent (Cell Signaling Technology, #8112). Biotin conjugated secondary donkey anti-rabbit antibodies (1:500, Jackson ImmunoResearch) were used prior to Vectastain Elite ABC immunoperoxidase detection kit (Vector Laboratories, PK6100). Visualization was performed using Signalstain DAB substrate kit (Cell Signaling Technology, #8049). Counterstaining

was performed with 50% Gill No. 1 hematoxylin solution (Sigma-Aldrich, GHS116). Images were acquired using an Olympus BX43 microscope with 4x and 10x objectives (Olympus UPlanSApo).

Histologic Evaluation

H&E stained sections of liver tissue from 6, 12, and 15-month diet cohorts as well as identified tumor samples were reviewed in a blinded-manner by Dr. Vikram Deshpande, clinical hepatopathologist in the Department of Pathology at MGH and Beth Israel Deaconess Medical Center.

Sirius Red

For collagen staining, 5 μ m paraffin-deparaffinized sections were hematoxylin stained, washed in running tap water, and then stained with Picro Sirius Red for 1 hour (0.1% Direct Red 80 in picric acid).

Oil Red O

10 μ m frozen sections were briefly fixed, rinsed in isopropanol, and incubated in Oil Red O for 15min with hematoxylin counterstain.

Glucose tolerance

To measure systemic insulin resistance, intraperitoneal glucose tolerance testing was performed in 6-hours fasted mice as described.²¹⁷

IHC (CK8/18)

Staining was performed as described²¹³ with the following modifications. Antigen retrieval was in buffer TE (pH=9) in a pressure cooker (Biocare Medical Decloaking Chamber, DC2002). A primary antibody already targeting K8/18 was used instead of mixing K8 & K18 1:1 (Progen, GP11; 1:400), detected with ImmPACT AMEC Red per manufacturer instructions (Vector Laboratories, SK-4285), and counterstained with hematoxylin.

Multiplexed Immunofluorescence

Iterative, multiplexed immunofluorescence was conducted as previously described²¹⁸ with the following modifications. Briefly, samples were deparaffinized and antigen retrieved in TE (pH=9) in a pressure cooker (Biocare Medical Decloaking Chamber, DC2002). The following antibodies were used: anti-HNF4A (Abcam, ab201460; 1:400), anti-CXCR6 (Thermo Fisher, PA5-79117; 1:125), anti-TOX (Abcam, ab237009; 1:50), anti-CD4 (Abcam, ab183685; 1:500), and anti-CD8A (Abcam, ab217344; 1:500), anti-A1CF (ThermoFisher, PA5-60608, 1:100), anti-ALB (Abcam, ab106582, 1:400), anti-APOA1 (ThermoFisher, PA5-20721, 1:300), anti-EGFR (Abcam, ab52894, 1:250), anti-F10 (Abcam, ab196023, 1:500), anti-GLUL (BD, BDB610518, 1:1000). Imaging was conducted on a confocal Nikon Ti2 microscope with a Yokogawa CSU-W1. Primaries were incubated overnight at 4°C in a humidified slide box. For quantitative measurement of nuclear-localized HNF4A, Cellpose²⁰⁹ was used to segment nuclei based on DAPI signal with mean targeted diameter of 9 μ m (Cellpose optimized for hepatocyte nuclei). Nuclear masks from Cellpose were imported into CellProfiler²¹⁹ along with HNF4A channels. Nuclei with area < 20 μ m² were discarded to enrich for hepatocyte nuclei, and then mean fluorescence of nuclear segmentations in the HNF4A channel were computed using the MeasureObjectIntensity module.

Immunoblotting

Snap frozen liver tissue was crushed into a fine powder before elution with RIPA buffer and tissue homogenizer. After centrifuging at 10,000 rpm for 15 minutes at 4°C, the resulting supernatant was quantified using the Pierce BCA protein assay kit (23225, Thermo Fisher Scientific) then suspended in Laemmli SDS sample buffer (J61337-AC, VWR) at a concentration of 2 μ g/ μ l and boiled for 5 minutes at 95°C. 20 μ g protein per sample was separated on 4-12% gradient gel (NuPAGE Bis-Tris Mini Protein, Invitrogen, NP0336BOX) and transferred onto a 0.45 micron PVDF membrane (Immobilon-P transfer, Millipore, ipvh00010) and analyzed by immunoblotting using mouse monoclonal anti-beta-catenin (1:1000, BD Biosciences, #610154), rabbit monoclonal anti-Cyclin D1 (1:1000, Cell Signaling Technology, #2978), rabbit monoclonal anti-vinculin (1:1000, Cell Signaling Technology, #13901) followed by horseradish peroxidase (HRP)-conjugated IgG secondary antibodies (1:3000, Cell Signaling Technology, #7076, #7074) and ECL detection kit (ECL K-12045-D20 and Sirius K-12043-D20, Advansta). Quantitative chemiluminescent imaging was performed using the ImageQuant LAS 4000 (GE Healthcare).

Lipidomics

Isolated liver tissue was snap frozen in liquid nitrogen. 50mg of homogenized tissue was then sent to UCLA Lipidomics in 2mL homogenizer tubes pre-loaded with (6) 2.8mm ceramic beads (Omni #19-628). 750 μ l PBS was added to the tube and homogenized in the Omni Bead Ruptor Elite (3 cycles of 10 seconds at 5 m/s with a 10 second dwell time). Homogenate containing 1-8mg of original tissue was transferred to a glass tube for extraction. A modified Bligh and Dyer extraction was carried out on all samples.²²⁰ Prior to biphasic extraction, a standard mixture of 75 lipid standards based on Avanti Ultimate Splash ONE mix was used (Avanti 330820, 861809, 330729, 330727, 791642, 330726). Following two successive extractions, pooled organic layers were dried down in a Thermo SpeedVac SPD300DDA using ramp setting 4 at 35°C for 45 minutes with a total run time of 90 minutes. Lipid samples were resuspended in 1:1 methanol/dichloromethane with 10mM Ammonium Acetate and transferred to robovials (Thermo 10800107) for analysis. Samples were analyzed on the Sciex 6500+ with DMS device with an expanded targeted acquisition list consisting of 1450 lipid species across 17 subclasses. Differential Mobility Device was tuned with EquiSPLASH LIPIDOMIX (Avanti 330731). Data analysis was performed on an in-house data analysis platform comparable to the Lipidizer Workflow Manager.²²¹ Instrument method including settings, tuning protocol, and MRM list available in Su et al.²²¹ Quantitative values were normalized to mg of tissue.

Live-Tissue Dissociation for scRNA-seq

Two-step collagenase liver perfusion was performed as previously described.¹¹³ 15,000 cells were loaded onto a Seq-Well array for experimental processing. Samples were sequenced on an Illumina NextSeq 500/500, NextSeq 2000, or NovaSeq 6000.

Nuclei Isolation for snRNA-seq + snATAC-seq

For tandem Seq-Well snRNA-seq and 10x v1.1 snATAC-seq, the following solutions and volumes were prepared for isolation of nuclei from each sample. All solutions were pre-chilled and kept on ice except when actively handling the tissue or nuclei solution, and then immediately returned to ice.

- Base buffer: 100μL of 1M Tris-HCl pH 7.4, 20μL of 5M NaCl, 30μL of 1M MgCl₂, 1000μL of 10% bovine serum albumin
- Wash buffer + RNase inhibitor: 230μL of base buffer, 20μL of 10% Tween-20, 1.7mL of nuclease-free water, 50μL of Sigma-Aldrich Protector
- Wash buffer + digitonin: 230μL of base buffer, 20μL of 10% Tween-20, 1.746mL of nuclease-free water, 4μL of 5% digitonin
- 1X lysis buffer: 230μL of base buffer, 20μL of 10% Tween-20, 20μL of 10% Nonidet P40 substitute, 1.736mL nuclear-free water
- Lysis dilution buffer: 230μL of base buffer, 1.77mL of nuclease-free water
- 0.1X lysis buffer: 200μL of 1X lysis buffer, 1.75mL of lysis dilution buffer, 50μL of Sigma-Aldrich Protector
- Diluted nuclei buffer: 48.75μL of 20X Nuclei Buffer (from 10x v1.1 scATAC-seq reagent kit), 926.25μL of nuclease-free water, 25μL of Sigma-Aldrich Protector
- PBS + 1% BSA + RNase inhibitor: 875μL PBS, 100μL of 10% BSA, 25μL of Sigma-Aldrich Protector

Flash-frozen pieces of liver tissue were kept on dry ice; if needed, a smaller piece of tissue (~2-3mm diameter) was cut using a scalpel on a petri dish on dry ice. The tissue piece was placed into a Miltenyi C tube containing 2 mL of 0.1X lysis buffer, then homogenized using 2 iterations of the *m_spleen_01* program on the gentleMACS tissue dissociator. Half of the solution was passed through a 40μm filter pre-wet with 1mL wash buffer + RNase inhibitor, and the other half of the solution was passed through a separate 40μm filter prewet with 1mL wash buffer + digitonin. The C tube was washed with 1mL of wash buffer + RNase inhibitor to capture remnant nuclei stuck to the tube side or lid, and 500μL was transferred to each separate tube. Each solution was transferred to a separate 15mL Falcon tube and centrifuged for 10min at 500g and 4°C with brake set to 5 (out of a maximum of 10). The supernatant was aspirated, and each pellet was resuspended in 100μL of diluted nuclei buffer. Two 35μm filters were pre-wet with diluted nuclei buffer, and flow-through from the pre-wetting solution was removed to leave the tubes empty; each nuclei solution was then passed through the separate pre-wet 35μm filters using a P200 pipette. Each set of nuclei was counted using a hemocytometer, followed by snRNA-seq and snATAC-seq as previously described:

- 15,000 nuclei in 200μL of PBS + 1% BSA + RNase inhibitor as input to Seq-Well S³, as described in Hughes et al.²²²
- 7,000 × 1.53 nuclei in 5μL of diluted nuclei buffer as input to 10x snATAC-seq, as described in Chromium Next GEM Single Cell ATAC Reagent Kits v1.1 User Guide, CG000209 Rev F

A similar protocol was followed for nuclei isolation for snRNA-seq (i.e., without tandem snATAC-seq). The following modifications were made:

- As snATAC-seq was not conducted, the splitting of nuclei into a tube containing wash buffer + digitonin and subsequent parallel processing steps were omitted; all nuclei were passed through a single 40μm filter pre-wet with 1mL wash buffer + RNase inhibitor.
- At the 35μm filter step, instead of diluted nuclei buffer, the filter was pre-wet with PBS + 1% BSA + RNase inhibitor solution.

Samples were sequenced on an Illumina NextSeq 500/500, NextSeq 2000, or NovaSeq 6000.

Lentiviral Production

Plasmids encoding TF ORFs were obtained as generated in Joung et al.¹³⁹ (deposited in Addgene MORF Collection).

600,000 Lenti-X 293T cells were plated in 2mL of media (DMEM + 10% FBS + 1% penicillin-streptomycin) in a 6-well plate and incubated overnight at 37°C and 5% CO₂ (Day 1). The following afternoon (Day 2), 1μg of psPAX2, 0.33μg of pMD2.G, 1.33μg of ORF plasmid, 5.3μL of P3000 reagent, and 125μL of Opti-MEM were mixed, added to a solution of 6.7μL of L3000 and 125μL of Opti-MEM (not mixed), and incubated for 15min at room temperature. The mixture was added to the Lenti-X 293T cells and incubated overnight. The following morning (Day 3), Lenti-X 293T media was replaced. The following afternoon (Day 4), Lenti-X 293T media was harvested and replaced; the overnight media was passed through a 0.45μm low protein binding filter, combined with Lenti-X Concentrator at a 3:1 media:Lenti-X ratio, and stored overnight at 4°C. The following afternoon (Day 5), Lenti-X 293T media was again harvested and added to the previous day's media and Lenti-X Concentrator solution at 4°C for 30min. The media was spun at 1500g for 45min at 4°C. Supernatant was aspirated, and the pellet was resuspended in 160μL for storage at -80°C in 20μL aliquots.

Lentiviral Transduction

For each TF ORF, 500,000 HepG2 cells were plated in 2 mL of media (Advanced DMEM/F12 + 10% FBS + 1% penicillin-streptomycin) in a 6-well plate and incubated overnight at 37°C and 5% CO₂ (Day 1). The following morning (Day 2), lentiviral stocks of each TF ORF and polybrene were thawed to room temperature. Cells' media was replaced with a 10 µg/mL solution of polybrene in 2 mL of HepG2 media, followed by addition of 8 µL of lentivirus to separate HepG2 wells (i.e., arrayed format; 1 TF ORF per well). Cells were incubated with lentivirus overnight, and media was replaced the following morning (Day 3). The following afternoon (Day 4), media was replaced with a 1 µg/mL solution of puromycin in HepG2 media.

To produce stable, arrayed HepG2 lines overexpressing each TF, cells were maintained in puromycin-containing media to select for successful transduction, with puromycin-containing media being changed every 2–3 days. Upon expanding to reach confluency in a 6-well plate, each TF ORF-overexpressing HepG2 line was passaged and replated in a 10 cm dish. Upon reaching confluency in a 10 cm dish, each TF ORF-overexpressing HepG2 line was passaged to freeze down cell stocks (1,000,000 cells in 1 mL of 90% FBS + 10% DMSO at -80°C in a Mr. Frosty Freezing Container), followed by subsequent functional and transcriptomic assays in the metabolic stress of lipid-rich media.

Liver Cell Lipid Culture

HepG2 cells overexpressing each TF ORF were seeded in a 96-well plate at a density of 4,000 cells/well. As lipid-rich, metabolically-stressful media, TF ORF-overexpressing cells were cultured in Advanced DMEM/F12 media containing 10% FBS, 500 µM palmitic acid, 100 µM oleic acid, and 1% penicillin-streptomycin. As controls, BFP-transduced HepG2 cells and non-transduced HepG2 cells were each cultured in lipid-rich media or control media (Advanced DMEM/F12 media containing 10% FBS, 600 mM BSA control, and 1% penicillin-streptomycin). Each well received 200 µL of its respective media condition (i.e., TF ORF-overexpressing HepG2's in lipid-rich puromycin-containing media; BFP-transduced HepG2's in lipid-rich or control puromycin-containing media; non-transduced HepG2's in lipid-rich or control puromycin-free media). Media was changed 2 and 4 days after seeding, with assays occurring 7 days after seeding.

Liver Cell scRNA-seq and Functional Imaging

For scRNA-seq, HepG2 cells for each condition were seeded across triplicate wells. 7 days after seeding, cells were passaged, and triplicate wells for each condition were pooled and used as input for scRNA-seq using Seq-Well S³.

For functional imaging, cells were stained with BODIPY (2 µM final concentration), CellROX Deep Red (1:500 final dilution), and Hoechst 33342 (5 µg/mL final concentration) in PBS at 37°C and 5% CO₂. After 30 min incubation, cells were washed 3 times with PBS, followed by imaging on an Opera Phenix at 37°C and 5% CO₂.

After functional imaging, cells were fixed in a solution of 4% paraformaldehyde in PBS for 10 minutes, followed by a 3X wash with PBS. Cells were then permeabilized with 0.1% Tween (KI67 imaging wells) or 0.3% Triton-X (total p53 imaging wells) in PBS with 1% BSA for 10 min, followed by overnight incubation at room temperature with the primary antibody (anti-KI67 ThermoFisher SolA15 at 1:100 dilution, anti-p53 Cell Signaling Technology 7F5 at 1:800 dilution). The following morning, cells were incubated with secondary antibody and imaged on an Opera Phenix.

In the liver, p53 is known to have roles beyond its canonical anti-cancer genoprotective and antiproliferative functions. For instance, p53 modulates a variety of metabolic pathways (e.g., lipid processing, gluconeogenesis) in the liver, suggesting potential additional relevance for this TF in the context of metabolic overload.^{223,224} Towards metabolic functions of p53 during lipid overload, liver knockout of p53 in mice can produce steatosis, and p53 upregulation (via degradation of an upstream inhibitor) can alternatively attenuate fat accumulation.²²⁵ Additionally, elevated p53 activity has also been linked to progenitor-associated hepatocyte phenotypes. Multiple studies have shown that p53 can repress hepatocytes' lineage-determining transcription factor HNF4A, across reductions of HNF4A protein levels and promoter activity.^{226,227} p53 stabilization (e.g., via MDM2 deletion) can induce progenitor markers *in vivo*; proposed mechanisms include p53 inducing inflammation that in turn promotes progenitor states or microRNA-mediated inhibition of HNF4A.^{228,229}

As literature support for KI67 accumulation and hepatocyte cycling despite elevated p53, both p53 and KI67 can increase with HCC grade. As specific examples, these genes were upregulated in tumors from a cohort of HBV-associated HCC patients relative to paired normal liver tissue²³⁰; in a separate human HCC cohort, p53 levels correlated with the abundance of PCNA (another common cell cycle marker), and each associated with worse tumor grade.¹⁹⁷ Additionally, in liver regeneration models using *in vitro* primary human hepatocytes and *in vivo* mouse livers, WNT signaling activation and AP-1 transcription factors can each suppress p53's anti-proliferative effects and abrogate p53-mediated cell cycle inhibition in hepatocytes^{231,232}; our transcriptional and epigenetic datasets support increased activities of each of these pathways over the course of MASLD progression. Thus, in particular cases in the liver, p53 and KI67 are not necessarily anti-correlated and can exhibit concordant regulation upon cellular perturbations.

Human Liver Tissue Microarray Construction

Explant tissue was fixed in 4% paraformaldehyde at 4°C for a minimum of 3 days, then embedded into paraffin at Massachusetts General Hospital's Pathology Histology Lab. Upon study participant selection for inclusion in the tissue microarray, individual FFPE blocks were melted, and regions of interest (based on matched hematoxylin and eosin staining) were scored for re-embedding into a collective tissue microarray.

Human Liver Xenium Spatial Transcriptomics

For Xenium spatial transcriptomics, the tissue microarray was sectioned (5µm), and sections were floated in a 37°C water bath onto a Xenium slide. The Xenium slide was air dried at room temperature for 10 minutes, followed by incubation at 42°C for 3 hours and desiccation for up to 4 weeks prior to Xenium spatial transcriptomics according to manufacturer specifications (10x Genomics, CG000578 Rev E). Specifically, spatial transcriptomics was conducted with Xenium In Situ Gene Expression and Multimodal Cell Segmentation (for improved cellular segmentation and transcript assignment), using the Human Multi-Tissue and Cancer base panel supplemented with a custom 100-gene panel focused on key markers of hepatocyte stress responses and intercellular signaling.

QUANTIFICATION AND STATISTICAL ANALYSES

sc/snRNA-seq QC, Filtering, and Annotation

Bcl2fastq was used to convert sequencing reads into bcl files for alignment with either the DropSeq pipeline (live-tissue scRNA-seq samples) or STARsolo (frozen-tissue snRNA-seq and HepG2 scRNA-seq samples). Mouse samples were aligned to the mm10 reference genome, and human HepG2 transcription factor overexpression samples were aligned to the Hg38 reference genome with the BFP sequence appended (to validate successful transduction via BFP positive control samples).

Cells were first filtered based on number of detected genes, detected UMIs, and percent of mitochondrial counts. The number of principal components was chosen based on an automated elbow-based selection criterion, followed by construction of nearest-neighbor and shared nearest-neighbor graphs and UMAP visualization. Clustering was implemented using the Leiden algorithm, and resolution was chosen based on a parameter scan and maximization of silhouette coefficient. Clusters solely distinguished by quality-associated or doublet-associated features were removed: high expression of mitochondrial genes, high expression of ribosomal genes, markers of low-quality cells (e.g., MALAT1, NEAT), significantly lower numbers of detected genes or features, high expression of long non-coding RNAs or unannotated transcripts, co-expression of markers for mutually-exclusive lineages (e.g., immune and epithelial cells). Cells were annotated based on canonical markers established in prior liver atlases, and clusters corresponding to broad lineages were merged for further subclustering (i.e., epithelial, immune, structural/stromal). Within each lineage, variable gene selection, PCA, nearest-neighbor graph construction, clustering, and UMAP visualization were re-performed; clusters distinguished by markers of other lineages were removed as doublets. Lineage-specific subclustering and doublet removal was repeated 2-3 times within each lineage to ensure robust heterotypic doublet identification.

snATAC-seq QC, Filtering, and Annotation

CellRanger was used for processing and alignment to mm10-2020-A_arc_v2.0.0. Cells were filtered based on number of detected peaks, percent of reads in peaks, nucleosome signal, and TSS enrichment. The number of latent semantic indexing components was chosen based on an automated elbow-based selection, followed by construction of nearest-neighbor and shared nearest-neighbor graphs and UMAP visualization. Iterative subclustering and doublet removal were implemented as described in “scRNA-seq and snRNA-seq QC, Filtering, and Annotation”, using chromatin-based gene activity scores instead of canonical marker genes.

Gene Program Derivation

At each timepoint for each of the scRNA-seq and snRNA-seq hepatocyte datasets, the average $\log_2$ (fold-change) was calculated across all genes detected in at least 25% of cells (chosen based on differential expression benchmarking analyses examining robust parameter estimates and statistical outputs as a function of average expression and detection rate²³³). To promote prioritization of generalizable gene programs across species, we additionally incorporated differential expression information from Govaere et al.’s human bulk RNA-seq cohort, using limma-trend to model gene expression as a function of MASLD stage. See below for qualitative descriptions of the temporal patterns and trajectories captured by each gene program (defined quantitatively further below):

- Sustained Upregulation program: Genes whose elevation is maintained over time
- Sustained Downregulation program: Genes whose lessening is maintained over time
- Longitudinal Increase program: Genes progressively elevated with long-term chronic stress exposure
- Longitudinal Decrease program: Genes progressively lessened with long-term chronic stress exposure

Different genes are preferentially retained and measured in live-tissue scRNA-seq (nuclear + cytoplasmic mRNA) vs. frozen-tissue scRNA-seq (nuclear mRNA only). As a result, a gene may have a large fold-change in one dataset, but non-detection or sparse detection in the other dataset (in turn causing fold-changes that are inestimable or equal to 0). To maximize insights from both our live-tissue scRNA-seq dataset (higher molecular capture given the retention of cytoplasmic mRNA) and frozen-tissue snRNA-seq dataset (higher hepatocyte abundance), we sought to leverage complementary information from each dataset during gene program derivation. We followed the principle that genes should follow a given temporal trajectory (e.g., Sustained Upregulation) in at least one dataset, while allowing for non-detection/sparse detection in the other dataset (e.g., positive fold-changes in one dataset and non-negative fold-changes in the other dataset). As a result, gene programs were derived by filtering to retain genes matching the general structure shown below:

$$(Condition_{Mouse\ scRNA} OR Condition_{Mouse\ snRNA}) AND Condition_{Human\ Bulk\ RNA}$$

See below for each gene program's definitions of $Condition_{Mouse\ scRNA}$, $Condition_{Mouse\ snRNA}$, and $Condition_{Human\ Bulk\ RNA}$, where $\log_2 FC$ represents average $\log_2$ (fold-change) at the noted timepoint between hepatocytes from high fat vs. control diet mice in live-tissue scRNA-seq or frozen-tissue snRNA-seq datasets, and $\beta_{Human\ Bulk\ RNA}$ represents the regression coefficient of gene expression as a function of disease stage in Govaere et al.

Sustained Upregulation

$Condition_{Mouse\ scRNA}$: Across all timepoints, consistent upregulation in live-tissue scRNA-seq and non-negative fold-changes in frozen-tissue snRNA-seq

$$\log_2 FC_{scRNA,6mo} > 0 AND \log_2 FC_{scRNA,12mo} > 0 AND \log_2 FC_{scRNA,15mo} > 0 AND \log_2 FC_{snRNA,6mo} \geq 0 AND \log_2 FC_{snRNA,12mo} \geq 0 AND \log_2 FC_{snRNA,15mo} \geq 0$$

$Condition_{Mouse\ snRNA}$: Across all timepoints, consistent upregulation in frozen-tissue snRNA-seq and non-negative fold-changes in live-tissue scRNA-seq

$$\log_2 FC_{snRNA,6mo} > 0 AND \log_2 FC_{snRNA,12mo} > 0 AND \log_2 FC_{snRNA,15mo} > 0 AND \log_2 FC_{scRNA,6mo} \geq 0 AND \log_2 FC_{scRNA,12mo} \geq 0 AND \log_2 FC_{scRNA,15mo} \geq 0$$

$Condition_{Human\ Bulk\ RNA}$: Increased expression with disease stage

$$\beta_{Human\ bulk\ RNA} > 0$$

Sustained Downregulation program

$Condition_{Mouse\ scRNA}$: Across all timepoints, consistent downregulation in live-tissue scRNA-seq and non-positive fold-changes in frozen-tissue snRNA-seq

$$\log_2 FC_{scRNA,6mo} < 0 AND \log_2 FC_{scRNA,12mo} < 0 AND \log_2 FC_{scRNA,15mo} < 0 AND \log_2 FC_{snRNA,6mo} \leq 0 AND \log_2 FC_{snRNA,12mo} \leq 0 AND \log_2 FC_{snRNA,15mo} \leq 0$$

$Condition_{Mouse\ snRNA}$: Across all timepoints, consistent downregulation in frozen-tissue snRNA-seq and non-positive fold-changes in live-tissue scRNA-seq

$$\log_2 FC_{snRNA,6mo} < 0 AND \log_2 FC_{snRNA,12mo} < 0 AND \log_2 FC_{snRNA,15mo} < 0 AND \log_2 FC_{scRNA,6mo} \leq 0 AND \log_2 FC_{scRNA,12mo} \leq 0 AND \log_2 FC_{scRNA,15mo} \leq 0$$

$Condition_{Human\ Bulk\ RNA}$: Decreased expression with disease stage

$$\beta_{Human\ bulk\ RNA} < 0$$

Longitudinal Increase program

$Condition_{Mouse\ scRNA}$: Progressive increases from 6-month to 15-month timepoints in live-tissue scRNA-seq and non-decreases in frozen-tissue snRNA-seq

$$\log_2 FC_{scRNA,15mo} > \log_2 FC_{scRNA,6mo} AND \log_2 FC_{snRNA,15mo} \geq \log_2 FC_{snRNA,6mo}$$

$Condition_{Mouse\ snRNA}$: Progressive increases from 6-month to 15-month timepoints in frozen-tissue snRNA-seq and non-decreases in live-tissue scRNA-seq

$$\log_2 FC_{snRNA,15mo} > \log_2 FC_{snRNA,6mo} AND \log_2 FC_{scRNA,15mo} \geq \log_2 FC_{scRNA,6mo}$$

$Condition_{Human\ Bulk\ RNA}$: Increased expression with disease stage

$$\beta_{Human\ bulk\ RNA} > 0$$

Longitudinal Decrease program

$Condition_{Mouse\ scRNA}$: Progressive decreases from 6-month to 15-month timepoints in live-tissue scRNA-seq and non-increases in frozen-tissue snRNA-seq

$$\log_2 FC_{scRNA,15mo} < \log_2 FC_{scRNA,6mo} AND \log_2 FC_{snRNA,15mo} \leq \log_2 FC_{snRNA,6mo}$$

$Condition_{Mouse\ snRNA}$: Progressive decreases from 6-month to 15-month timepoints in frozen-tissue snRNA-seq and non-increases in live-tissue scRNA-seq

$$\log_2 FC_{snRNA,15mo} < \log_2 FC_{snRNA,6mo} AND \log_2 FC_{scRNA,15mo} \leq \log_2 FC_{scRNA,6mo}$$

Condition_{Human Bulk RNA}: Decreased expression with disease stage

$$\beta_{\text{Human bulk RNA}} < 0$$

This work's stress response gene programs were derived based on our mouse live-tissue scRNA-seq dataset, our mouse frozen-tissue snRNA-seq dataset, and Govaere et al.'s human bulk RNA-seq dataset. These stress response programs were then scored and evaluated in further external datasets (e.g., mouse tumor snRNA-seq, human bulk RNA-seq, human snRNA-seq, human bulk proteomics, human cancer survival outcomes, human spatial transcriptomics, human tumor copy number variation, human future tumor incidence). Module scores for each program and dataset were calculated using AddModuleScore in Seurat, and TCGA patient stratification and visualization were conducted with the survival and survminer packages in R.

Cancer Signature Induction in Stress Response

To evaluate whether and which cancer and developmental phenotypes are mirrored in the spontaneous HCC tumors in our chronic metabolic stress mouse model, we began by conducting differential gene expression testing between tumor cells and hepatocytes in matched adjacent normal tissue. As a broad view of potential cancer phenotypes, we conducted gene set enrichment analysis using the fgsea R package, comparing our model's tumor differential expression against gene sets from: 1) MSigDB's "Chemical and Genetic Perturbations" (3,405 genesets spanning diverse prior studies' mutational and signaling perturbations); 2) mutation-specific mouse liver tumor models¹⁰⁶; and 3) liver development and regenerative states.^{107–111} Thus, all gene sets considered during gene set enrichment analysis were defined externally to this study, providing a complementary framework (to the previously-described response program derivation) for uncovering phenotypes accentuated with tumorigenesis in our model.

We then sought to understand which tumorigenesis-linked gene sets were also differentially regulated during hepatocytes' progressive stress responses (i.e., before tumorigenesis). Seurat's AddModuleScore was used to score each dataset for leading-edge genes from statistically-significantly enriched cancer and developmental gene sets.

Longitudinal Epigenetic Alterations & Priming

Differential chromatin accessibility was calculated by pseudobulking snATAC-seq hepatocytes from each mouse, then calculating differential expression within each timepoint between high fat vs. control diet pseudobulked hepatocyte profiles using edgeR. chromVAR scores were calculated using the JASPAR2020 core motif collection and the Signac wrapper function RunChromVAR.¹¹⁴ Chromatin peak co-accessibility links were calculated using the Signac wrapper function run_cicero.^{122,234}

For higher-resolution inference of hepatocytes' epigenetic response trajectories, we conducted pseudotime analyses. To avoid confounding effects of diet and time, we derived separate pseudotime trajectories for hepatocytes from high fat and control diet hepatocytes, following concepts in previous analyses on allergic inflammation and stem cell differentiation.¹⁷⁸ Ordering hepatocytes according to smooth gradients in chromatin accessibility, we prioritized gene loci dynamically altered by chronic metabolic stress but not control diet. Pseudotime trajectories were calculated for each diet condition using chromatin peaks that were differentially accessible with age within each diet condition. To control for varying cell counts across samples, each timepoint was downsampled to equal cell counts (matching the timepoint with the fewest cells). Latent semantic indexing and UMAP visualization (on LSI components 2 to 30) were implemented for each diet condition, and Slingshot was used to find pseudotime trajectories.²³⁵

For epigenetic priming analyses, we began by linking distal peaks to genes based on co-accessibility with peaks in a gene's promoter or gene body (only considering peaks within 100 kilobases of the gene and with Cicero co-accessibility score greater than 0.1). To identify genes whose putative distal chromatin regulators collectively exhibit differential accessibility, we then compared: 1) the observed distribution of differential accessibility fold-changes of actual co-accessible peaks; against, 2) 50 random background peaksets with matched average accessibility and GC bias. Finally, towards genes that may exhibit evidence of epigenetic priming in hepatocytes, we identified genes where:

1. Early chromatin accessibility changes exhibited the same directionality as transcriptional changes across longitudinal stress response and tumorigenesis

Epigenetic comparison: 6-month high fat vs. control diet snATAC-seq

Transcriptional comparison: [15-month tumor vs. adjacent normal snRNA-seq] – [6-month high fat vs. control diet snRNA-seq]

2. Late chromatin accessibility changes (15-month high fat vs. control diet snATAC-seq) exhibited the same directionality as tumorigenesis transcriptional changes (15-month tumor vs. adjacent normal snRNA-seq)

Epigenetic comparison: 15-month high fat vs. control diet snATAC-seq

Transcriptional comparison: 15-month tumor vs. adjacent normal snRNA-seq

3. Tumorigenesis incurred a large shift in gene expression (15-month tumor vs. adjacent normal snRNA-seq)

Transcriptional comparison: 15-month tumor vs. adjacent normal snRNA-seq

4. Early stress drove large changes in chromatin accessibility (6-month high fat vs. control diet snATAC-seq)

Epigenetic comparison: 6-month high fat vs. control diet snATAC-seq differential accessibility fold-changes at gene-linked co-accessible peaks, relative to matched random background control

MATCHA Transcription Factor Inference

MATCHA (<https://github.com/ctzouanas/MATCHA>) seeks to prioritize transcription factors regulating arbitrary, user-specified gene programs, while leveraging multi-omic information on context-specific regulatory relationships (e.g., cell type- or tissue-specific gene-enhancer regulation). Due to the sparse nature of single-cell transcriptional and especially epigenetic measurements, correlation values in a variety of studies combining single-cell RNA-seq and ATAC-seq tend to have low absolute values (e.g., ~0.1–0.2).^{236–238} Instead, relative rankings of transcription factors and statistical testing enable their prioritization for more thorough targeted analyses and validation experiments.²³⁹

In brief, MATCHA links gene programs to cell-type-specific distal enhancers by identifying chromatin regions co-accessible with the gene program's promoters or gene bodies. MATCHA then prioritizes causal regulators by determining TF motifs whose accessibility at program-coaccessible enhancers likewise covaries with program transcriptional expression. MATCHA further optionally incorporates: 1) concordance across datasets towards robust regulatory inference (e.g., across species, single-cell vs. bulk measurements, etc.); and, 2) identification of TFs co-regulating multiple gene programs. MATCHA therefore enables prioritization of TFs driving arbitrary gene programs while also modeling context-dependent functions via cell-type- and tissue-specific gene regulatory landscapes (see [resource availability](#)).

While a wide variety of computational methods exist to estimate TF abundance or activity, the goal of identifying TFs that specifically regulate particular phenotypic gene programs remains an open problem. Below, we highlight generally-related approaches and distinctions from MATCHA's goal of mapping gene programs, enhancers, and program-specific TFs. We also note that we do not intend the following descriptions to represent a comprehensive or all-encompassing review (e.g., see Badia-i-Mompel et al.,²⁴⁰ Prata et al.,²⁴¹ Akers and Murali,²⁴² and Bellot et al.²⁴³ for examples of prior reviews and benchmarking efforts).

- Examining differentially-expressed TFs (at the transcriptional level) or genome-wide TF motifs with differential accessibility (at the chromatin level) can prioritize TFs with generally altered activity, but not connect them to specific cellular phenotypes.
- Genome-wide transcriptomic profiles can be translated into predicted TF activities through tools that leverage prior knowledge (e.g., response signatures, inferred interactomes, etc.; see Ding et al.,²⁴⁴ Badia-i-Mompel et al.,²⁴⁵ Holland et al.,²⁴⁶ and Garcia-Alonso et al.²⁴⁷ for examples). However, these tools provide a single TF score for each transcriptomic profile, but do not associate TFs with specific programs or distinguish their effects (or lack thereof) on specific cellular phenotypes.
- Construction of gene regulatory networks can connect transcription factors to inferred downstream genes (with optional incorporation of multi-omic information).^{241,248–251} However, these tools most often focus on inference of TFs distinguishing distinct cell types (e.g., differentiation from one developmental stage to another), or simulated transitions across genome-wide transcriptomic profiles (e.g., represented as regions of density on a low-dimensional visualization such as UMAP or t-SNE). Thus, frameworks to connect TF regulons to user-specified gene programs of interest remain less clear. While gene regulatory network inference connects TFs to potential targets, the ability to define gene programs of interest and prioritize causal TFs has received less exploration. It remains difficult to identify TFs that target and modulate specific disease-associated phenotypes, rather than affecting the global cell state.

In contrast, MATCHA begins from a user-specified gene program and enables prioritization and nomination of TFs regulating that specific gene program, towards TFs driving particular aspects of cellular physiology. In this way, MATCHA enables discovery of tradeoffs or co-regulation across disease-linked axes of variation with distinct functions and repercussions (e.g., this work's Longitudinal Increase and Decrease programs, with opposing enriched processes, temporal trajectories, and disease connections). We highlight that MATCHA: 1) leverages cell type- and/or tissue-specific transcriptomic and epigenetic relationships, towards capturing context-specific regulatory interactions, 2) can emphasize robust and generalizable gene program drivers by incorporating datasets spanning studies, cohorts and species, and, 3) can uncover core, co-regulatory TFs capable of activating or repressing each of multiple gene programs simultaneously.

As inputs, MATCHA accepts: 1) one or more arbitrary gene programs; 2) a TF motif database (e.g., JASPAR 2020); 3) multi-omic sc/snRNA-seq data; and optionally, 4) external datasets with relevance to the user's context (e.g., prior bulk or sc/snRNA-seq atlases).

As outputs, MATCHA provides: 1) prioritization scores of the predicted strength and directionality of a TF's regulatory effect on each arbitrary gene program (along with contributions of each input dataset to the overall prioritization score); 2) a bipartite network of which TFs regulate which gene programs (and in what direction, along with TF and gene program network centrality metrics); and, 3) rankings of which TFs may co-regulate multiple gene programs simultaneously (if multiple gene programs were provided).

Towards inference of gene program – co-accessible enhancer – causal TF triads, MATCHA is based on the principle that robust, strong regulatory relationships should be reflected across multiple -omic layers and across datasets. Therefore, MATCHA follows the following steps:

1. If a particular TF regulates the user-specified gene program(s), then it is plausible that the regulatory relationship should be reflected in accessibility changes at program-associated chromatin regions containing the TF's motif. Therefore, MATCHA

begins by identifying chromatin regions that are co-accessible with a gene's promoter or gene body (based on Cicero), then filters peaks based on genomic distance and co-accessibility strength towards plausible regulatory relationships. For each TF motif, MATCHA creates a program-specific motif score by evaluating accessibility at program-coaccessible peaks containing each TF motif (following chromVAR's framework within program-coaccessible peaks). Finally, MATCHA calculates the correlation between the program-specific motif score and transcriptional expression of the gene program itself. In this way, MATCHA connects epigenetic alterations at program-linked, TF motif-containing peaks to transcriptional levels of the gene program.

2. If a particular TF regulates the user-specified gene program(s), then it is plausible that the regulatory relationship should be reflected in concordant changes between TF abundance and gene program transcription. Therefore, MATCHA calculates the correlation between expression level of each TF in the user-input TF motif database and transcriptional expression of the gene program, thereby connecting transcriptional TF abundance to transcriptional levels of the gene program.
3. If a particular TF regulates the user-specified gene program(s) robustly, then it is plausible that the regulatory relationship should be reflected not only in a single dataset, but across studies. Therefore, if the user provided multiple input datasets (whether bulk or single-cell), MATCHA calculates similar correlations as described above between gene program transcriptional level and either TF motif accessibility at program-linked peaks or TF transcriptional abundance. In this way, MATCHA connects each TF (motif) to transcriptional levels of the gene program across wide-ranging contexts (e.g., spanning species, disease severities, experimental designs, measurement technologies, etc.).
4. To create a single prioritization score for each TF that incorporates information across -omic measurements and studies, MATCHA aggregates each correlation (as calculated in preceding steps). To account for different correlation distributions across -omic measurement modalities and studies, correlations calculated in each previous step are scaled from -1 (most negative association between TF and gene program, indicative of repression) to 1 (most positive association between TF and gene program, indicative of activation). Scaled correlations are averaged, and TFs are ranked by the average of scaled correlations.
5. In addition to identifying which TFs regulate a particular gene program, it is important to understand whether TFs may co-regulate multiple gene programs, towards essential couplings of cellular phenotypes encapsulated by different programs or potential opportunities to disentangle otherwise-associated phenotypes. Therefore, if the user provided multiple input gene programs, MATCHA will create rankings of each TF's cross-dataset association with each gene program (as described in preceding steps), and calculate network centrality metrics for TFs and gene programs (e.g., out-degree for TFs to identify the number of gene programs that they strongly regulate, in-degree for gene programs to identify the number of TFs potentially regulating them). For concise summarization, MATCHA will filter to retain TFs that are ranked within the top TFs in at least a minimum number of gene programs (Figure 5F generated with the top 10 TFs for each gene program, showing TFs linked to at least 2 gene programs). Optionally, users can further filter to retain only TFs whose regulatory relationships match observed transcriptional correlations between gene programs (e.g., if a given TF is linked to program₁ and program₂ which are in turn negatively correlated with each other at the transcriptional level, the TF must activate one of the programs but repress the other). In this way, MATCHA enables nomination and prioritization of TFs with strong regulatory effects on wide-ranging, but specific cellular phenotypes of particular interest to the user (e.g., this work's stress response gene programs, with distinct temporal trends, functional enrichments, and prognostic stratification of human HCC survival).

Control Analyses of RELB CNVs on Programs

We approached control analyses of RELB CNVs on stress response gene programs through two complementary approaches:

1. How likely are RELB copy number variations to regulate matched, randomly-generated gene programs? These analyses serve as a control for RELB having regulatory effects specifically on stress response gene programs (i.e., as opposed to non-specific effects on genes throughout the genome).

For each of our stress response programs, we began by generating random gene programs with matched expression based on the procedure in Tirosh et al.²⁵² We then scored TCGA hepatocellular carcinoma samples for these matched, randomly-generated gene programs using the same AddModuleScore approach as in RELB analyses. For each gene program, we quantified RELB CNVs' regulatory effects as the signed Cohen's D effect size of gene program expression differences between tumors with RELB copy number amplifications and RELB copy number deletions. Finally, to summarize RELB's effects across all programs into a single score, we calculated an aggregate RELB CNV regulation score as the difference between [sum of RELB CNV regulatory effects on the Longitudinal Increase, Sustained Upregulation, Human HCC S1: WNT Activation, and Development-Associated Hepatocyte programs] and the [sum of Sustained Downregulation and Longitudinal Decrease programs]. To build a null distribution of RELB's effects across matched, randomly-shuffled gene programs, this procedure was repeated across a total of $n = 250$ iterations in order to provide both statistical rigor and computational tractability.

RELB's aggregate CNV regulation score on the bona fide, original submission stress response gene programs was 2.05; in contrast, no matched, randomly-generated gene program exhibited a higher aggregate CNV regulation score across any of the $n = 250$ iterations (Figure S6O; N.B., we found highly similar results across choices of random seeds). As an additional check on

validity of this random shuffling procedure, we found that the null background distribution of aggregate RELB regulation score exhibited a median very close to 0, in line with RELB CNVs not having strong regulatory effects across the genome indiscriminately.

From these analyses, we conclude that RELB copy number variations support RELB's regulatory effects on our original submission's stress response gene programs, as compared to the baseline of matched, randomly-generated background gene programs.

2. How much do copy number variations in any transcription factor regulate stress response gene programs? These analyses serve as a control for RELB having heightened activity compared to randomly-selected transcription factors.

Using the TCGA hepatocellular carcinoma dataset, we began by considering all transcription factors satisfying the following two conditions: 1) copy number amplifications at the transcription factor's genomic locus in at least 10 study participants; and, 2) copy number deletions at the transcription factor's genomic locus in at least 10 study participants. For each stress response gene program, we quantified transcription factor CNVs' regulatory effects as the signed Cohen's D effect size of gene program expression differences between tumors with copy number amplifications and copy number deletions. Finally, to summarize each transcription factor's effects across all programs into a single score, we calculated an aggregate transcription factor CNV regulation score as the difference between [sum of transcription factor CNV regulatory effects on the Longitudinal Increase, Sustained Upregulation, Human HCC S1: WNT Activation, and Development-Associated Hepatocyte programs] and the [sum of Sustained Downregulation and Longitudinal Decrease programs]. We additionally sought to prioritize transcription factors with relevance to the liver/hepatocellular carcinoma context based on average expression, reasoning that high average expression would prioritize transcription factors active in hepatocyte-derived tumor cells.

We found that RELB CNVs served as one of the strongest hits for *positive* regulation of stress response gene programs (i.e., increasing the programs that increase with chronic metabolic stress, decreasing the programs that decrease with chronic metabolic stress; Figure S6P). Specifically, only 1.2% of transcription factors exhibited higher aggregate CNV regulation scores and higher average expression in hepatocellular carcinoma than RELB, positioning RELB CNVs as having stronger regulatory effects than would be expected from a typical randomly-selected transcription factor.

As a check on these analyses, we found that HNF4A and THRB CNVs served as some of the strongest hits for *negative* regulation of stress response gene programs (i.e., decreasing the programs that increase with chronic metabolic stress, and vice versa). As context, HNF4A is the master transcription factor for mature adult hepatocyte lineage specification and function, and THRB is the target of the first approved treatment for MASH (approved while this work was in revision).^{137,138} As such, our finding that HNF4A and THRB CNVs serve to blunt or revert hepatocytes' stress response gene programs is in line with canonical hallmarks of cancer (e.g., reversion to dedifferentiated cell states), THRB therapeutic efficacy in MASH, and our biological interpretations of stress response gene programs (i.e., chronic metabolic stress driving cellular responses that involve activation of pro-cell survival, fetal-like cell states and concomitant decreases in contributions to tissue function). More broadly, these findings on HNF4A and THRB provide confidence in further interpretation of RELB's regulatory effects.

Therefore, these control analyses further support RELB CNVs as one of the strongest drivers of stress response gene programs as compared to all other transcription factors; other transcription factors nominated through these analyses may represent bona fide drivers of or protectors against disease dysfunction and could merit investigation in future work.

Xenium Spatial Transcriptomics

Counts matrices, cell coordinates, and cell segmentation were loaded from Xenium outputs. Iterative subclustering and celltype annotation were implemented as described in "scRNA-seq and snRNA-seq QC, Filtering, and Annotation".

Towards defining recurrent spatially-structured multicellular compositions (Figure 7B, right), MULTISPATI PCA was run as implemented through the adespatial and Voyager packages.²⁵³⁻²⁵⁵ The cellular composition within each cell's spatial microenvironment was calculated (radius=50µm), with inverse distance weighting of cellular neighbors' proximities followed by normalization to a total neighbor weight sum of 1. The cells × distance-weighted multicellular spatial composition matrix underwent principal component analysis, neighborhood graph construction, and Leiden-based clustering with silhouette coefficient optimization (as described in "scRNA-seq and snRNA-seq QC, Filtering, and Annotation") to produce spatial neighborhoods in Figure 7B (right).

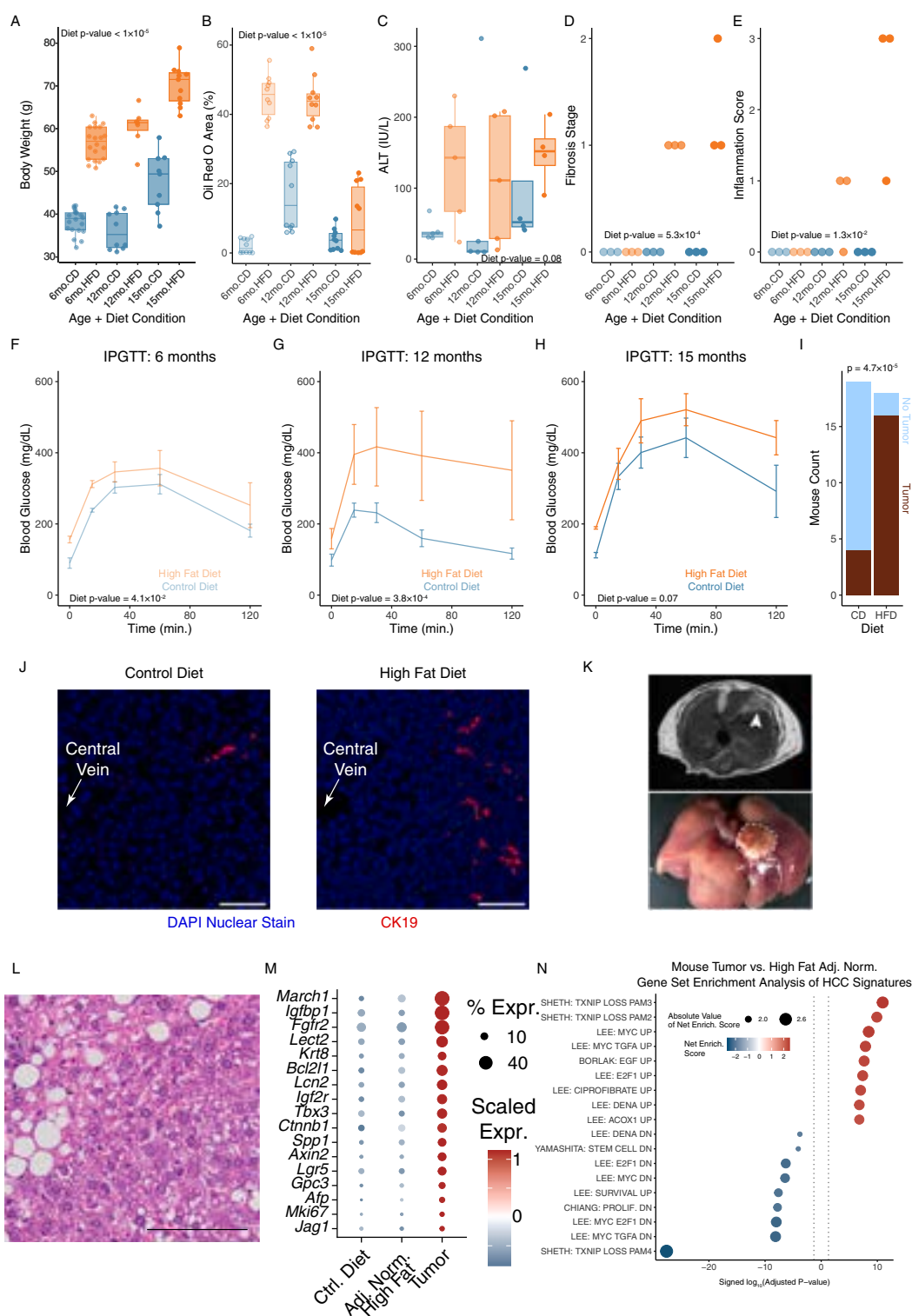
We additionally leveraged natural variation in our human spatial transcriptomics cohort to provide initial indications on the contributions of adipose-liver signaling, as compared to direct liver tissue microenvironments, in the human *in vivo* context. While all MASH study participants had matched liver pathology (i.e., MASH cirrhosis), we purposefully selected study participants with lean MASH (BMI mean=26.6, standard deviation=0.81) vs. MASH with elevated BMI (BMI mean=35.7, standard deviation=0.80). With 5'3" and 5'9" as typical heights for U.S. women and men (1.60 and 1.75 meters),¹⁴⁹ respectively, this difference in mean BMI corresponds to a weight difference of 51.4-61.5 pounds (23.3-27.9 kilograms), likely to correspond to meaningful shifts in adipose tissue mass and adipose-derived signals in human adults. As such, we reasoned that this cohort with matched liver disease severity, but highly disparate BMIs, could provide information on the dependency of hepatocyte stress responses on adipose-liver signaling in the human *in vivo* context. We found strong concordance of hepatocyte gene expression changes during MASH regardless of BMI (Spearman correlation=0.64, p-value < 2.2×10⁻¹⁶); this result was robust to controlling for potentially confounding covariates such as age (Spearman correlation=0.56, p-value < 2.2×10⁻¹⁶) or biological sex (Spearman correlation=0.59, p-value < 2.2×10⁻¹⁶) (Figure S7E). These human *in vivo* findings were further supported by data from *in vitro* human liver organoids, where palmitic

acid treatment alone was sufficient to upregulate hepatocyte *RELB*, and TGFB1 alone upregulated *SOX4*¹³¹ (Figure S7F). Likewise, conducting comparative analyses across MASLD mouse models, we found that methionine-choline-deficient mice (which do not become obese but develop liver steatosis, inflammation, and fibrosis) exhibited the greatest transcriptional similarity to our 15mo.HFD timepoint; in contrast, *db/db* mice (which spontaneously overeat to obesity but retain non-inflamed and non-fibrotic livers) exhibited transcriptional similarity to our earlier HFD timepoints^{150–152} (Figures S7G and S7H). Thus, across human *in vivo*, human *in vitro*, and mouse *in vivo* experiments and analyses, hepatocyte chronic metabolic stress responses do not seem to be predominantly driven by adipose-liver crosstalk alone, with at least select aspects arising from liver-intrinsic lipid responses and intra-liver intercellular signaling.

For multicellular neighborhood spatial regression (Figures 7E–7G), a composite stress score was calculated for each hepatocyte as the difference of module scores of the increased and decreased gene programs (gene programs as in Figure 7C). The cellular composition within each hepatocyte's spatial microenvironment was calculated as described above (with cell types annotated to capture both canonical lineages and fine-level cell states; Figure S7). Hepatocytes' composite stress score was linearly modeled as a function of the distance-weighted multicellular composition in their spatial microenvironment. Outputs of this linear modeling indicate neighboring cell types with differential abundances in the microenvironments of hepatocytes exhibiting extreme manifestations of stress response programs (i.e., positive linear modeling coefficients indicate neighbor cell types enriched in hepatocytes with extreme manifestations of stress response programs; negative linear modeling coefficients indicate depleted neighbor cell types).

For intercellular signaling – gene regulatory spatial regression (Figures 7H and 7I), we conducted negative binomial/Gamma-Poisson generalized linear modeling of hepatocyte gene expression as a function of signaling protein transcripts in hepatocytes' spatial microenvironment. To quantify signaling protein transcripts in hepatocytes' spatial microenvironments, we began by subsetting our spatial transcriptomics dataset to genes annotated as secreted (based on OmniPath annotations, Human Protein Atlas annotations, and manual curation^{256,257}) and detected in at least one fine-level cell type in our dataset at a level of at least 1% (fine-level cell type annotations shown in Figure S7B). To calculate a matrix of hepatocytes × signaling protein transcript abundances in hepatocytes' surrounding spatial microenvironment, we multiplied: 1) hepatocyte × distance-weighted adjacency matrix of neighboring cells (radius=50μm; matrix dimensions=hepatocytes × neighboring cells); and, 2) neighboring cells × signaling protein transcript abundance matrix (matrix dimensions=neighboring cells × signaling protein transcripts). To then capture spatially structured, intercellular signaling effects on hepatocyte gene expression, we then modeled hepatocyte gene expression (i.e., hepatocyte × gene expression matrix) as a function of spatially proximal signaling protein transcripts (i.e., matrix of hepatocytes × signaling protein transcript abundances in hepatocytes' surrounding spatial microenvironment), using a negative binomial/Gamma-Poisson generalized linear model to account for the count-based data structure of spatial transcriptomics. The output of this spatial regression is a matrix of signaling protein transcripts × hepatocyte gene expression, indicating signaling protein interactions that spatially co-localize with particular patterns of hepatocyte gene expression (i.e., positive linear modeling coefficients indicate signaling proteins enriched near hepatocytes with high expression of a particular gene; negative linear modeling coefficients indicate signaling proteins enriched near hepatocytes with low expression of a particular gene). Spatial regression generalized linear modeling was implemented using the glmGamPoi package.²⁵⁸

Supplemental figures



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Figure S1. Characterization of mouse functional, organ-level, and tissue-level phenotypes induced by metabolic stress, related to Figure 1

(A–E) Body weight (A; $n = 77$ mice), oil red O (B; $n = 40$ mice), circulating alanine transaminase concentrations (C; $n = 28$ mice), histologic fibrosis stage (D; $n = 18$ mice), and histologic inflammation score (E; $n = 18$ mice) across chronic metabolic stress progression, and each dot is an independent mouse.

(F–H) Intraperitoneal glucose tolerance test across diet conditions at 6-month (F), 12-month (G), and 15-month (H) time points ($n = 3$ –5 per age $\times$ diet condition), and bars indicate standard error of the mean.

(I) Spontaneous liver tumor occurrence counts in the orthogonal HFD mouse validation cohort at an independent mouse facility.

(J) Immunofluorescence staining for CK19. Blue, DAPI nuclear stain; red, CK19 protein. Scale bar, 100 μm .

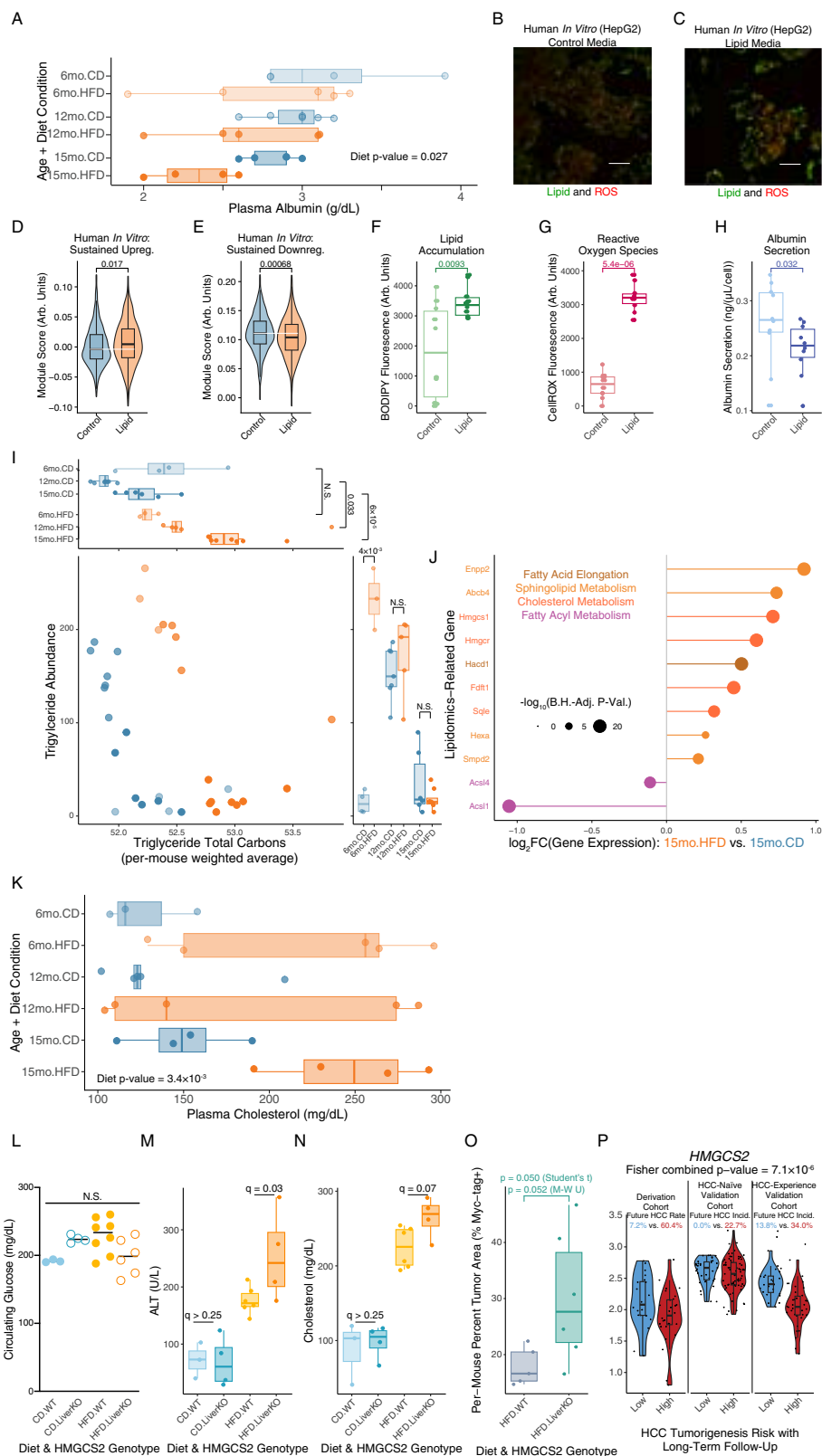
(K) MRI (top) and gross imaging (bottom) of mouse spontaneous HCC. The dashed line indicates a tumor, and the dotted line indicates adjacent normal.

(L) H&E of mouse spontaneous HCC (scale bar, 100 μm).

(M) HCC marker expression in mouse spontaneous tumors vs. matched samples ($n = 9$ samples).

(N) GSEA of HCC signatures among differentially expressed genes between mouse HFD tumor and adjacent normal samples.

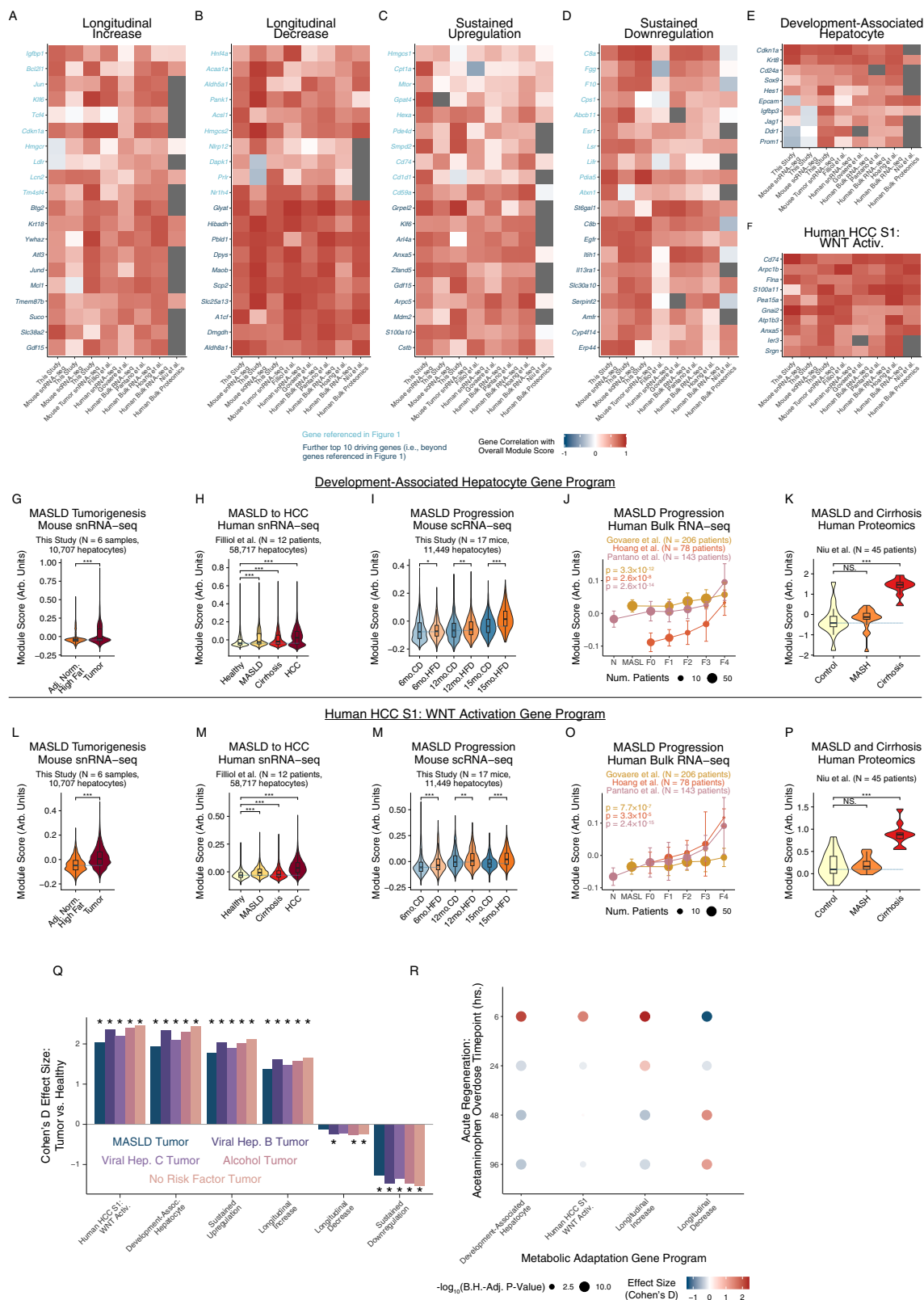
p values in (A)–(H) were calculated through two-way ANOVA, and the p value in (I) was calculated through Fisher's exact test. GSEA through the fgsea package.



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Figure S2. Human *in vitro* lipid culture, mouse *in vivo* bulk lipidomics, and mouse *in vivo* liver HMGCS2 knockout support hepatocyte stress adaptations, related to Figures 2 and 3

- (A) Mouse *in vivo* circulating plasma albumin concentration, and each dot is an independent mouse.
- (B and C) HepG2 lipid accumulation (BODIPY 493) and ROS accumulation (CellROX) in control media (B) or lipid media (C) (scale bar, 100 μ m).
- (D and E) Sustained Upregulation program (D) and Sustained Downregulation program (E) expression in HepG2 cells ($n = 891$ cells).
- (F–H) HepG2 lipid accumulation (F; microscopy), ROS accumulation (G; microscopy), and albumin secretion (H; ELISA). Dots indicate independent wells; $n = 10$ wells/condition.
- (I) Long-term diet-induced shifts in abundances and total carbons of triglyceride species.
- (J) Changes in snRNA-seq gene expression of lipid-related genes in hepatocytes after 15 months of high-fat vs. CD ($n = 6,913$ hepatocytes for 15-month CD; $n = 5,962$ hepatocytes for 15-month HFD).
- (K) Mouse *in vivo* circulating cholesterol concentration, and each dot is an independent mouse.
- (L–N) Circulating glucose (L), ALT (M), and cholesterol (N) in the HMGCS2 knockout cohort.
- (O) Tumor area per mouse after 2 weeks on HFD followed by oncogene introduction (per-mouse %Myc-tag+ liver area; $n = 11$ mice).
- (P) HMGCS2 expression across patient cohorts and HCC risk in Fujiwara et al.,⁹⁷ showing increased HCC tumorigenesis with downregulated HMGCS2. ALT and cholesterol statistics were calculated using Student's *t* test with Benjamini-Hochberg multiple-testing correction. All other *p* values were calculated using a Mann-Whitney U test.



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Figure S3. Driver genes of metabolic stress gene programs, related to Figure 4

(A–F) Spearman correlation of individual genes with overall program scores across datasets: (A) Longitudinal Increase program, (B) Longitudinal Decrease program, (C) Sustained Upregulation program, (D) Sustained Downregulation program, (E) Development-Associated hepatocyte program, and (F) Human HCC S1: WNT activation program.

(G–K) Development-Associated program expression in mouse snRNA-seq tumor cells and adjacent normal hepatocytes (G), human snRNA-seq hepatocytes and tumor cells (H), mouse scRNA-seq hepatocytes (I), human bulk liver RNA-seq (J), or human bulk liver proteomics (K).

(L–P) Human HCC S1 WNT activation program expression, following (G)–(L).

(Q) Tumor-vs.-healthy expression differences of this work's hepatocyte adaptation programs, split by tumor etiology.

(R) Effect size of change in expression levels of this work's stress adaptation programs during acute regeneration (comparing time points after acetaminophen overdose to expression at $t = 0$).

p values in (J) and (O) were calculated with Student's t test on the linear regression slope coefficient. All other p values were calculated with a Mann-Whitney U test with Benjamini-Hochberg correction. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

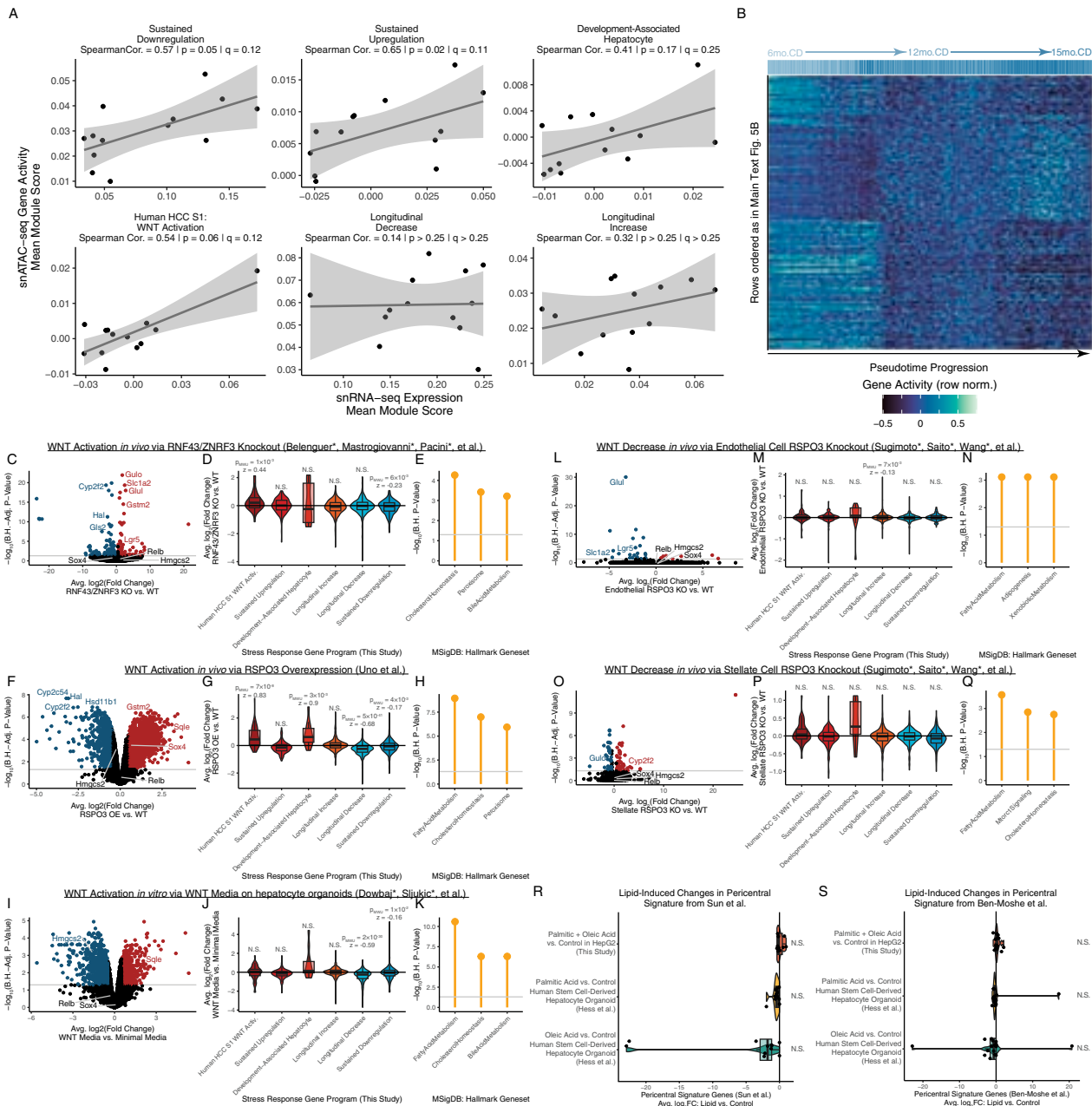


Figure S4. Epigenetic trajectories of hepatocytes adapting to chronic metabolic stress and contextualization to WNT pathway perturbations, related to Figure 5

(A) Association between transcriptomic expression (x axis) and chromatin accessibility (y axis) of gene programs uncovered in this work (pseudobulked tandem snRNA-seq and snATAC-seq datasets).

(B) Heatmap of chromatin accessibility-based gene activity scores in CD hepatocyte pseudotime progression, and rows have matched order with Figure 5B.

(C) *In vivo* liver bulk RNA-seq differential expression induced by WNT pathway activation via RNF43/ZNRF3 knockout.

(D) Differential expression of our work's stress response programs with WNT activation via RNF43/ZNRF3 knockout.

(E) Over-represented Hallmark gene sets among genes with concordant expression change directions between RNF43/ZNRF3 knockout and our work's stress response programs.

(F–H) Same format as (C)–(E), but for *in vivo* WNT activation via RSPO3 overexpression.

(I–K) Same format as (C)–(E), but for *in vitro* hepatocyte organoid WNT activation via WNT media.

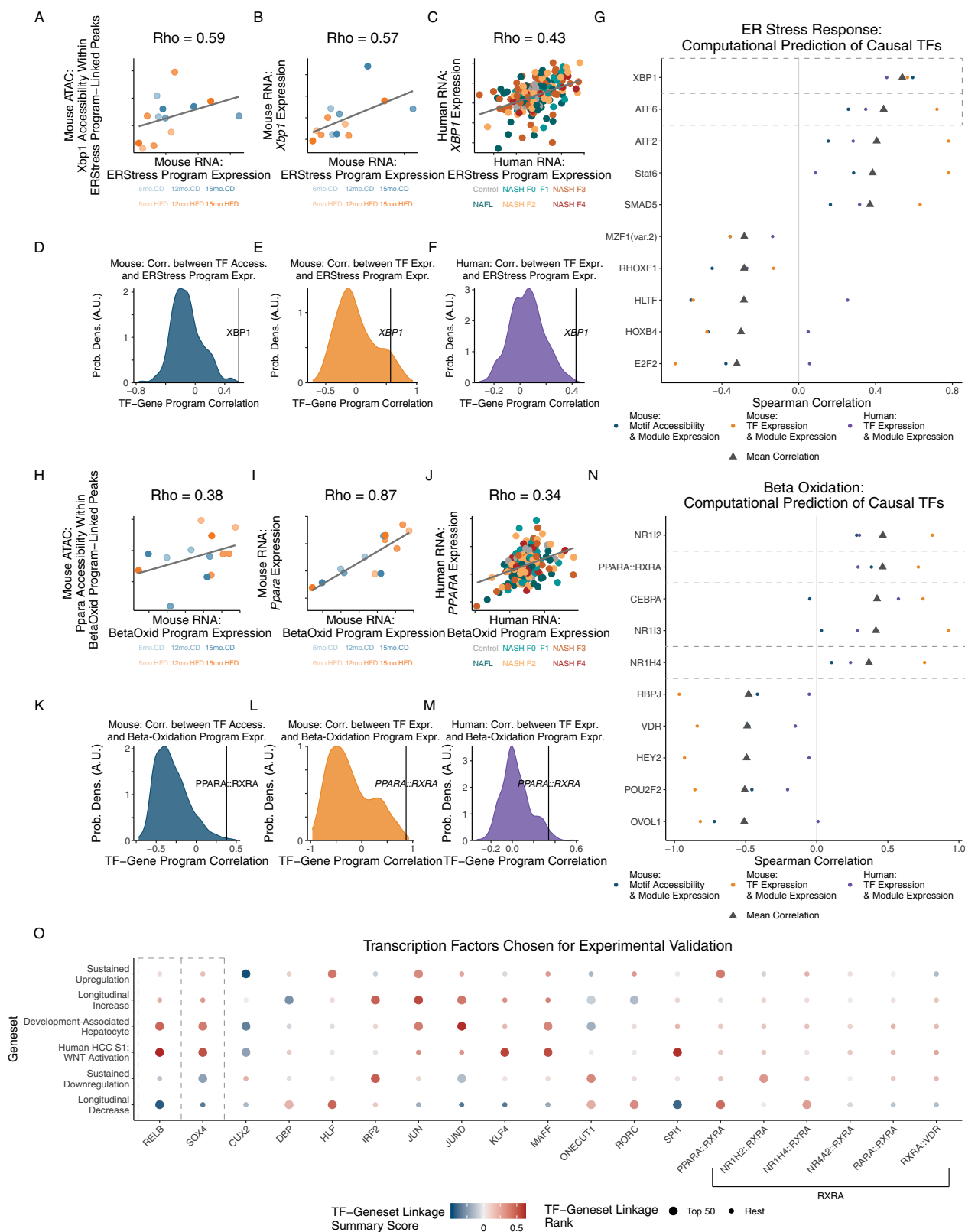
(L–N) Same format as (C)–(E), but for *in vivo* WNT decrease via endothelial cell-specific RSPO3 knockout.

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(O–Q) Same format as (C)–(E), but for *in vivo* WNT decrease via stellate cell-specific RSPO3 knockout.

(R) Changes in pericentral gene signature from Sun et al.¹³⁰ with lipid treatment across HepG2's (this study) and human adult hepatocyte-like cells from pluripotent stem-cell-derived liver organoids (Hess et al.¹³¹).

(S) Same format as (R), but for pericentral signature from Ben-Moshe et al.¹²⁹



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Figure S5. MATCHA enables prioritization of TFs regulating arbitrary user-defined gene programs and specific cellular phenotypes, related to Figure 5

- (A) Spearman correlation between transcriptomic expression of the ER stress response gene program (defined externally to this study through the public GO:BP database) and Xbp1 motif accessibility within program-coaccessible linked peaks (this study's natural progression tandem snRNA-seq and snATAC-seq datasets).
- (B) Spearman correlation between transcriptomic expression of the ER stress response gene program and Xbp1 transcriptomic expression (this study's natural progression snRNA-seq datasets).
- (C) Spearman correlation between transcriptomic expression of the ER stress response gene program and XBP1 transcriptomic expression (Govaere et al.'s human patient cohort⁸²).
- (D) Distribution of Spearman correlations between ER stress response gene program transcriptomic expression and accessibility of all TF motifs at program-coaccessible linked peaks (this study).
- (E) Distribution of Spearman correlations between ER stress response gene program transcriptomic expression and expression of all TFs (this study).
- (F) Distribution of Spearman correlations between ER stress response gene program transcriptomic expression and expression of all TFs (Govaere et al.⁸²).
- (G) Maximally prioritized TFs inferred as driving (top 5) or repressing (bottom 5) the ER stress response gene program.
- (H) Spearman correlation between transcriptomic expression of the β -oxidation response gene program (defined externally to this study through the public GO:BP database) and PPARA::RXRA motif accessibility within program-coaccessible linked peaks (this study's natural progression tandem snRNA-seq and snATAC-seq datasets).
- (I) Spearman correlation between transcriptomic expression of the β -oxidation gene program and PPARA transcriptomic expression (this study's natural progression snRNA-seq datasets).
- (J) Spearman correlation between transcriptomic expression of the β -oxidation gene program and PPARA transcriptomic expression (Govaere et al.'s human patient cohort⁸²).
- (K) Distribution of Spearman correlations between β -oxidation gene program transcriptomic expression and accessibility of all TF motifs at program-coaccessible linked peaks (this study).
- (L) Distribution of Spearman correlations between β -oxidation gene program transcriptomic expression and expression of all TFs (this study).
- (M) Distribution of Spearman correlations between β -oxidation gene program transcriptomic expression and expression of all TFs (Govaere et al.⁸²).
- (N) Maximally prioritized TFs inferred as driving (top 5) or repressing (bottom 5) the β -oxidation gene program.
- (O) Summary of inferred regulatory relationships between TFs chosen for experimental validation and each stress-adaptation-associated gene program.

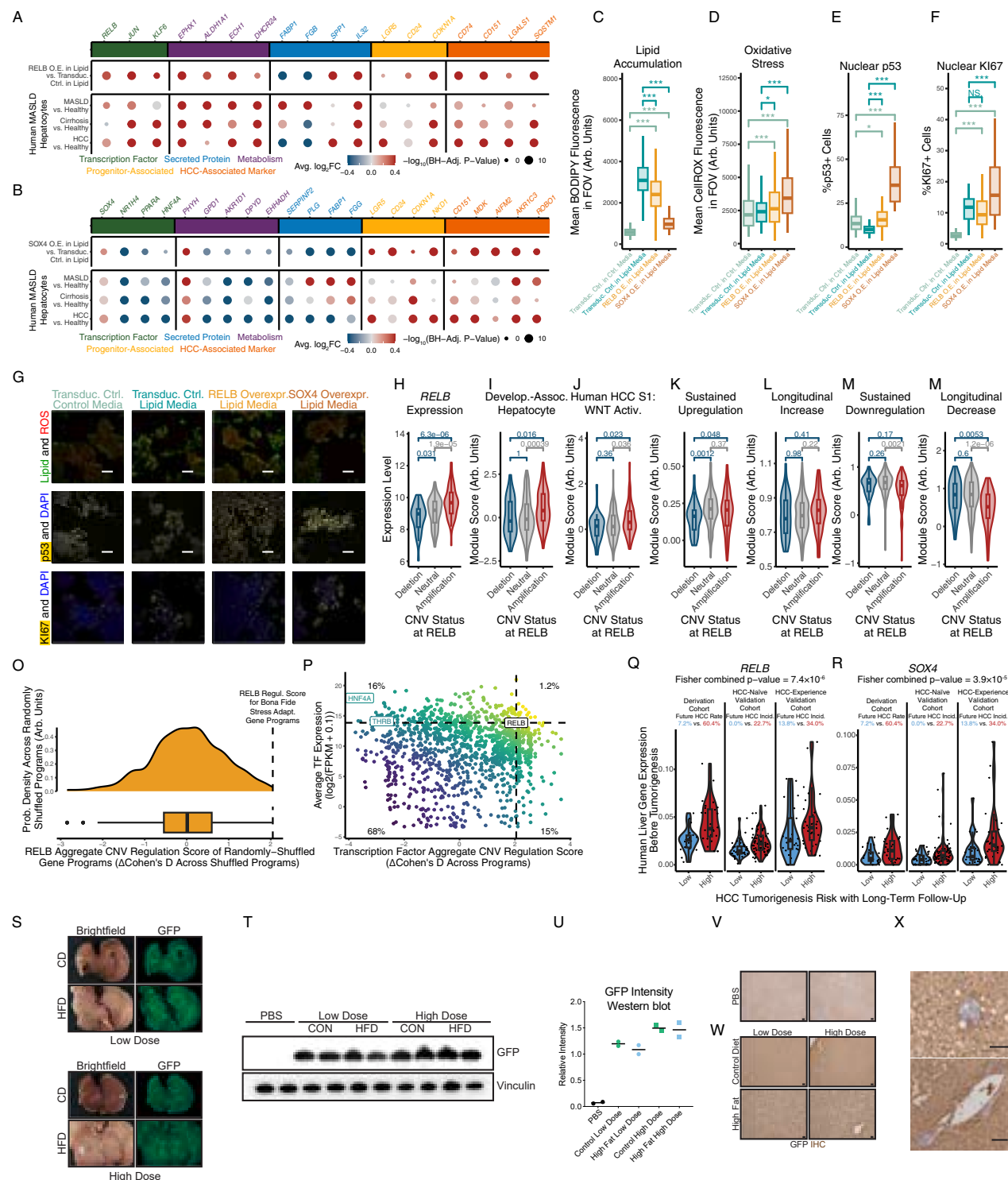


Figure S6. Contextualization of regulatory effects of RELB and SOX4 on hepatocytes' metabolic adaptation programs, connections to human *in vivo* disease progression, and human future tumorigenesis, related to Figure 6

(A and B) Gene expression change induced by RELB (A) or SOX4 (B) overexpression in lipid-rich media (compared with BFP transduction control in lipid-rich media) (top row), or gene expression change with MASLD/cirrhosis/HCC progression in human patients' hepatocytes (Filliol et al.⁹⁸ human snRNA-seq dataset). (C–F) Lipid accumulation (C; BODIPY 493), ROS accumulation (D; CellROX), nuclear p53 (E), and nuclear Ki67 (F). (G) Representative microscopy images supporting (C)–(F) (scale bar, 100 μm).

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(H–N) TCGA HCC samples stratified by CNV status at the RELB gene locus, with expression of (H) RELB itself, (I) Development-Associated hepatocyte gene program, (J) Human HCC S1: WNT Activation gene program, (K) Sustained Upregulation gene program, (L) Longitudinal Increase gene program, (M) Sustained Downregulation gene program, and (N) Longitudinal Decrease gene program.

(O) RELB CNV regulation of bona fide stress adaptation gene programs relative to a null background of matched, randomly shuffled gene programs.

(P) RELB average expression and CNV regulation of stress adaptation gene programs relative to corresponding values for all other TFs. All values were calculated from TCGA HCC datasets ($n = 371$ study participants).

(Q) *RELB* expression across patient cohorts and HCC risk in Fujiwara et al.,⁹⁷ showing increased HCC tumorigenesis with upregulated *RELB*.

(R) *SOX4* expression across patient cohorts and HCC risk in Fujiwara et al.,⁹⁷ showing increased HCC tumorigenesis with upregulated *SOX4*.

(S) Gross images of overexpression efficiency throughout the liver across diet conditions.

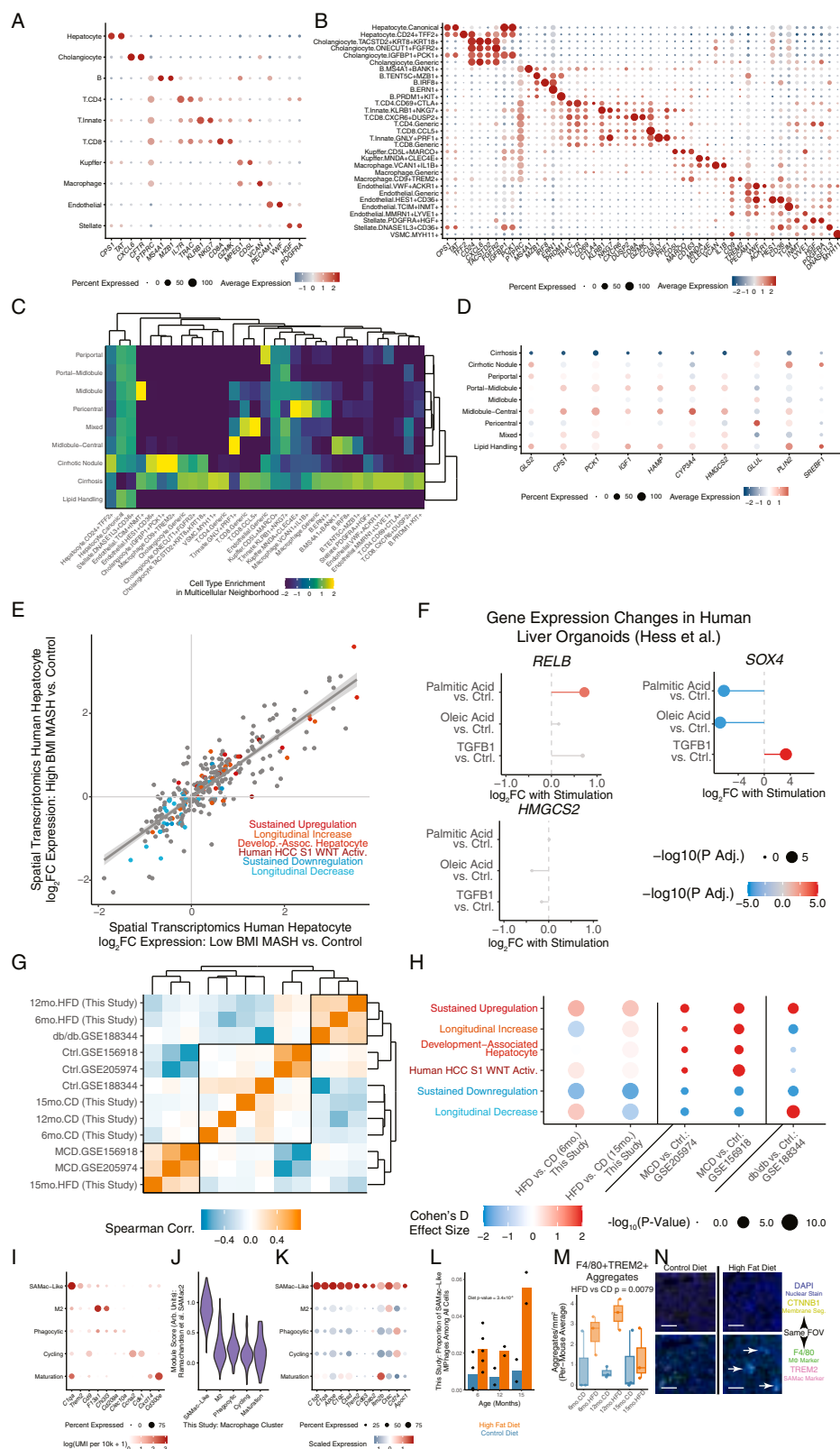
(T and U) Western blot (T) and quantification (U) of protein expression intensity.

(V) Immunohistochemistry of protein intensity in PBS-injected animals (i.e., vehicle control).

(W) Immunohistochemistry of protein intensity in AAV-injected animals across diet conditions.

(X) Regions of interest supporting specificity of protein expression in hepatocytes but not immune cells or other cell types comprising the portal triad.

Scale bars, 100 μm . All p values were calculated using a Mann-Whitney U test with Benjamini-Hochberg correction. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.



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Figure S7. Contextualization of hepatocyte stress responses across human liver spatial organization, human BMI, lipid-intrinsic responses, inflammation vs. obesity, and immune effects, related to Figure 7

- (A and B) Cell-type markers at coarse (A) and fine (B) levels of annotation in human liver spatial transcriptomics data.
- (C) Compositional enrichment of fine-level cell type in multicellular spatial neighborhoods.
- (D) Gene expression of hepatocytes within each multicellular spatial neighborhood.
- (E) Hepatocyte gene expression changes in MASH vs. control study participants from this study's spatial transcriptomics dataset, stratified by BMI cohort ($n = 18$ patients, 320,694 hepatocytes).
- (F) Human liver organoid scRNA-seq expression of RELB, SOX4, or HMGCS2 in culture when stimulated with palmitic acid, oleic acid, or TGF β 1.
- (G) Global expression similarity of mouse models in this study (natural progressive response to chronic metabolic stress), methionine-choline-deficient (MCD) diet models (exhibit liver pathology but do not become obese), or *db/db* mouse models (become obese without nutrient compositional imbalance or liver inflammation).
- (H) Expression changes in stress response gene programs across mouse models and studies.
- (I) Dotplot of marker genes distinguishing this study's macrophage subclusters.
- (J) Module score of SAMac2 markers, split by macrophage subcluster.
- (K) Dotplot of SAMac marker genes highly expressed in this study's SAMac-like subcluster.
- (L) Compositional enrichment of SAMac-like macrophages with HFD in scRNA-seq.
- (M) Compositional enrichment of F4/80+TREM2+ SAMac aggregates with HFD in multiplexed immunofluorescence ($n = 18$ mice).
- (N) Representative images of F4/80+TREM2+ SAMac aggregates (scale bar, 100 μ m).