Supplemental Information

Wnt signaling stimulates cooperation between GREB1 and HNF4α to promote proliferation in hepatocellular carcinoma

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Supplementary Methods

Immunohistochemical analysis

Tissue microarrays (TMAs) were constructed from formalin-fixed paraffin-embedded (FFPE) tumor tissue for immunohistochemistry. Three 2-mm tissue cores were randomly taken from each case. Antigen retrieval was performed using a decloaking chamber (Biocare Medical, Walnut Creek, CA, USA). Endogenous peroxidase activity was blocked using Dako REAL^{TM} Peroxidase Blocking Solution (Dako, Carpentaria, CA, USA) for 15 min. The sections were then incubated with Blocking One Histo (NACALAI, Kyoto, Japan) for 15 min to block nonspecific antibody binding sites. Tissue specimens were incubated with primary antibodies for 2 h at room temperature and then with HRP Horse Anti-Mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h. Staining was visualized using diaminobenzidine (Nichirei Biosciences, Tokyo, Japan) as the chromogen. Tissue sections were counterstained with 0.1% (w/v) hematoxylin.

For subsequent double staining, tissue specimens were treated with antibody stripping buffer (10 mM sodium citrate [pH 6.0]) at 95°C for 5 min using a decloaking chamber. Tissue specimens were incubated with mouse anti-β-catenin (1:100) antibody for 1 h at room temperature, followed by AP Horse Anti-Mouse IgG (Vector Laboratories, Burlingame, CA, USA) for 1 h. Staining was detected using the Warp Red Chromogen Kit (Biocare Medical, Walnut Creek, CA, USA). Tissue sections were counterstained with 0.1% (w/v) hematoxylin. Tumors were considered β-catenin or GREB1 positive if > 30% of the total area of a tumor lesion was stained. Two investigators assessed the sections independently in a blinded fashion.

Identification of general Wnt signaling target genes

In order to search for general Wnt target genes, correlation of gene expression with AXIN2 in each of 33

TCGA tumors were calculated. AXIN2 was chosen as the most established general Wnt target gene because its expression is widely used as a reporter for various tissues (1, 2). For each gene, the number of tumors where the gene expression correlates significantly ($R > 0.5$, $p < 0.05$) with AXIN2 were counted. As shown in Supplemental Table. 1, these genes were sorted in descending order by the count, and the top 10 genes which have previously been presented as Wnt target gene were defined as general Wnt target genes (3-13).

Knockdown of protein expression by siRNA or ASO

The siRNA and ASO sequences used in this study are shown in Supplemental Material 2. Hep3B, JHH7, and Huh7 cells were transfected with the siRNA (10-20 nM) or ASO (10-20 nM) against the genes of interest using RNAiMAX (Thermo Fisher Scientific, Waltham, MA, USA). The transfected cells were used for experiments 48–72 h post-transfection.

Plasmid construction and generation of stable transfectants

Standard recombinant DNA techniques were used to generate plasmids harboring full-length GREB1 or dominant negative (DN)-TCF4. Lentiviral vectors were constructed by subcloning EGFP-GREB1 or EGFP-DN-TCF4 into CSII-CMV-MCS-IRES2-Bsd, kindly provided by Dr. H. Miyoshi (RIKEN BioResource Center, Ibaraki, Japan) (14). CS-RfA-EVBsd containing stem-loop cassettes encoding shRNAs targeted to scramble (targeting sequence: CAGTCGCGTTTGCGACTGG) for control, human GREB1 (targeting sequence 5029: GTCACGAACATGGGCTCTTTA, 6839: GGTAAATACCCTACCTCTTAG), human HNF4α (targeting sequence 1190: CGAGCAGATCCAGTTCATCAA, 1882: ATGACTTGAGGCCTTACTTAA), and human FOXA2 (targeting sequence 249: GGGTGATTGCTGGTCGTTTGT, 2233: GGTCTCGGGTCCGATTAAT) were

used for shRNA. Plasmids were transfected with packaging vectors, pMD2.G and psPAX2, into X293T cells using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) to generate lentiviruses. To generate Hep3B cells stably expressing GFP-DN-TCF4, PLC/PRF/5 cells stably expressing GFP-GREB1, or Hep3B and JHH7 cells stably expressing control or GREB1 shRNA, parental cells (1×10^5/well) were plated in 12-well plates and transduced with lentiviruses and 10 μg/ml polybrene. After the infection, the cells were centrifuged at 1200 × *g* for 30 min and then incubated for 24 h.

Quantitative RT-PCR

Total RNA (1 μg) was reverse transcribed using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan). Quantitative RT-PCR was performed as previously described (15). The primers used for quantitative RT-PCR are listed in Supplemental Material 3.

RNA-seq data analysis

Sequenced reads were preprocessed by Trim Galore! v0.6.7 and quantified by Salmon v1.4.0 with the flags gcBias and validateMappings. GENCODE v36 (GRCh38.p13) annotation was used as the transcript reference. All procedures were implemented using the RNA-seq pipeline ikra v2.0.1 [http://doi.org/10.5281/zenodo.4718200] with default parameters. The quantified transcript-level scaled TPM was summarized into a gene-level scaled TPM using tximport v1.22.0. Downstream analysis was conducted using iDEP.951 [http://bioinformatics.sdstate.edu/idep/]. Low expression genes that had less than 0.5 counts per million (CPM) in at least one sample were filtered. Differential expression analysis with DESeq2 was performed in iDEP using the default parameters (false discovery rate < 0.1). The signature score was defined as the mean z-score of the z-score transformed data.

Gene Set Enrichment Analysis (GSEA)

Filtering of low expression genes was performed as described in the "RNA-seq data analysis" section. Normalization was conducted using DESeq2, and the normalized counts were used as the input data. FDR cutoff was set to 0.01. GSEA was conducted using GSEApy v0.10.7, and hallmark gene sets from the Molecular Signatures Database (MSigDB) were used.

Single-cell RNA-seq data analysis

The GSE151530 dataset consisted of 46 liver tumor samples from 37 patients. Malignant cells (17164 cells) that were already defined in previous studies (16) were extracted from the total cells, and 13187 cells, including 20 HCC and ten intrahepatic cholangiocarcinoma samples, met the quality control metrics as follows: doublets detected using Scrublet v0.2.1, cells with a high percentage of counts in the mitochondrial genes (≥ 15%), cells that had less than 200 genes expressed. Genes that were detected in less than three cells were filtered out. Pre-processing was conducted by sc.pp.normalize total (target_sum = 1e4) and sc.pp.log1p implemented in Scanpy. Batch-effect correction was performed using the BBKNN algorithm. UMAP was used for dimensionality reduction. Scanpy v1.6.0 and python 3.7.8 were used to analyze and visualize data.

Generation of knockout cells

To establish GREB1 or GREB1 enhancer (En1) knockout cells, plasmid pRP [CRISPR] expressing hCas9 and guide RNA were designed and synthesized by VectorBuilder Inc. (Guangzhou, China). The target sequences for human GREB1 and GREB1 enhancer (En1) were as follows: human GREB1, 5'-TAAAGCCGATGGTCGCGGCA -3'; En1-5', 5'-AAAGATCATTCTCGTAGCAC-3'; En1-3', 5'-CATCAGTCTCTTTTGTTCTG-3'. The plasmid pRP [CRISPR] with the gRNA sequences was

introduced into Hep3B or JHH7 cells, and then single colonies were picked, mechanically disaggregated, and replated into the individual wells of 24-well plates.

Colony formation assay

Hep3B or JHH7 cells (5000 cells) were seeded into 100-mm dishes and cultured for 10–14 days in 10% serum culture medium. The cells were fixed with 4% PFA for 10 min, and the colonies were stained with 0.2% crystal violet for 5 min. The areas of the colonies were measured using Image Pro software (Media Cybernetics, Silver Spring, MD, USA).

Cell proliferation assay

Cells (2.0×10^4/ml) were transfected with the indicated siRNA. Four days after plating, the medium was replaced with 10% serum culture medium. The cells were counted every 48 h for eight days. The assay was performed in triplicate for each experiment. CyQUANT NF assays (Life Technologies/Thermo Fisher Scientific, Waltham, MA, USA) were performed according to the manufacturer's instructions. Fluorescence was measured using a Synergy HTX Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA).

Subcutaneous xenografts

Male BALB/cAJcl-nu/nu mice (6-8 weeks old; CLEA Japan, Tokyo, Japan) were subcutaneously injected dorsally with Hep3B or JHH7 cells (1×10^7 cells) resuspended in150 µl of Matrigel (Corning, NY, USA) or PLC/PRF5 cells (5×10^6 cells) resuspended in 100 µl of 3D Ready Atelocollagen (Koken Inc., Tokyo, Japan), and euthanized 15, 46, 35 days after inoculation, respectively. Tumor tissue was harvested, and the tumors were measured and weighed.

Supplementary References

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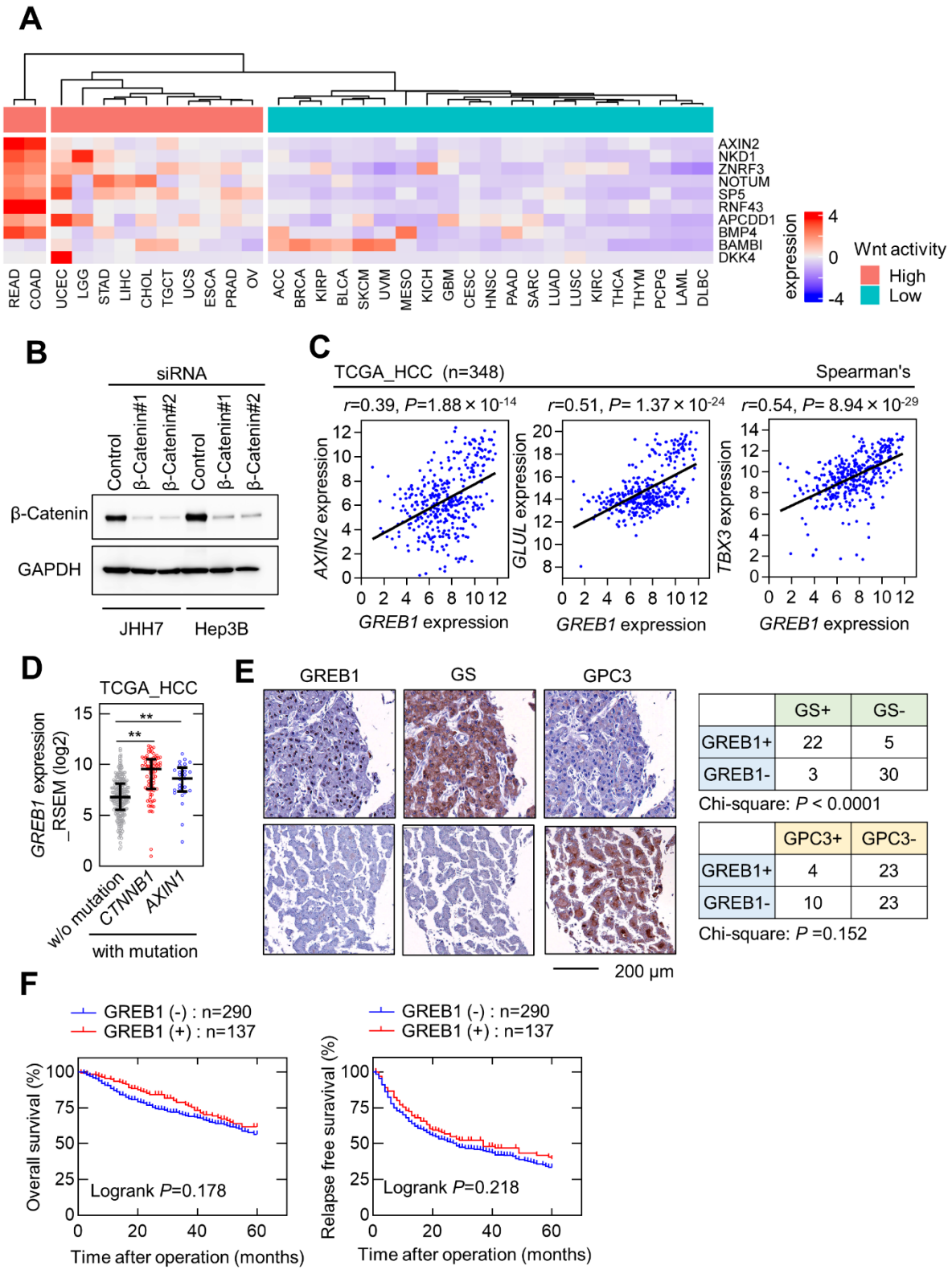
Matsumoto et al., Supplemental Figure 1



Supplemental Figure 1| Activation of Wnt signaling and expression of GREB1 in HCC

(A) Heatmap of mean expression value for general Wnt target genes across 33 cancers from TCGA is shown. General Wnt signaling target genes were defined as described in Supplemental Table 1. Z-score was calculated for each row (gene), and k-mean clustering (k = 3) was performed on cancers. Clustered cancer groups were divided into two groups, Wnt high activity and low activity cancers.

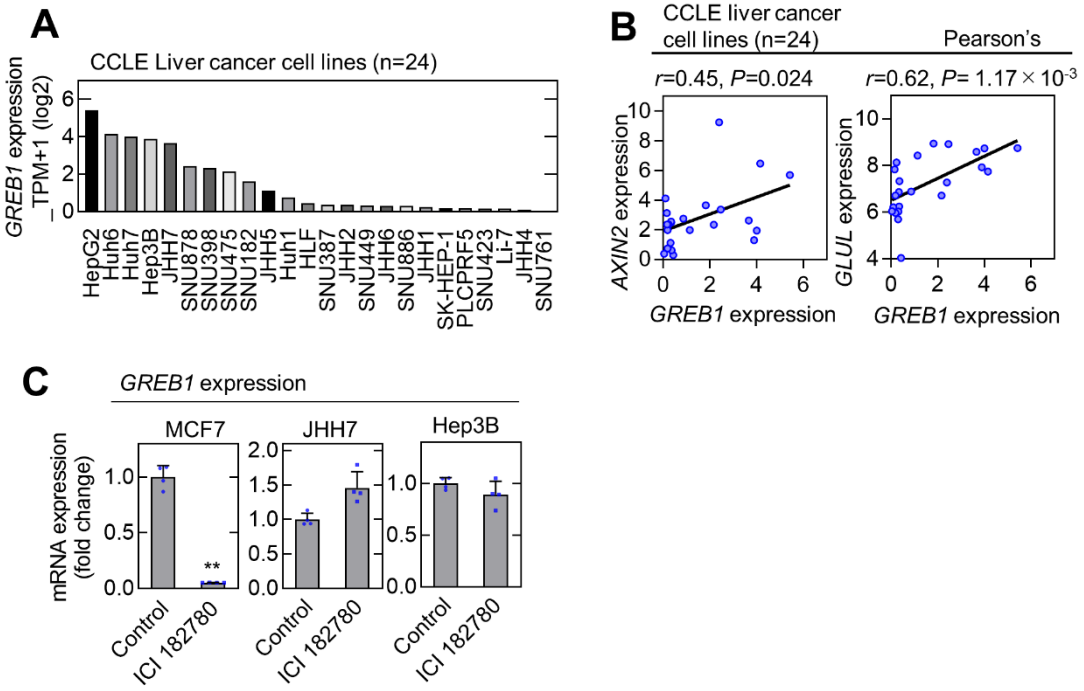
(B) JHH7 and Hep3B cells were transfected with control or two independent β-catenin siRNAs and the lysates were probed with the indicated antibodies. GAPDH was used as a loading control.

(C) Scatter plots showing the correlation between the expression of Wnt signaling target genes (y-axis) and *GREB1* gene expression (x-axis) in the liver tumors were obtained from a TCGA HCC patient dataset (n = 348). The Spearman *r*- and *P*-values were calculated with GraphPad Prism.

(D) Scatter plots showing *GREB1* expression in patients without mutations in the *CTNNB1* or *AXIN1* gene (234 patients) and those with driver mutations in the *CTNNB1* (89 patients) or *AXIN1* (25 patients) gene were obtained from a TCGA HCC patient dataset using the cBioPortal platform. The *P*-values were calculated using the Mann-Whitney test. w/o; without.

(E) HCC specimens (n = 60) were stained with anti-GREB1, anti-GS, and anti-GPC3 antibodies and counterstained with hematoxylin (left panel). The contingency table for the number of positive and negative cases for each of GREB1 and GS or GREB1 and GPC3 is shown in the right panel. Scale bar, 200 μm.

(F) The relationship between overall or relapse-free survival and GREB1 mRNA expression in HCC patients was analyzed. The log-rank test is used for statistical analysis.



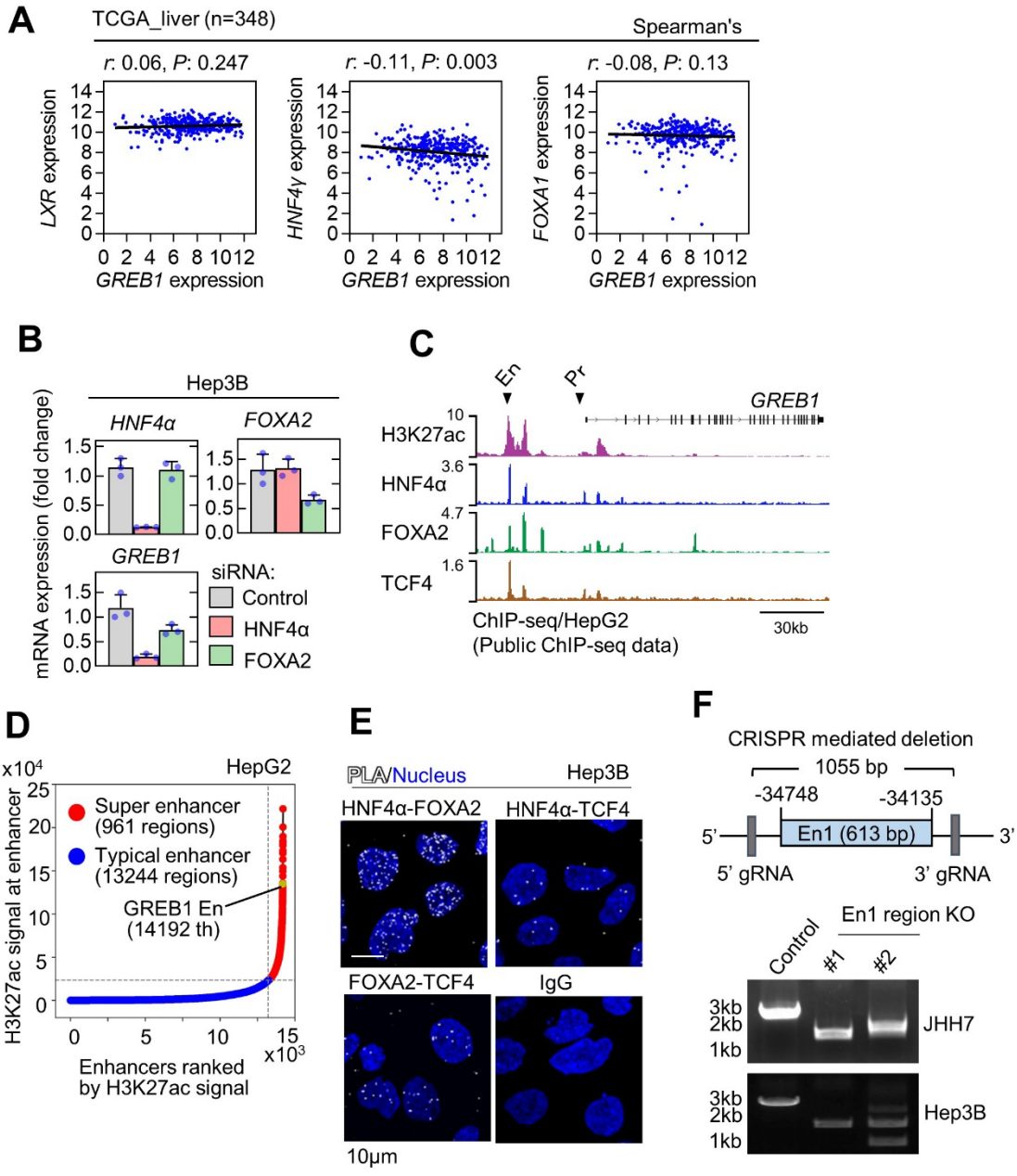
Supplemental Figure 2| GREB1 expression in HCC cell lines

(A) *GREB1* mRNA expression levels in 24 hepatocellular carcinoma cell lines. The expression data were obtained from the CCLE (https://depmap.org/portal/).

(B) Scatter plots showing the correlation between the expression of the target genes of Wnt signaling (y-axis) and the *GREB1* gene (x-axis) were obtained from the CCLE (n = 24). The Pearson *r*- and *P*-values were calculated with GraphPad Prism.

(C) MCF7, JHH7, or Hep3B cells were treated with 10 μM ICI 182780 for 48 hours, then RNA was extracted and GREB1 mRNA levels were measured using real-time PCR (**, P < 0.01, t-test).

Matsumoto et al., Supplemental Figure 3



Supplemental Figure 3| GREB1 expression is regulated in HCC through a super-enhancer involving HNF4α and FOXA2

(A) Scatter plots showing the correlation between LXR, HNF4γ, and FOXA1 gene expression (y-axis) and GREB1 gene expression (x-axis) were obtained from a dataset of TCGA HCC patients (n=348). Spearman *r* and *p* values are calculated using GraphPad Prism.

(B) Hep3B cells were transfected with the indicated siRNAs and mRNA levels of HNF4α, FOXA2, and GREB1 were measured by real-time PCR. The results are expressed as fold-change of the mRNA levels by HNF4α and FOXA2 siRNAs compared with those by control siRNA and shown as mean ± SD.

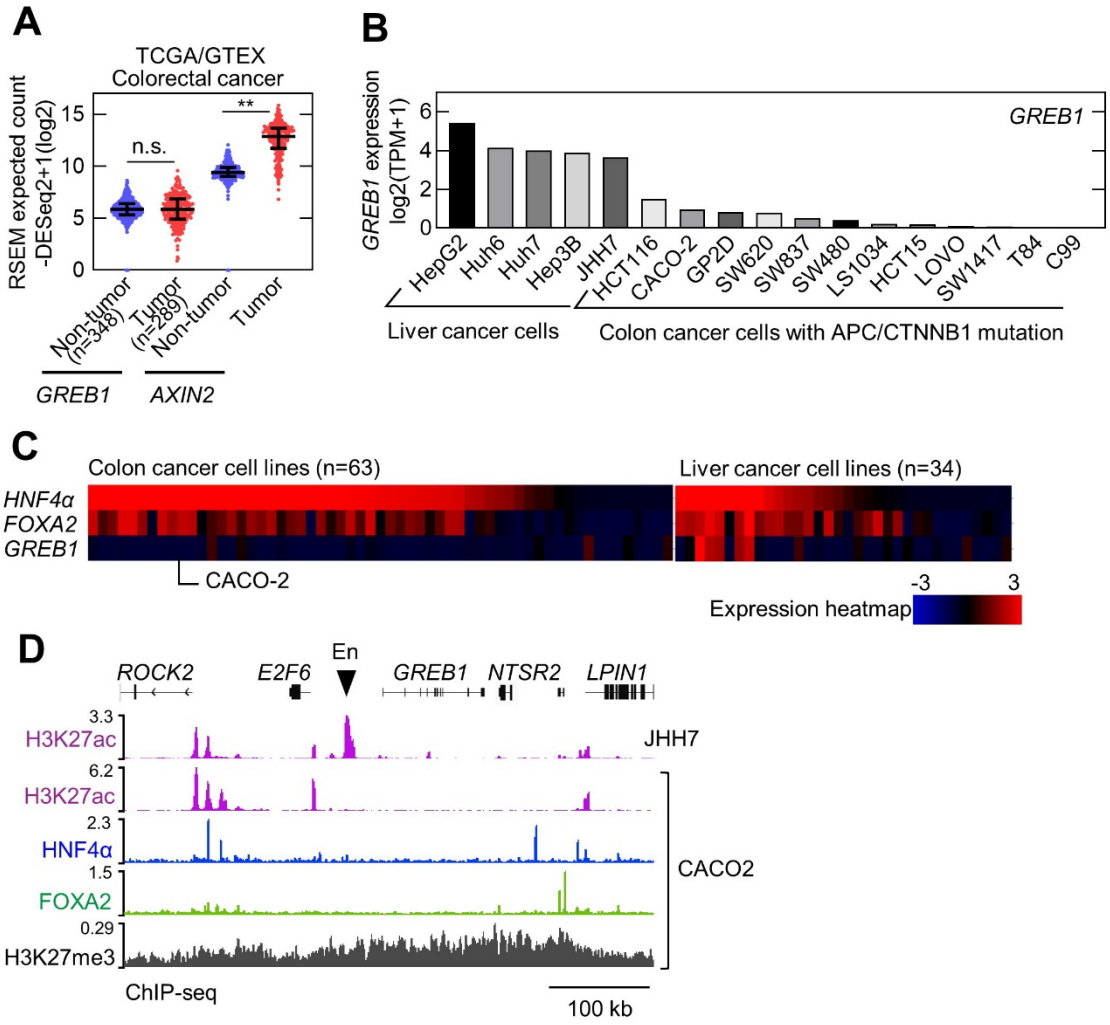
(C) All data of ChIP-seq of the *GREB1* locus in HepG2 cells were obtained and visualized using Cistrome Data Browser (http://cistrome.org/db/#/). The data shows the binding peaks of H3K27 acetylation (ac) and the indicated transcription factors.

(D) Based on ChIP-seq data of H3K27ac in HepG2 cells, a super-enhancer prediction analysis was performed. 14206 enhancer regions were identified and ranked based on ascending order of the signal intensity of H3K27ac. Super-enhancers were defined as the enhancers above the inflection point of the curve (rank 13245) GREB1 En was predicted as a super-enhancer at rank 14192.

(E) Hep3B cells were incubated with the indicated antibodies, followed by the secondary PLA probe. Interaction events are indicated by white dots.

(F) To generate En1 knockout (KO) cells, gRNAs on the 5'- and 3'-sides of the En1 region were designed (top panel) and introduced into JHH7 and Hep3B cells with Cas9. The deletion of the En1 region was confirmed by PCR using primers flanking the deleted region for the two clones (#1 and #2) (bottom panels).

Matsumoto et al., Supplemental Figure 4



Supplemental Figure 4| GREB1 is not expressed in colorectal cancer

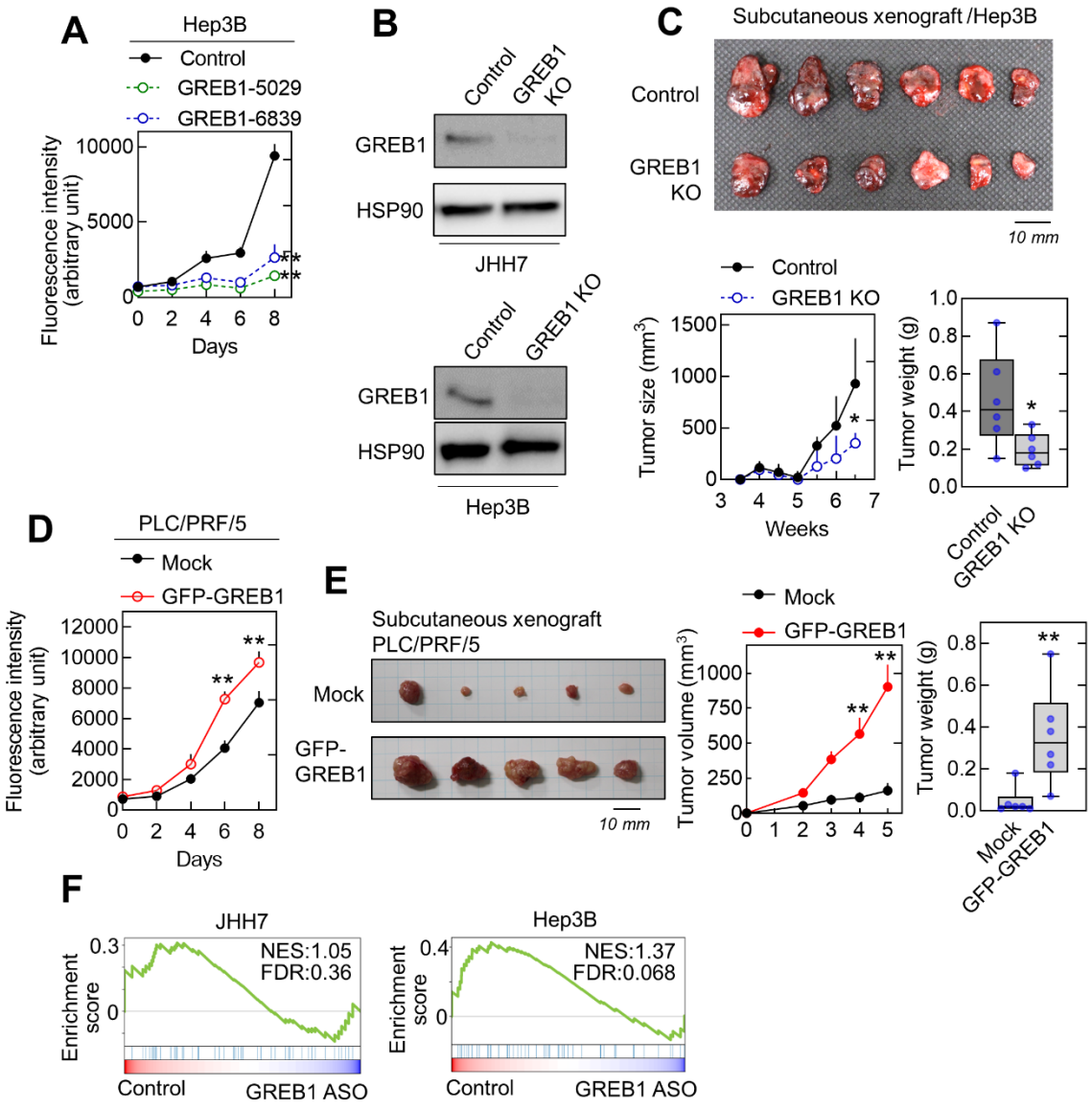
(A) *GREB1* and *AXIN2* mRNA levels in tumor and non-tumor tissues of colorectal cancer are compared using the UCSC Xena platform for analyzing TCGA TARGET GTEx datasets (https://xenabrowser.net/). **, $P < 0.001$.

(B) The expression levels of *GREB1* mRNA in 5 liver cancer and 12 colorectal cancer cell lines harboring the mutation in *APC* or *CTNNB1* are shown. Expression data are obtained from DepMap portal (https://depmap.org/portal/).

(C) Heat maps showing mRNA expression of *HNF4α*, *FOXA2*, and *GREB1* in human colon cancer (n=63) and liver cancer (n=34) cell lines were obtained from cBioPortal. The heatmaps were sorted in descending order based on *HNF4α* expression per cell line. The location of the expression data for CACO-2 cells is indicated.

(D) ChIP-seq analysis was performed using H3K27 trimethylation (me3), H3K27 acetylation (ac), HNF4α and FOXA2 antibodies in CACO-2 cells, and profile of the *GREB1* and the surrounding locus are shown. All ChIP-seq data are obtained using Cistrome Data Browser (http://cistrome.org/db/#/) and visualized. En and Pr represent regions as described in Fig. 2D.

Matsumoto et al., Supplemental Figure 5



Supplemental Figure 5| GREB1 expressed in HCC is involved in cell proliferation and tumorigenesis

(A) Hep3B cells stably expressing control or GREB1 shRNAs were subjected to a 2D cell proliferation assay. Relative cell numbers were quantified using the CyQUANT assay. Results are expressed as an arbitrary unit and shown as mean ± SD. **, $P < 0.01$.

(B) Control and GREB1 knockout (KO) cells were generated in JHH7 (top panels) and Hep3B (bottom panels) cells. Whole cell lysates were probed with the indicated antibodies. HSP90 is used as a loading control.

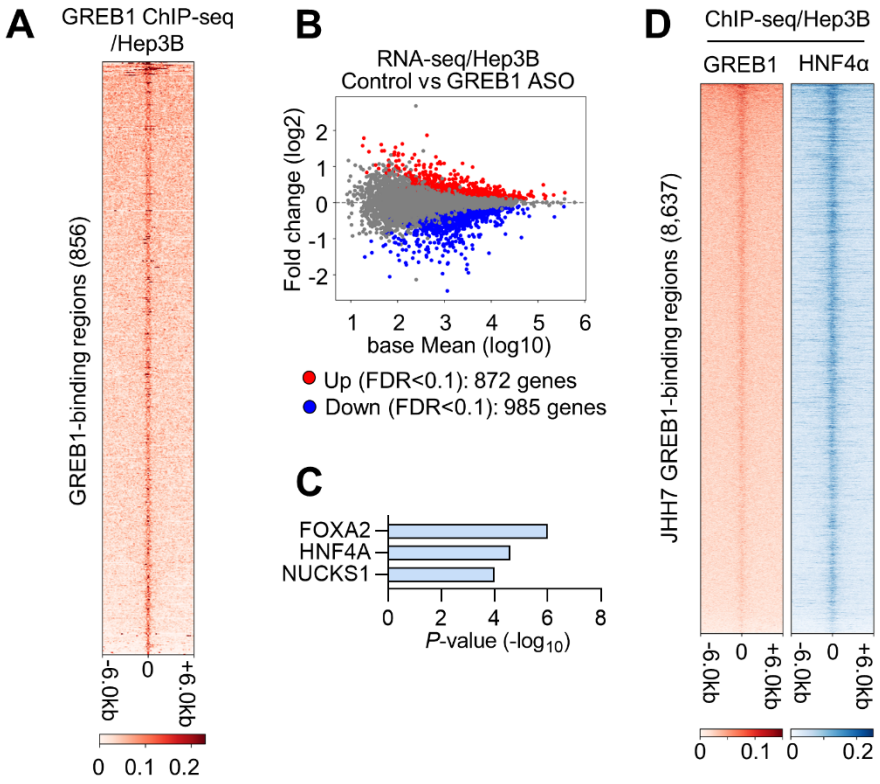
(C) Control or GREB1 KO Hep3B cells were subcutaneously implanted into nude mice (n =6), and the mice were euthanized after 6.5 weeks. Representative appearances of extirpated xenograft tumors are shown (top panel). The sizes and weights (bottom panels) of the xenograft tumors were measured. Results are shown as mean ± SD. *, $P < 0.05$.

(D) PLC/PRF/5 cells stably expressing control vector or GFP-GREB1 were subjected to a 2D cell proliferation assay. Relative cell numbers were quantified using the CyQUANT assay. Results are expressed in arbitrary units and expressed as mean ± SD. **, $P < 0.01$.

(E) PLC/PRF/5 cells stably expressing mock or GFP-GREB1 were subcutaneously transplanted into nude mice (n =6) and mice were euthanized after 5 weeks.

Representative appearances of extirpated xenograft tumors are shown (left panel). The size (middle panel) and weight (right panel) of the xenograft tumors were measured. Results are shown as mean ± SD. **, $P < 0.01$.

(F) GSEA plots show the enrichment of the TGF-β signaling genes from HALLMARK between the control and GREB1 ASO-treated JHH7 (top panel) and Hep3B (bottom panel) cells. NES, normalized enrichment score.



Supplemental Figure 6| GREB1 cooperates with HNF4α to regulate gene expression in Hep3B cells.

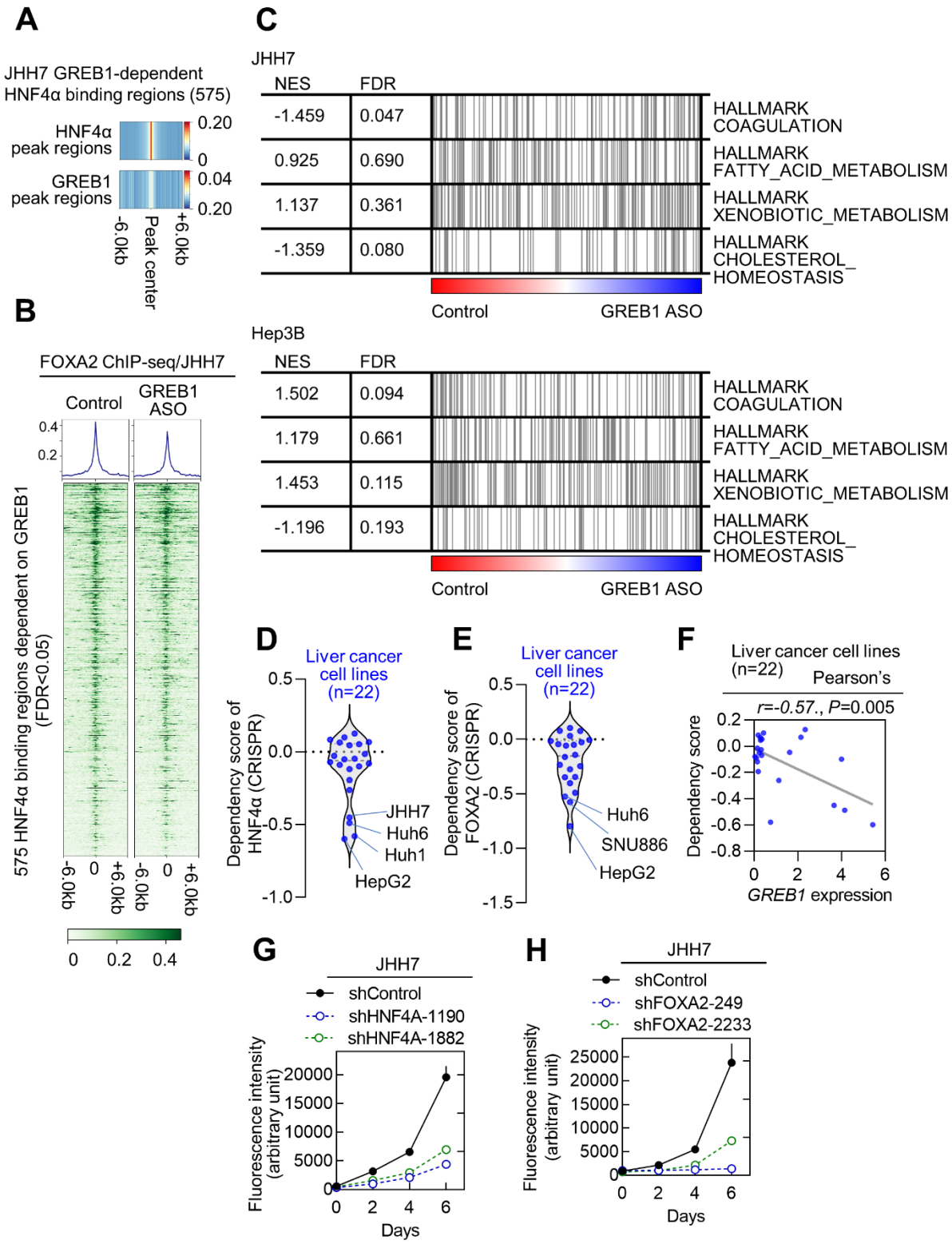
(A) ChIP-seq analysis was performed on Hep3B cells using anti-GREB1 antibody. Peak signals in the 856 GREB1 binding regions are shown in a range ± 6 kb from the peak center.

(B) Total RNA was extracted from Hep3B cells transfected with control or GREB1 ASO, and RNA sequencing analysis was performed. GREB1 knockdown resulted in significantly increased expression of 872 genes and decreased expression of 985 genes in Hep3B cells (FDR < 0.1). The MA plot for differential expression analysis between control (n = 2) and GREB1 (n = 2) ASO-treated samples is shown. The red dots indicate significantly upregulated genes, and the blue dots represent the downregulated genes in the GREB1 ASO-treated cells. FDR, false discovery rate.

(C) ChEA was performed on the GREB1 target genes in Hep3B. The top three significantly enriched terms for ChEA are listed based on their P-values.

(D) ChIP-seq analysis was performed on Hep3B cells using anti-GREB1 and anti-HNF4α antibodies. Peak signals in the 8637 GREB1 binding regions defined in JHH7 cells (Fig. 4A) are shown in a range ± 6 kb from the peak center.

Matsumoto et al., Supplemental Figure 7



Supplemental Figure 7| HNF4α and FOXA2 is involved in cell proliferation in liver cancer cells

(A) The average Hep3B ChIP-seq signal for HNF4α and GREB1 in the GREB1-dependent HNF4α binding regions in JHH7 cells which was identified in Fig. 5B is visualized in the heatmap.

(B) ChIP-seq was performed using anti-FOXA2 antibody in JHH7 cells treated with control or GREB1 ASO. The FOXA2 signal for the 575 regions described in Fig. 5B is shown in a range of ± 6 kb from the peak center. Heatmaps are sorted in descending order by intensity of the HNF4α peak signal in control ASO-treated condition.

(C) Enrichment score of the indicated gene sets between the control and GREB1 ASO-treated JHH7 (top panel) and Hep3B (bottom panel) cells was calculated by GSEA. Barcode plot shows the enrichment of each gene sets. Each vertical line in the plot represents a gene in the gene set.

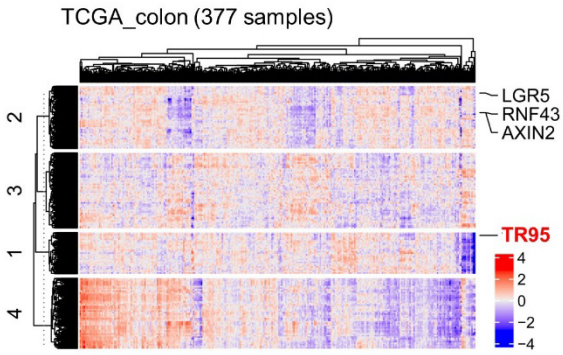
(D) Dependency scores for HNF4α in the liver cancer cell lines were obtained from DepMap and are shown by dot-and-box plots. Negative scores indicate that deletion of HNF4α suppressed cell proliferation.

(E) Dependency scores for FOXA2 in the liver cancer cell lines were obtained from DepMap and are shown by dot-and-box plots. Negative scores indicate that deletion of FOXA2 suppressed cell proliferation.

(F) Scatter plots showing the correlation between the HNF4α dependency score (y-axis) and GREB1 expression (x-axis) were obtained from the DepMap portal (n = 22). The Pearson r- and P-values were calculated with GraphPad Prism.

(G) JHH7 cells stably expressing control or HNF4α shRNA were cultured in 2D plastic dishes for the indicated number of days. Relative cell numbers were quantified using the CyQUANT assay. The results are presented as the mean ± SD.

(H) JHH7 cells stably expressing control or FOXA2 shRNA were cultured in 2D plastic dishes for the indicated number of days. Relative cell numbers were quantified using the CyQUANT assay. The results are presented as the mean ± SD.



Supplemental Figure 8| Expression profile of TR95 genes is different from Wnt/β-catenin target gene in colon cancer

Heatmap was built for highly variable and/or cancer-interesting genes (3516 genes) across TCGA COAD samples, and TR95 signature score. Z-score was calculated for each row (gene) and k-mean clustering (k=4) was performed on genes.

Supplemental Table 1

Wnt target	Gene	Count
○	AXIN2	33
○	NKD1	20
○	ZNRF3	18
○	NOTUM	14
○	SP5	13
○	RNF43	13
○	APCDD1	13
○	BMP4	12
	ASPSCR1	10
	TSPOAP1-AS1	9
	ABHD12B	9
○	BAMBI	9
	FGF3	8
	HUNK	8
	LY6G6F	8
○	DKK4	8
○	TNFRSF19	8
○	MSX2	8
○	TCF7	8
	MYH7B	7

Supplemental Table 1: Candidates of general Wnt target genes.
The "count" column shows the number of tumors where the expression of indicated gene correlates significantly with *AXIN2* in 33 TCGA tumors. Genes were sorted in descending order by the count. The open circle in the "Wnt target" column shows that the indicated gene has been previously reported as a Wnt target gene.

Supplemental Table 2

Candidates of HCC-specific Wnt target genes

Rank	Genes	LIHC	COAD	difference
1	MAP3K8	0.8286	0	0.8286
2	RHBG	0.8255	0	0.8255
3	GLUL	0.8041	0	0.8041
4	TMEM245	0.7145	0	0.7145
5	TRIB2	0.6948	0	0.6948
6	PANX1	0.6825	0	0.6825
7	CPPED1	0.83	0.1534	0.6766
8	INSIG2	0.6755	0	0.6755
9	GREB1	0.6454	0	0.6454
10	FOXRED2	0.6397	0	0.6397
11	CDK6	0.6204	0	0.6204
12	GNAI1	0.612	0	0.612
13	FAM8A1	0.6007	0	0.6007
14	HLF	0.7961	0.1957	0.6004
15	GLT8D1	0.5959	0	0.5959
16	PRKAA2	0.5952	0	0.5952
17	MINPP1	0.5913	0	0.5913
18	ACSS3	0.5662	0	0.5662
19	ITGA6	0.6679	0.1556	0.5123
20	UGGT1	0.6279	0.1284	0.4995
21	GRK3	0.7159	0.2274	0.4885
22	STARD13	0.6869	0.1987	0.4882
23	TMCC1	0.6127	0.1367	0.476
24	ITPR2	0.5537	0.1187	0.435
25	FBXO31	0.6696	0.257	0.4126
26	LGR5	0.649	0.2365	0.4125
27	PARD3B	0.5321	0.1505	0.3816
28	PHYHIPL	0.6562	0.2766	0.3796
29	HABP4	0.5627	0.2004	0.3623
30	ROCK2	0.4717	0.1285	0.3432
31	SMYD2	0.5947	0.255	0.3397
32	NEK3	0.6275	0.2922	0.3353
33	CYB5B	0.5594	0.2263	0.3331
34	TBX3	0.4619	0.1564	0.3055
35	PREB	0.5984	0.2976	0.3008
36	AMACR	0.5974	0.3106	0.2868
37	WASHC3	0.6548	0.3804	0.2744
38	TMEM150C	0.6423	0.3754	0.2669
39	HSDL1	0.4161	0.1661	0.25
40	ZNF503	0.4547	0.2366	0.2181
41	TNFRSF19	0.4253	0.225	0.2003
42	SLC22A11	0.4668	0.2815	0.1853
43	ACSL5	0.465	0.2894	0.1756
44	TCF7	0.4896	0.384	0.1056
45	TSC22D1	0.4369	0.3314	0.1055
46	ASPSCR1	0.3805	0.3366	0.0439

Supplemental Table 2: Rank of candidates of HCC-specific Wnt signaling target gene.

Table shows 46 candidates of HCC-specific Wnt signaling target genes ranked by the difference of specificity score between HCC and colon cancer.

Supplemental Table 3

Rank	Term	*P*-value
1	HNF4A 19822575 ChIP-Seq HepG2 Human	4.99E-26
2	FOXA2 19822575 ChIP-Seq HepG2 Human	1.64E-16
3	LXR 22158963 ChIP-Seq LIVER Mouse	1.03E-06
4	CTNNB1 20460455 ChIP-Seq HCT116 Human	6.39E-06
5	PPARA 22158963 ChIP-Seq LIVER Mouse	1.52E-05
6	RXR 22158963 ChIP-Seq LIVER Mouse	1.52E-05
7	GATA4 25053715 ChIP-Seq YYC3 Human	1.52E-05
8	SMAD4 21799915 ChIP-Seq A2780 Human	1.80E-05
9	TCF7L2 21901280 ChIP-Seq H4IIE Rat	3.38E-05
10	FOXA1 21915096 ChIP-Seq LNCaP-1F5 Human	3.52E-05

Supplemental Table 3: Prediction of transcription factors regulating GREB1 expression in HCC by ChEA.

Using Ualcan (http://ualcan.path.uab.edu/cgi-bin/ualcan-res.pl), the top 200 genes positively correlated with GREB1 expression were obtained from the TCGA HCC dataset. Using that gene set, ChIP-Enrichment Analysis (ChEA) (https://maayanlab.cloud/Enrichr/) analysis was performed to predict upstream regulatory transcription factors. A list of the top 10 significantly enriched transcription factors was presented. Results based on the liver-related ChIP-seq dataset are marked in light blue.

Supplemental Table 4

GSEA analysis: enriched in Control (FDR<0.01)

Rank	Hallmark	NES	FDR q-val
1	HALLMARK_MYC_TARGETS_V1	2.20	0
2	HALLMARK_E2F_TARGETS	1.88	0.0025
3	HALLMARK_MYC_TARGETS_V2	1.86	0.0020
4	HALLMARK_UNFOLDED_PROTEIN_RESPONSE	1.76	0.0052
5	HALLMARK_G2M_CHECKPOINT	1.70	0.0070
6	HALLMARK_MTORC1_SIGNALING	1.67	0.0068

GSEA analysis: enriched in GREB1 ASO (FDR<0.01)

Rank	Hallmark	NES	FDR q-val
1	HALLMARK_INTERFERON_ALPHA_RESPONSE	-2.80	0
2	HALLMARK_INTERFERON_GAMMA_RESPONSE	-2.62	0
3	HALLMARK_INFLAMMATORY_RESPONSE	-2.08	0
4	HALLMARK_TNFA_SIGNALING_VIA_NFKB	-2.00	0
5	HALLMARK_ALLOGRAFT_REJECTION	-1.94	0.00017
6	HALLMARK_IL6_JAK_STAT3_SIGNALING	-1.69	0.0055
7	HALLMARK_MYOGENESIS	-1.69	0.0048
8	HALLMARK_APICAL_JUNCTION	-1.63	0.0086
9	HALLMARK_COMPLEMENT	-1.61	0.0098
10	HALLMARK_APICAL_SURFACE	-1.49	0.035
11	HALLMARK_CHOLESTEROL_HOMEOSTASIS	-1.41	0.059
12	HALLMARK_COAGULATION	-1.41	0.056
13	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	-1.39	0.060
14	HALLMARK_APOPTOSIS	-1.37	0.071

Supplemental Table 4: Rank of annotation terms that are significantly enriched as a result of GSEA of GREB1 knockdown RNA-seq data.

In JHH7 cells, GREB1 expression was suppressed by antisense oligonucleotides (ASO) to perform RNA-seq analysis and gene set enrichment analysis (GSEA) of its transcriptional profile was conducted. Using the Hallmark gene set, the top ranks of annotation terms with FDR<0.01 are shown in descending order based on absolute values of normalized enrichment score (NES). Fourteen annotation terms were significantly enriched in the control condition and six in the GREB1 knockdown condition.

Supplemental Table 5

Term	Overlap	*P*-value
HNF4A 19822575 ChIP-Seq HepG2 Human	447/6083	1.49E-52
FOXA2 19822575 ChIP-Seq HepG2 Human	248/2968	1.93E-32
CREM 20920259 ChIP-Seq GC1-SPG Mouse	367/5776	1.99E-25
KDM5B 21448134 ChIP-Seq MESCs Mouse	261/3724	2.78E-22
ZNF217 24962896 ChIP-Seq MCF-7 Human	137/1522	7.65E-20
MITF 21258399 ChIP-Seq MELANOMA Human	340/5578	1.07E-19
LXR 22158963 ChIP-Seq LIVER Mouse	163/2000	2.59E-19
NUCKS1 24931609 ChIP-Seq HEPATOCYTES Mouse	72/588	1.99E-17
MYC 19030024 ChIP-ChIP MESCs Mouse	243/3868	1.59E-14
EKLF 21900194 ChIP-Seq ERYTHROCYTE Mouse	103/1239	8.95E-13
YAP1 20516196 ChIP-Seq MESCs Mouse	161/2329	1.25E-12
WT1 25993318 ChIP-Seq PODOCYTE Human	216/3464	2.05E-12
MYCN 18555785 ChIP-Seq MESCs Mouse	156/2261	3.61E-12
TFAP2C 20629094 ChIP-Seq MCF-7 Human	99/1203	4.76E-12
RXR 22158963 ChIP-Seq LIVER Mouse	140/2000	2.20E-11
E2F1 18555785 ChIP-Seq MESCs Mouse	244/4172	3.73E-11
SMARCA4 23332759 ChIP-Seq OLIGODENDROCYTES Mouse	165/2522	5.38E-11
PRDM5 23873026 ChIP-Seq MEFs Mouse	86/1029	6.38E-11
RUNX1 21571218 ChIP-Seq MEGAKARYOCYTES Human	283/5071	8.44E-11
FLI1 21571218 ChIP-Seq MEGAKARYOCYTES Human	316/5834	1.09E-10

Supplemental Table 5: Prediction of regulatory transcription factors of GREB1-dependent 800 genes in HCC by ChEA.

A ChIP-Enrichment Analysis (ChEA) (https://maayanlab.cloud/Enrichr/) analysis was performed on a set of 800 genes bound and transcriptionally regulated by GREB1 on chromatin to predict regulatory transcription factors. A list of the top 20 significantly enriched transcription factors was presented. Results based on the liver-related ChIP-seq dataset are marked in light blue.

Supplemental Table 6

MSigDB Hallmark 2020

Term	Overlap	*P*-value
Myc Targets V1	44/200	1.06E-20
mTORC1 Signaling	40/200	2.21E-17
Unfolded Protein Response	22/113	6.20E-10
G2-M Checkpoint	29/200	1.90E-09
Hypoxia	27/200	3.22E-08
E2F Targets	27/200	3.22E-08
Mitotic Spindle	26/199	1.13E-07
Xenobiotic Metabolism	24/200	1.64E-06
Oxidative Phosphorylation	21/200	5.48E-05
Glycolysis	21/200	5.48E-05
Myc Targets V2	10/58	9.06E-05
IL-2/STAT5 Signaling	20/199	1.49E-04
Adipogenesis	20/200	1.60E-04
UV Response Dn	16/144	2.14E-04
Fatty Acid Metabolism	16/158	6.11E-04
Estrogen Response Early	18/200	0.001161
Cholesterol Homeostasis	9/74	0.002705
Epithelial Mesenchymal Transition	17/200	0.002878
PI3K/AKT/mTOR Signaling	11/105	0.003196
UV Response Up	14/158	0.004509

Supplemental Table 6: List of the top 20 annotated terms significantly enriched by pathway analysis of GREB1-dependent 800 genes.

Pathway analysis (https://maayanlab.cloud/Enrichr/) was performed on a set of 800 GREB1-dependent genes that are bound and transcriptionally regulated by GREB1 on chromatin. A list of the top 20 significantly enriched annotation terms from the analysis using the Hallmark gene set is shown.

Supplemental Table 7

Rank	Term	p-value
1	ZNF217 24962896 ChIP-Seq MCF7 Human	3.50E-11
2	SMAD4 21799915 ChIP-Seq A2780 Human	6.74E-09
3	WT1 25993318 ChIP-Seq PODOCYTE Human	4.00E-08
4	RUNX2 22187159 ChIP-Seq PCA Human	7.09E-07
5	STAT3 23295773 ChIP-Seq U87 Human	7.44E-07
6	FOXA2 19822575 ChIP-Seq HepG2 Human	1.07E-06
7	PPARD 21283829 ChIP-Seq MYOFIBROBLAST Human	3.42E-06
8	NR3C1 21868756 ChIP-Seq MCF10A Human	2.07E-05
9	HNF4A 19822575 ChIP-Seq HepG2 Human	2.40E-05
10	ESR2 21235772 ChIP-Seq MCF-7 Human	9.48E-05
11	NUCKS1 24931609 ChIP-Seq HEPATOCYTES Mouse	1.11E-04
12	EGR1 20690147 ChIP-Seq ERYTHROLEUKEMIA Human	1.16E-04
13	SOX2 21211035 ChIP-Seq LN229 Gbm	1.47E-04
14	AR 22383394 ChIP-Seq PROSTATE CANCER Human	1.61E-04
15	VDR 24787735 ChIP-Seq THP-1 Human	1.76E-04

Supplemental Table 7: Prediction of regulatory transcription factors of GREB1-dependent genes in Hep3B cells by ChEA.

To predict regulatory transcription factors, ChIP-Enrichment Analysis (ChEA) (https://maayanlab.cloud/Enrichr/) analysis was performed on a set of genes whose regulatory regions are occupied by GREB1 (identified by ChIP-seq) and whose expression are regulated by GREB1 (identified by RNA-seq). A list of the top 15 significantly enriched transcription factors was presented. Results based on the liver-related ChIP-seq dataset are marked in light blue.

Supplemental Table 8

Rank	Terms	Known motif	*P*-value
1	RAR:RXR(NR),DR0/ES-RAR-ChIP-Seq(GSE56893)	AGGTCAAGGTCA	1.00E-38
2	COUP-TFII(NR)/K562-NR2F1-ChIP-Seq(Encode)	GKBCARAGGTCA	1.00E-26
3	HNF4a(NR),DR1/HepG2-HNF4a-ChIP-Seq(GSE25021)	CARRGKBCAAAGTYCA	1.00E-26
4	RARa(NR)/K562-RARa-ChIP-Seq(Encode)	TTGAMCTTTG	1.00E-23
5	RARg(NR)/ES-RARg-ChIP-Seq(GSE30538)	AGGTCAAGGTCA	1.00E-22
6	EAR2(NR)/K562-NR2F6-ChIP-Seq(Encode)	NRBCARRGGTCA	1.00E-22
7	COUP-TFII(NR)/Artia-Nr2f2-ChIP-Seq(GSE46497)	AGRGGTCA	1.00E-22
8	Foxa3(Forkhead)/Liver-Foxa3-ChIP-Seq(GSE77670)	BSNTGTTTACWYWGN	1.00E-22
9	Foxa2(Forkhead)/Liver-Foxa2-ChIP-Seq(GSE25694)	CYTGTTTACWYW	1.00E-22
10	Elk4(ETS)/Hela-Elk4-ChIP-Seq(GSE31477)	NRYTTCCGGY	1.00E-20
11	CEBP:AP1(bZIP)/ThioMac-CEBPb-ChIP-Seq(GSE21512)	DRTGTTGCAA	1.00E-20
12	Fli1(ETS)/CD8-FLI-ChIP-Seq(GSE20898)	NRYTTCCGGH	1.00E-20
13	ERRg(NR)/Kidney-ESRRG-ChIP-Seq(GSE104905)	GTGACCTTGRVN	1.00E-19
14	Esrrb(NR)/mES-Esrrb-ChIP-Seq(GSE11431)	KTGACCTTGA	1.00E-19
15	RORg(NR)/Liver-Rorc-ChIP-Seq(GSE101115)	WAABTAGGTCAV	1.00E-18
16	ETV4(ETS)/HepG2-ETV4-ChIP-Seq(ENCODE)	ACCGGAAGTG	1.00E-18
17	Fox:Ebox(Forkhead,bHLH)/Panc1-Foxa2-ChIP-Seq(GSE47459)	NNNVCTGWGYAAACASN	1.00E-17
18	Elk1(ETS)/Hela-Elk1-ChIP-Seq(GSE31477)	HACTTCCGGY	1.00E-17
19	FOXA1(Forkhead)/MCF7-FOXA1-ChIP-Seq(GSE26831)	WAAGTAAACA	1.00E-17
20	Erra(NR)/HepG2-Erra-ChIP-Seq(GSE31477)	CAAAGGTCAG	1E-17

Supplemental Table 8: List of known motif analysis results for GREB1 binding regions identified by ChIP-seq analysis.

HOMER known motif analysis was performed on the 8637 GREB1 binding regions identified. A list of the top 20 significantly enriched binding motifs is shown. Results based on the liver-related ChIP-seq dataset are marked in light blue.

Supplemental Table 9

GO Biological Process_2021

Term	*P*-value
positive regulation of cell migration (GO:0030335)	1.61E-06
positive regulation of cellular process (GO:0048522)	1.68E-06
negative regulation of cell differentiation (GO:0045596)	4.03E-06
regulation of cell migration (GO:0030334)	4.72E-06
regulation of Wnt signaling pathway (GO:0030111)	4.91E-06
positive regulation of cell population proliferation (GO:0008284)	5.12E-06
heart development (GO:0007507)	7.44E-06
cellular response to glucocorticoid stimulus (GO:0071385)	8.81E-06
cellular protein modification process (GO:0006464)	1.38E-05
positive regulation of intracellular signal transduction (GO:1902533)	1.6E-05
organonitrogen compound biosynthetic process (GO:1901566)	1.84E-05
regulation of apoptotic process (GO:0042981)	2.1E-05
Fc-epsilon receptor signaling pathway (GO:0038095)	2.39E-05
Fc receptor signaling pathway (GO:0038093)	2.64E-05
negative regulation of cellular macromolecule biosynthetic process (GO:2000113)	3.93E-05
cellular response to cytokine stimulus (GO:0071345)	4.55E-05
negative regulation of signal transduction (GO:0009968)	4.75E-05
negative regulation of nucleic acid-templated transcription (GO:1903507)	5.47E-05
mitochondrial respiratory chain complex assembly (GO:0033108)	6.77E-05

Supplemental Table 9: List of top 20 annotated terms significantly enriched by GO analysis of 590 GREB1-dependent HNF4α target genes.

Gene Ontology (GO) enrichment analysis was performed on the 590 gene set assigned to 575 HNF4α binding peaks that were significantly attenuated by GREB1 knockdown (https://maayanlab.cloud/Enrichr/). A list of the top 20 annotated terms that were significantly enriched in the analysis using the GO Biological Process_2021 gene set is shown.

Supplemental Table 10

No.	Gene symbol	Dependency Score
1	GINS1	-2.35
2	SF3B6	-1.66
3	SEC13	-1.40
4	TXN	-1.37
5	PSMG4	-1.16
6	RBMX	-1.11
7	HK2	-1.04
8	HNRNPU	-1.03
9	FARS2	-0.97
10	MRPS23	-0.89
11	CHCHD3	-0.70
12	SLC25A3	-0.68
13	NUP35	-0.66
14	BUD13	-0.63
15	NDUFA10	-0.54
16	AK2	-0.54
17	TBCA	-0.51
18	IGF2	-0.49
19	SCD	-0.49
20	CWF19L2	-0.43
21	MAP4K4	-0.28
22	LIN28B	-0.28
23	SDC1	-0.24
24	ASS1	-0.21
25	RAPGEF1	-0.20
26	DAB1	-0.20
27	DIAPH3	-0.16
28	PUM2	-0.15
29	PRDX6	-0.13
30	MSRB3	-0.13
31	DPYD	-0.11
32	RRAS2	-0.11
33	SNTB1	-0.10
34	FAH	-0.10
35	ACSL4	-0.08
36	CDK14	-0.08
37	PAH	-0.08
38	ANKRD50	-0.07
39	MLLT10	-0.07
40	ARID1B	-0.06
41	KLF15	-0.06
42	GPAM	-0.06
43	GUCY1A2	-0.06
44	PREP	-0.05
45	LPP	-0.04
46	PTDSS1	-0.04
47	MPP6	-0.04
48	CDKAL1	-0.03
49	TBC1D22A	-0.03
50	CDC42EP1	-0.03
51	NFIA	-0.03
52	ID2	-0.02
53	LMAN1	-0.02
54	MAOB	-0.02
55	ARRDC3	-0.01
56	PLD5	-0.01
57	SDC2	-0.01
58	EXOC2	-0.01
59	IVD	0.00
60	STT3B	0.01
61	MGLL	0.01
62	ACOX2	0.01
63	PID1	0.02
64	MAGI1	0.02
65	SLC22A23	0.02
66	CYP24A1	0.03
67	HACD2	0.03
68	ERI3	0.04
69	YWHAH	0.05
70	PARD3	0.05
71	DUSP9	0.05
72	FRAS1	0.06
73	CACUL1	0.06
74	PRLR	0.06
75	RAPH1	0.06
76	PDXP	0.08
77	GALNT7	0.08
78	BRD9	0.08
79	WWOX	0.09
80	MAN1A1	0.09
81	HEG1	0.10
82	GPC3	0.11
83	DDAH1	0.11
84	GMDS	0.12
85	TGFBR2	0.13
86	TMEM43	0.13
87	APOB	0.14
88	CWC27	0.15
89	LRIG1	0.15
90	MACF1	0.15
91	GALNT2	0.20
92	RBM26	0.21
93	ERRFI1	0.27
94	AKR1C1	N.A
95	SIRPA	N.A

Supplemental Table 10: List of 95 GREB1-dependent HNF4α target genes (TR95) and the dependency scores

A list of the dependency scores for the 95 target genes of HNF4α (TR95) is shown. Data were obtained from DepMap portal (https://depmap.org/portal/).