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## In Vivo Screen Identifies Liver X Receptor Alpha Agonism Potentiates Sorafenib Killing of Hepatocellular Carcinoma

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Hepatocellular carcinoma (HCC) is the most prevalent cancer of the liver and is a leading cause of cancer deaths worldwide. The multikinase inhibitor sorafenib has long been used to treat HCC because it was shown to extend survival by 3 months.<sup>1</sup> Although other drugs have recently been approved for therapy of HCC based on slightly improved outcomes,<sup>2</sup> there is still a pressing need for new and effective treatments. Sequencing technologies have identified hundreds of prognostic genes in HCC.<sup>3</sup> Nevertheless, correlation does not equate to functionality. We used an innovative approach to perform genetic screens in the native environment of the mouse liver to interrogate gene function in the presence or absence of sorafenib, designed to identify potential combination treatments for HCC. We validate a drug combination—sorafenib plus a Liver X Receptor alpha (LXR $\alpha$ ) agonist—with enhanced killing of HCC in multiple cell lines. LXR $\alpha$  agonists are currently in clinical trials and may have acceptable side effect profiles for patients with advanced HCC.

We engineered a plasmid library of 43 complementary DNA (cDNAs) important in liver growth and function to conduct genetic screening in the livers of *Fah*<sup>-/-</sup> mice, a model of liver injury and repopulation.<sup>4</sup> *Fah*<sup>-/-</sup> mice hydrodynamically injected into the tail vein with

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Supplementary Materials

Material associated with this article can be found in the online version at <https://doi.org/10.1016/j.gastha.2022.05.014>.

Conflicts of Interest:

The authors disclose no conflicts.

Ethical Statement:

The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

Data Transparency Statement:

Data, analytic methods, and study materials will be made available to other researchers on request to the corresponding author. The RNA-seq data set is available on the Gene Expression Omnibus (GEO, accession #GSE151412).

the plasmid library developed visible HCCs as early as 6 weeks postinjection, which were confirmed to be HCCs linked to plasmids from the library (Figure 1A and Figure A1).

We hypothesized that a screening approach could be used to discover genes that confer sensitivity or resistance to antitumor drug therapy with sorafenib. Therefore, we injected *Fah*<sup>-/-</sup> mice with the plasmid library and allowed the mice to form tumors for 6 weeks, then began daily treatment with either 30 mg/kg sorafenib or vehicle as a control for 2 months (Figure 1B and C). Sorafenib treatment significantly reduced the liver weight to body weight ratio and tumor burden compared with vehicle (Figure 1D and Table A1).

To identify genes correlated with sorafenib sensitivity, we analyzed cDNAs present or absent in HCCs that developed in the sorafenib-treated group. Strikingly, the oncogene *Myc* was linked to all tumors of vehicle- or sorafenib-treated mice. In contrast, the *Nr1h3* transgene, which encodes LXR $\alpha$ , was present in a number of vehicle-treated tumors as expected by chance but was completely absent from sorafenib-resistant tumors (Figure 1E and F). These results suggested that LXR $\alpha$  activity is incompatible with tumor growth in the presence of sorafenib.

Based on the result of the screen, we asked whether activating LXR $\alpha$  can increase the response to sorafenib. We treated 3 previously established human HCC cell lines (Hep3B and Huh7), a primary human HCC cell line (PGM898), and a murine hepatoma cell line (Hepa1-6) with varying concentrations of the small-molecule LXR $\alpha$  agonists GW3965 and T0901317 and sorafenib (Figure 2A and Figure A2). In each cell line, there was a 30%–50% reduction in cell viability with the combination treatment at a minimal concentration of 5  $\mu$ M GW3965 and 2  $\mu$ M sorafenib compared with sorafenib-only treatment, whereas treatment with LXR $\alpha$  agonist alone did not affect cell viability. At these concentrations, the combination treatment led to only a 20% reduction from baseline in a murine hepatocyte-like cell line (Figure A2B), suggesting treatment preferentially affects HCC cells. Taken together, the results indicate that LXR $\alpha$  activation improves the efficacy of sorafenib in multiple HCC cell lines.

We queried the mechanism by which the combination treatment targets HCC by performing RNA sequencing (RNAseq) of Hep3B and Huh7 cells treated with drugs for 24 hours (Figure 2B and Figure A3A). Principal component analysis of the RNAseq data indicated a clear separation between the cell lines and treatment groups (Figure A3B). Differential expression analysis comparing treatment to DMSO control groups showed marked changes in expression with the combination. Among genes altered congruently in both cell lines: 19 genes were differentially expressed in response to GW3965, 190 to sorafenib, and 915 to the combination treatment, reflecting a remarkable synergistic effect on gene expression. We confirmed the specificity of sorafenib and GW3965 treatment by assessing gene expression levels of known markers of drug activity (Figure A3C).

The gene expression changes with combined LXR $\alpha$  agonist and sorafenib treatment were linked to alterations in 472 gene ontology pathways. The top 10 altered pathways, based on gene ratio, are displayed in Figure 2C. The regulation of cell death was the most altered pathway, and cell cycle pathways were also altered (red boxes). When

investigating steady-state messenger RNA levels of cell cycle and apoptotic regulators, we found downregulation of many proliferation markers such as various cyclins (CCNE1 and CCND1), minichromosome maintenance complex proteins (MCM2, MCM4, MCM5, and MCM6), proliferating cell nuclear antigen, and cyclin-dependent kinase 2 (Figure 2B). Western blotting confirmed the downregulation of cell cycle regulators Cyclin D1 and proliferating cell nuclear antigen with combination therapy (Figure A3D). Differentially expressed genes associated with apoptosis included (BBC3, PMAIP1, JUN, and DDIT3; Figure 2B). Western blotting confirmed the upregulation of apoptosis regulator JUN with sorafenib and the combination treatment (Figure A3E). In short, combination treatment led to diminished expression of cell cycle genes and upregulation in apoptosis markers compared with sorafenib or LXR agonist alone, highlighting the cooperativity of these compounds.

In summary, our *in vivo* genetic screen documents that LXR $\alpha$  is a key signal to determine the response to sorafenib of MYC-driven tumors in a model of liver injury and repopulation. We show that LXR $\alpha$  agonists enhance the vulnerability of HCC cell cultures to sorafenib. The beneficial action of LXR $\alpha$  agonism we reported here is in agreement with LXR $\alpha$  expression correlating with better HCC prognosis<sup>5</sup> and with diminished LXR $\alpha$  expression in sorafenib-resistant HCC cells.<sup>6</sup> Recent preclinical assays in mice of LXR $\alpha$  agonists and sorafenib support the benefit of combinatorial treatment and showed enhanced antitumor activity in both xenograft- and oncogene-driven hydrodynamic tail vein injection models.<sup>7,8</sup> Further studies are needed to identify HCC subgroups that may benefit most from this combination. Collectively, our studies document how *in vivo* genetic screening in combination with drug therapies could identify cancer drug dependency and susceptibility patterns, which could be scaled to high-throughput screens.<sup>9</sup>

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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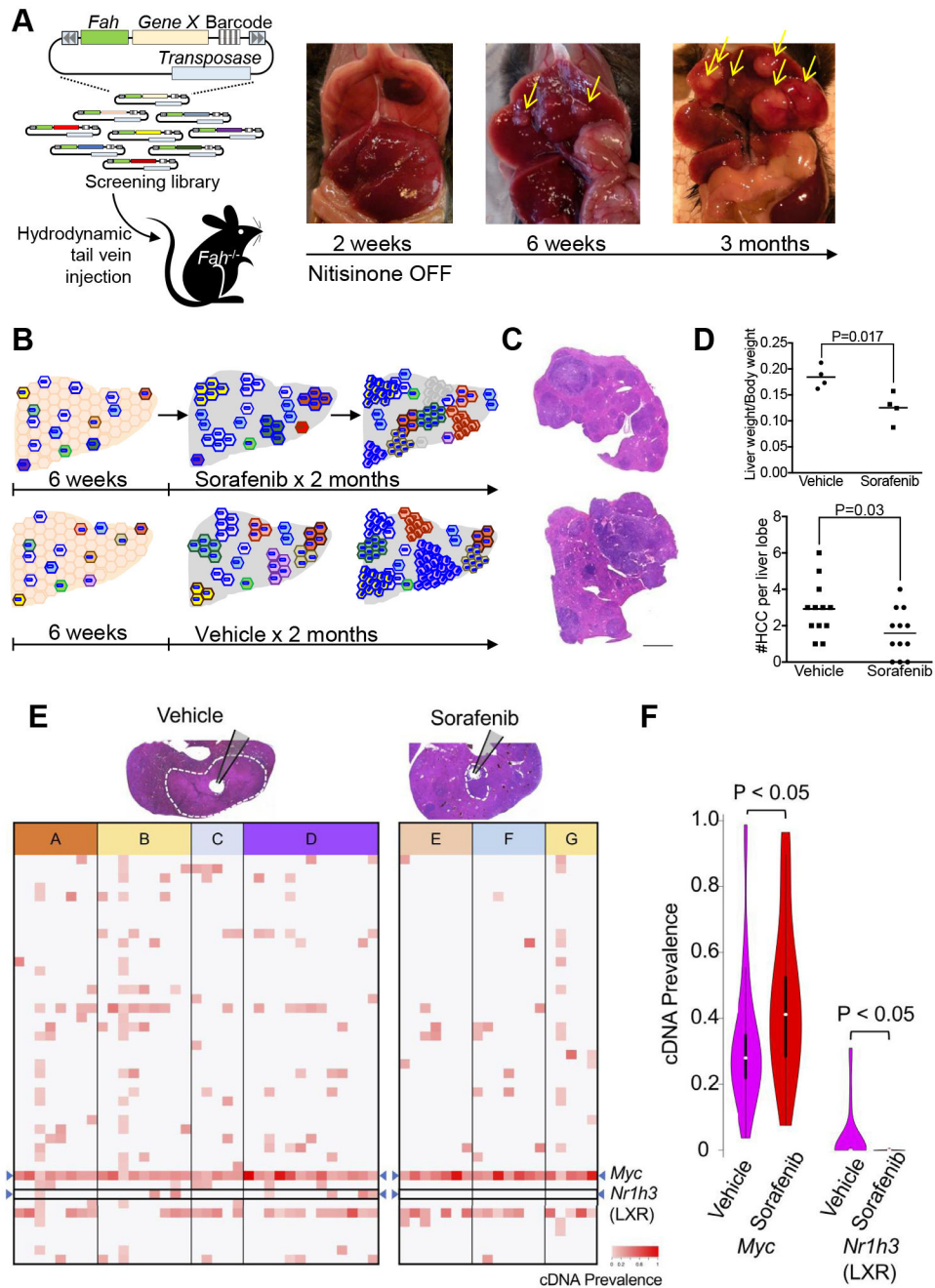
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## Abbreviations used in this paper:

<b>HCC</b>	hepatocellular carcinoma
<b>LXR<math>\alpha</math></b>	liver X receptor alpha
<b>RNAseq</b>	RNA sequencing

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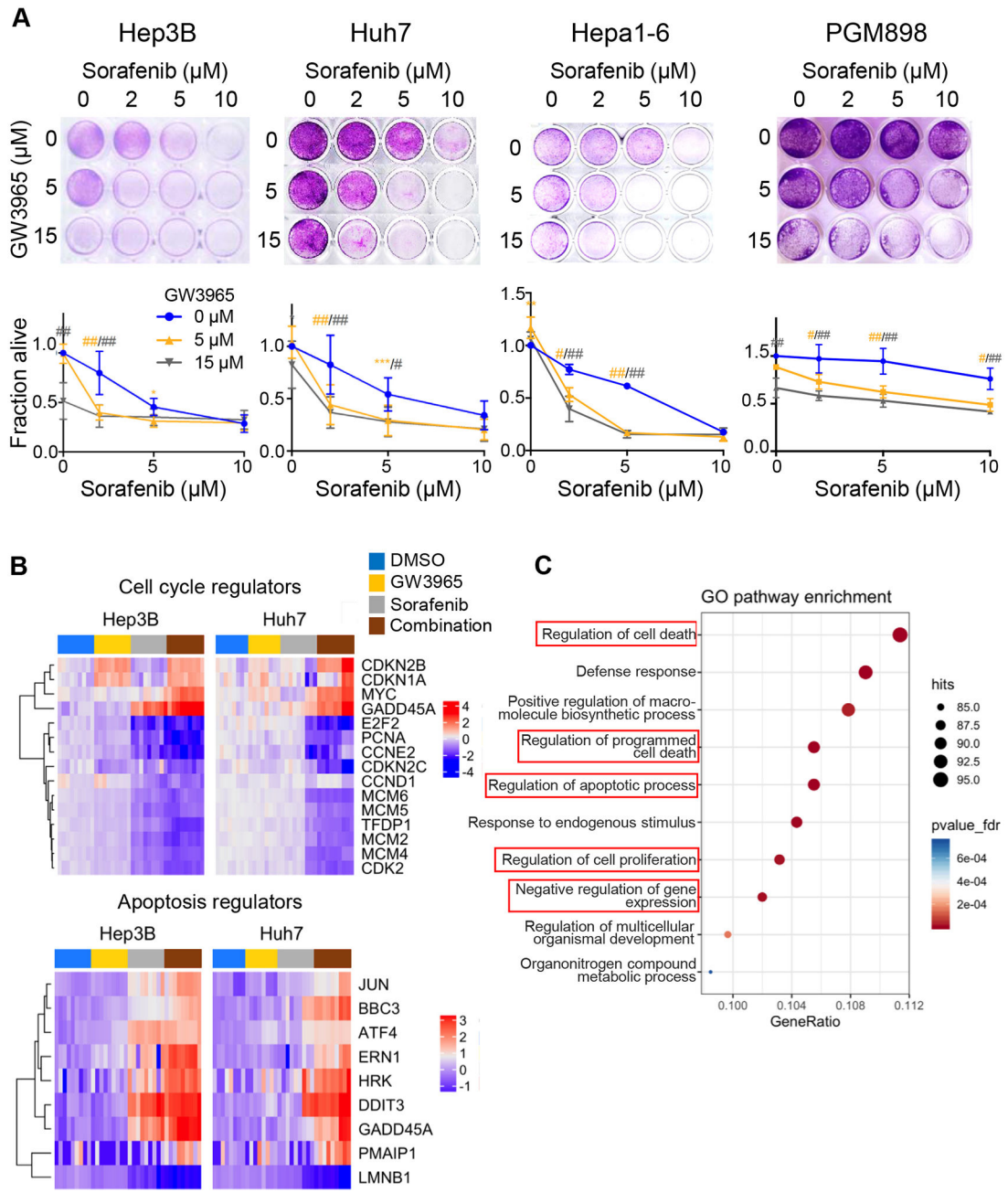
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**Figure 1.**

In vivo genetic screening for target genes associated with sorafenib activity. (A) *Fah*<sup>-/-</sup> mice are a model of liver injury and repopulation. When FAH expression is restored in subset of hepatocytes, these cells repopulate the injured liver upon removal of nitisinone from the drinking water (see Online Methods). FAH expression plasmids are delivered to a subset of hepatocytes using the hydrodynamic tail vein injection technique. The *Sleeping Beauty* Transposon system enables integration into the genome. We constructed 44 different plasmids and pooled them into a screening library (43 genes-of-interest and GFP). Each cDNA in the library has a unique 5-nucleotide barcode in the 3' untranslated region

to enable linkage of individual cDNAs or their combinations to specific tumors via high-throughput sequencing. *Fah*<sup>-/-</sup> mice were injected with the library of 43 genes-of-interest plus GFP, all linked to FAH expression. Tumors were visible by 6 weeks postinjection, and large tumors developed by 3 months (representative image shown, N = 3 mice at 6 weeks and N = 4 at 3 months). (B) Schematic of experimental design to generate tumors with the screening library, then treat with sorafenib or vehicle. (C) Representative H&E staining of sorafenib- and vehicle-treated tumors (scale bar = 5 mm). (D) Liver weight to body weight ratio and number of HCC tumors per liver lobe (N = 4 mice in each group). Statistics performed using Student's t-test. (E) HCC tumors were micro-dissected and sequenced to determine the linked cDNAs (N = 35 vehicle-treated and N = 19 sorafenib-treated HCCs). Linkage of *Myc* and *Nr1h3* cDNAs is indicated with arrowheads. *Nr1h3* was significantly depleted in the sorafenib group ( $P < .05$ , Fisher exact test). Headings A-G indicate separate animals. (F) Violin plots of sorafenib- and vehicle-treated tumors indicating the prevalence of *Myc* and *Nr1h3* cDNA among total sequence reads. ( $P < .05$  by Student's t-test).





**Figure 2.** Combination treatment with Sorafenib and LXR agonist GW3965 effectively targets HCC. (A) Representative images and quantification of crystal violet staining of Hep3B (n = 8) and Huh7 (n = 11), Hep1-6 (n = 3), and PGM898 cells (n = 4) treated with varying concentrations of DMSO control, GW3965, sorafenib, or GW3965 and sorafenib for 48 hours. Quantification was performed by addition of methanol to plates and measuring absorbance (right). Statistical analysis performed with two-way analysis of variance with Tukey's multiple comparison.  $P$  values are as follows: \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .005$ , # $P < .001$ , ## $P < .0001$ . (B) Heatmap of RNAseq transcriptomic analysis of Hep3B and

Huh7 cells treated with DMSO, 2  $\mu$ M GW3965, 5  $\mu$ M sorafenib, or GW3965 and sorafenib (Combo; N = 8–9 independent replicates) showing reduction of cell cycle regulators (top) and induction of apoptosis regulators (bottom). We selected an early time point of 24 hours and low drug dosage to capture the major expression changes that ultimately lead to cell death. (C) Top 10 significantly modulated gene ontology (GO) pathways from the RNAseq transcriptomic analysis. Red boxes highlight pathways associated with the cell cycle or apoptosis.