iScience, Volume *27*

Supplemental information

ALK inhibitors suppress HCC and synergize with anti-PD-1 therapy and ABT-263 in preclinical models

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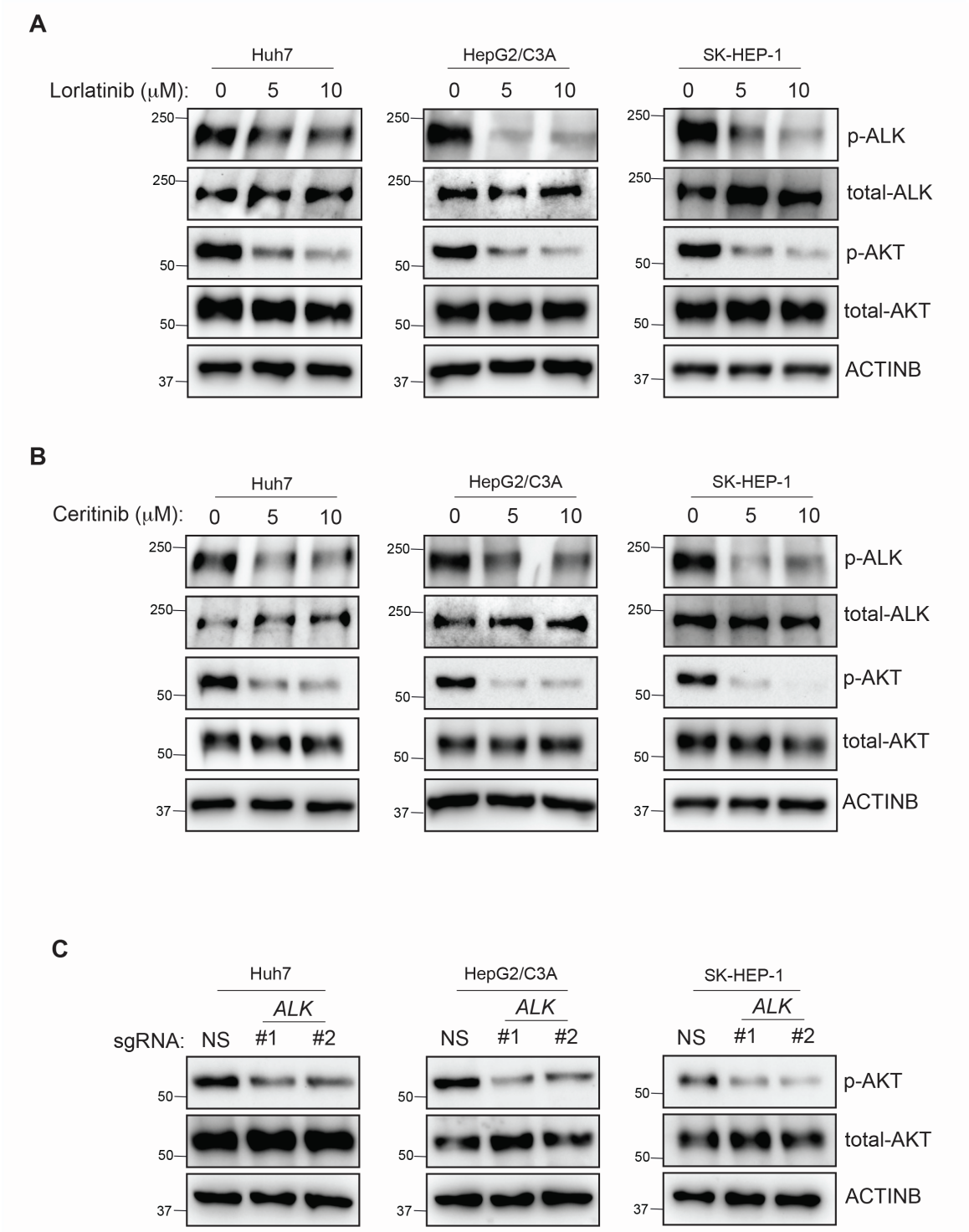

Figure S1. Pharmacological and genetic inhibition of ALK blocks the ALK signaling pathway. Related to Figure 1. A. Indicated HCC cell lines were treated with DMSO or indicated concentrations of lorlatinib for 3 h and analyzed for the indicated proteins using immunoblotting. ACTINB was used as a loading control. **B.** Indicated HCC cell lines were treated with DMSO or indicated concentrations of ceritinib for 3 h and analyzed for the indicated proteins using immunoblotting. ACTINB was used as a loading control. **C.** Indicated HCC cell lines expressing either non-specific sgRNA or *ALK*-specific

sgRNAs were analyzed for the indicated proteins using immunoblotting. ACTINB was used as a loading control.

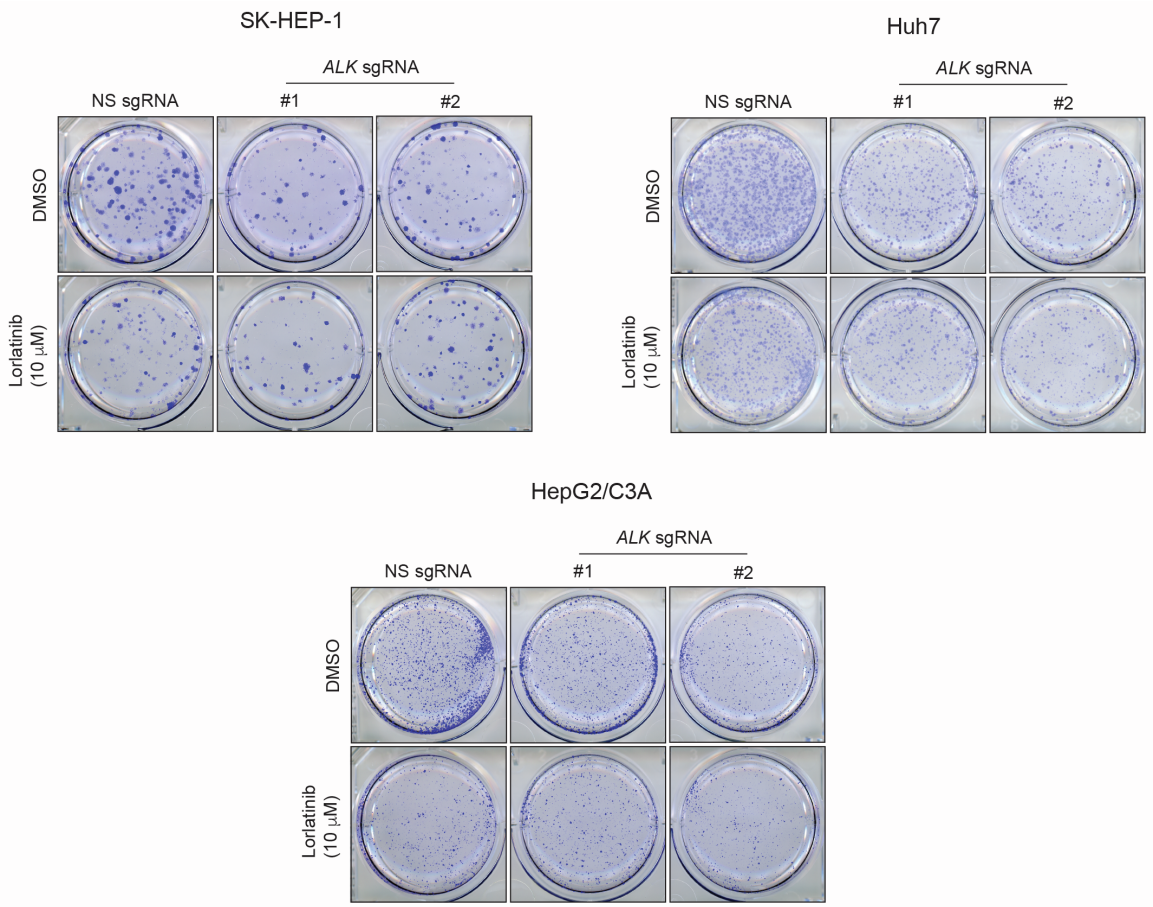

Figure S2. HCC suppressive effect of the ALK inhibitor lorlatinib is dependent upon ALK expression. Related to Figure 1. Indicated HCC cell lines expressing either non-specific sgRNA or *ALK*-specific sgRNAs were treated with DMSO or lorlatinib (10 μM) and analyzed by clonogenic assay. Representative wells for the indicated HCC cell lines under the indicated treatment conditions are shown.

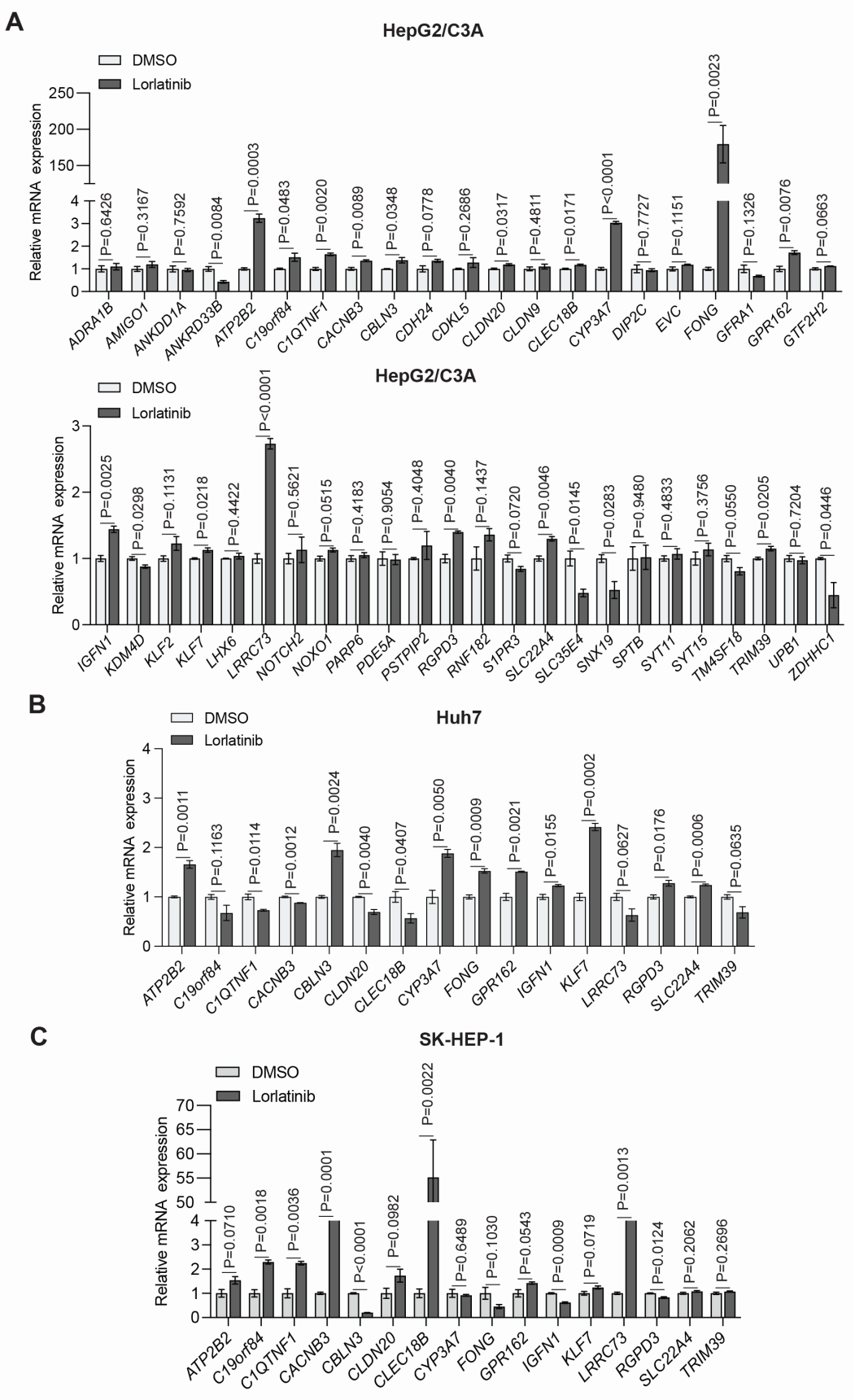
A
HepG2/C3A
DMSO
Lorlatinib
Relative mRNA expression
ADRA1B P=0.6426
AMIGO1 P=0.3167
ANKDD1A P=0.7592
ANKRD33B P=0.0084
ATP2B2 P=0.0003
C19orf84 P=0.0483
C1QTNF1 P=0.0020
CACNB3 P=0.0089
CBLN3 P=0.0348
CDH24 P=0.0778
CDKL5 P=0.2686
CLDN20 P=0.0317
CLDN9 P=0.4811
CLEC18B P=0.0171
CYP3A7 P<0.0001
DIP2C P=0.7727
EVC P=0.1151
FONG P=0.0023
GFRA1 P=0.1326
GPR162 P=0.0076
GTF2H2 P=0.0663
HepG2/C3A
DMSO
Lorlatinib
Relative mRNA expression
IGFN1 P=0.0025
KDM4D P=0.0298
KLF2 P=0.1131
KLF7 P=0.0218
LHX6 P=0.4422
LRRC73 P<0.0001
NOTCH2 P=0.5621
NOXO1 P=0.0515
PARP6 P=0.4183
PDE5A P=0.9054
PSTPIP2 P=0.4048
RGPD3 P=0.0040
RNF182 P=0.1437
S1PR3 P=0.0720
SLC22A4 P=0.0046
SLC35E4 P=0.0145
SNX19 P=0.0283
SPTB P=0.9480
SYT11 P=0.4833
SYT15 P=0.3756
TM4SF18 P=0.0550
TRIM39 P=0.0205
UPB1 P=0.7204
ZDHHC1 P=0.0446
B
Huh7
DMSO
Lorlatinib
Relative mRNA expression
ATP2B2 P=0.0011
C19orf84 P=0.1163
C1QTNF1 P=0.0114
CACNB3 P=0.0012
CBLN3 P=0.0024
CLDN20 P=0.0040
CLEC18B P=0.0407
CYP3A7 P=0.0050
FONG P=0.0009
GPR162 P=0.0021
IGFN1 P=0.0155
KLF7 P=0.0002
LRRC73 P=0.0627
RGPD3 P=0.0176
SLC22A4 P=0.0006
TRIM39 P=0.0635
C
SK-HEP-1
DMSO
Lorlatinib
Relative mRNA expression
ATP2B2 P=0.0710
C19orf84 P=0.0018
C1QTNF1 P=0.0036
CACNB3 P=0.0001
CBLN3 P<0.0001
CLDN20 P=0.0982
CLEC18B P=0.0022
CYP3A7 P=0.6489
FONG P=0.1030
GPR162 P=0.0543
IGFN1 P=0.0009
KLF7 P=0.0719
LRRC73 P=0.0013
RGPD3 P=0.0124
SLC22A4 P=0.2062
TRIM39 P=0.2696

Figure S3. RT-qPCR validation of mRNAs upregulated following lorlatinib treatment in HCC cells as identified by the RNA-seq analysis. Related to Figure 6. A. HepG2/C3A cells were treated with either DMSO, or Lorlatinib (1 μM) for 24 h. mRNA expression for the indicated genes was performed using RT-qPCR analysis. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n=3 each) for the indicated genes relative to DMSO is plotted. For HepG2/C3A for *ADRA1B* gene P=0.6426, t=0.5011, df=4, *AMIGO1* gene P=0.3167, t=1.143, df=4, *ANKDD1A* gene P=0.7592, t=0.3283, df=4, *ANKRD33B* gene P=0.0084, t=4.834, df=4, *ATP2B2* gene P=0.0003, t=11.89, df=4, *C19ORF84* gene P=0.0483, t=2.811, df=4, *C1QTNF1* gene P=0.0020, t=7.209, df=4, *CACNB3* gene P=0.0089, t=4.757, df=4, *CBLN3* gene P=0.0348, t=3.142, df=4, *CDH24* gene P=0.0778, t=2.359, df=4, *CDKL5* gene P=0.2686, t=1.284, df=4, *CLDN20* gene P=0.0317, t=3.240, df=4, *CLDN9* gene P=0.4811, t=0.7759, df=4, *CLEC18B* gene P=0.0171, t=3.928, df=4, *CYP3A7* gene P<0.0001, t=23.59, df=4, *DIP2C* gene P=0.7727, t=0.3091, df=4, *EVC* gene P=0.1151, t=2.008, df=4, *FONG* gene P=0.0023, t=6.923, df=4, *GFRA1* gene P=0.1326, t=1.885, df=4, *GPR162* gene P=0.0076, t=4.975, df=4, *GTF2H2* gene P=0.0663, t=2.506, df=4, *IGFN1* gene P=0.0025, t=6.756, df=4, *KDM4D* gene P=0.0298, t=3.305, df=4, *KLF2* gene P=0.1131, t=2.023, df=4, *KLF7* gene P=0.0218, t=3.647, df=4, *LHX6* gene P=0.4422, t=0.8521, df=4, *LRRC73* gene P<0.0001, t=16.29, df=4, *NOTCH2* gene P=0.5621, t=0.6314, df=4, *NOXO1* gene P=0.0515, t=2.748, df=4, *PARP6* gene P=0.4183, t=0.9016, df=4, *PDE5A* gene P=0.9054, t=0.1266, df=4, *PSTPIP2* gene P=0.4084, t=0.9226, df=4, *RGPD3* gene P=0.0040, t=5.968, df=4, *RNF182* gene P=0.1437, t=1.815, df=4, *S1PR3* gene P=0.0720, t=2.429, df=4, *SLC22A4* gene P=0.0046, t=5.720, df=4, *SLC35E4* gene P=0.0145, t=4.129, df=4, *SNX19* gene P=0.0283, t=3.362, df=4, *SPTB* gene P=0.9480, t=0.06940, df=4, *SYT11* gene P=0.4833, t=0.7718, df=4, *SYT15* gene P=0.3756, t=0.9960, df=4, *TM4SF18* gene P=0.0550, t=2.685, df=4, *TRIM39* gene P=0.0205, t=3.721, df=4, *UPB1* gene P=0.7204, t=0.3842, df=4, *ZDHHC1* gene P=0.0446, t=2.889, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. **B**. Huh7 cells were treated with either DMSO, or Lorlatinib (1 μM) for 24 h. mRNA expression for the indicated genes was performed using RT-qPCR analysis. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n=3 each) for indicated genes relative to DMSO is plotted. For Huh7 for *ATP2B2* gene P=0.0011, t=8.468, df=4, *C19ORF84* gene P=0.1163, t=1.998, df=4, *C1QTNF1* gene P=0.0114, t=4.433, df=4, *CACNB3* gene P=0.0012, t=8.218, df=4, *CBLN3* gene P=0.0024, t=6.862, df=4, *CLDN20* gene P=0.0040, t=5.941, df=4, *CLEC18B* gene P=0.0407, t=2.981, df=4, *CYP3A7* gene P=0.0050, t=5.594, df=4, *FONG* gene P=0.0009, t=8.786, df=4, *GPR162* gene P=0.0021, t=7.040, df=4, *IGFN1* gene P=0.0155, t=4.046, df=4, *KLF7* gene P=0.0002, t=13.44, df=4, *LRRC73* gene P=0.0627, t=2.559, df=4, *RGPD3* gene P=0.0176, t=3.898, df=4, *SLC22A4* gene P=0.0006, t=9.868, df=4, *TRIM39* gene P=0.0635, t=2.547, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. **C**. SK-HEP-1 cells were treated with either DMSO, or Lorlatinib (1 μM) for 24 h. mRNA expression for the indicated genes was performed using RT-qPCR analysis. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n=3 each) for the indicated genes relative to DMSO is plotted. For SK-HEP-1 for *ATP2B2* gene P=0.0710, t=2.443, df=4, *C19ORF84* gene P=0.0018, t=7.422, df=4, *C1QTNF1* gene P=0.0036, t=6.102, df=4, *CACNB3* gene P=0.0001, t=14.25, df=4, *CBLN3* gene P<0.0001, t=28.28, df=4, *CLDN20* gene P=0.0982, t=2.148, df=4, *CLEC18B* gene P=0.0022, t=6.999, df=4, *CYP3A7* gene P=0.6489, t=0.4913, df=4, *FONG* gene P=0.1030, t=2.106, df=4, *GPR162* gene P=0.0543, t=2.696, df=4, *IGFN1* gene P=0.0009, t=8.832, df=4, *KLF7* gene P=0.0719, t=2.431, df=4, *LRRC73* gene P=0.0013, t=8.029, df=4, *RGPD3* gene P=0.0124, t=4.329, df=4, *SLC22A4* gene P=0.2062, t=1.507, df=4, *TRIM39* gene P=0.2696, t=1.280, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. Data is presented as the mean ± SEM.

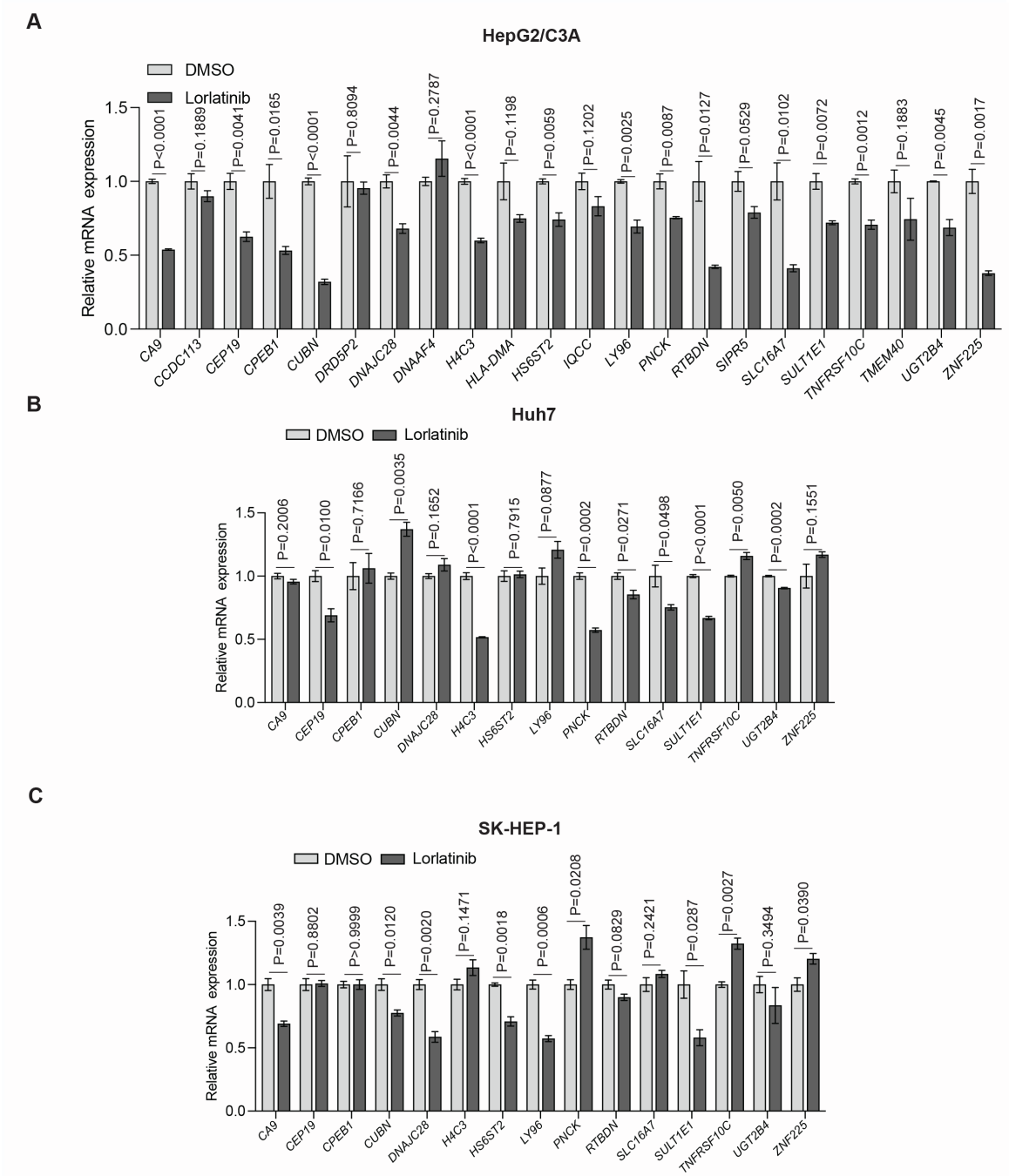

Figure S4. RT-qPCR validation of mRNAs downregulated following lorlatinib treatment in HCC cells as identified by the RNA-seq analysis. Related to Figure 6. A. HepG2/C3A cells were treated with either DMSO, or Lorlatinib (1 μM) for 24 h. mRNA expression for the indicated genes was performed using RT-qPCR analysis. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n =3 each) for the indicated genes relative to DMSO is plotted. For HepG2/C3A for *CA9* gene P<0.0001, t=29.38, df=4, *CCDC113* gene P=0.1889, t=1.582, df=4, *CEP19* gene P=0.0041, t=5.906, df=4, *CPEB1* gene P=0.0165, t=3.973, df=4, *CUBN* gene P<0.0001, t=23.27, df=4, *DRD5P2* gene P=0.8094, t=0.2576, df=4, *DNAJC28* gene P=0.0044, t=5.800, df=4, *DNAAF4* gene P=0.2787, t=1.252, df=4, *H4C3* gene P<0.0001, t=16.32, df=4, *HLA-DMA* gene P=0.1198, t=1.973, df=4, *HS6ST2* gene P=0.0059, t=5.356, df=4, *IQCC* gene P=0.1202, t=1.970, df=4, *LY96* gene P=0.0025,

t=6.778, df=4, *PNCK* gene P=0.0087, t=4.789, df=4, *RTBDN* gene P=0.0127, t=4.295, df=4, *SIPR5* gene P=0.0529, t=2.721, df=4. *SLC16A7* gene P=0.0102, t=4.580, df=4, *SULT1E1* gene P=0.0072, t=5.053, df=4, *TNFRSF10C* gene P=0.0012, t=8.279, df=4, *TMEM40* gene P=0.1883, t=1.584, df=4, *UGT2B4* gene P=0.0045, t=5.753, df=4, *ZNF225* gene P=0.0017, t=7.507, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. **B**. Huh7 cells were treated with either DMSO, or Lorlatinib (1 μM) for 24 h. mRNA expression for the indicated genes was performed using RT-qPCR analysis. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n=3 each) for the indicated genes relative to DMSO is plotted. For Huh7 for *CA9* gene P=0.2006, t=1.530, df=4, *CEP19* gene P=0.0100, t=4.602, df=4, *CPEB1* gene P=0.7166, t=0.3897, df=4, *CUBN* gene P=0.0035, t=6.192, df=4, *DNAJC28* gene P=0.1652, t=1.696, df=4, *H4C3* gene P<0.0001, t=17.45, df=4, *HS6ST2* gene P=0.7915, t=0.2826, df=4, *LY96* gene P=0.0877, t=2.249, df=4, *PNCK* gene P=0.0002, t=13.84, df=4, *RTBDN* gene P=0.0271, t=3.406, df=4, *SLC16A7* gene P=0.0498, t=2.780, df=4, *SULT1E1* gene P<0.0001, t=18.12, df=4, *TNFRSF10C* gene P=0.0050, t=5.587, df=4, *UGT2B4* gene P=0.0002, t=12.57, df=4, *ZNF225* gene P=0.1551, t=1.749, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. **C**. SK-HEP-1 cells were treated with either DMSO, or Lorlatinib (1 μM) for 24 h. mRNA expression for the indicated genes was performed using RT-qPCR analysis. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n= 3 each) for the indicated genes relative to DMSO is plotted. For SK-HEP-1 for *CA9* gene P=0.0039, t=5.993, df=4, *CEP19* gene P=0.8802, t=0.1606, df=4, *CPEB1* gene P>0.9999, t=0.000, df=4, *CUBN* gene P=0.0120, t=4.370, df=4, *DNAJC28* gene P=0.0020, t=7.156, df=4, *H4C3* gene P=0.1471, t=1.795, df=4, *HS6ST2* gene P=0.0018, t=7.403, df=4, *LY96* gene P=0.0006, t=9.848, df=4, *PNCK* gene P=0.0208, t=3.703, df=4, *RTBDN* gene P=0.0829, t=2.300, df=4, *SLC16A7* gene P=0.2421, t=1.372, df=4, *SULT1E1* gene P=0.0287, t=3.343, df=4, *TNFRSF10C* gene P=0.0027, t=6.620, df=4, *UGT2B4* gene P=0.3494, t=1.059, df=4, *ZNF225* gene P=0.0390, t=3.025, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. All quantitative data represent the mean ± SEM.

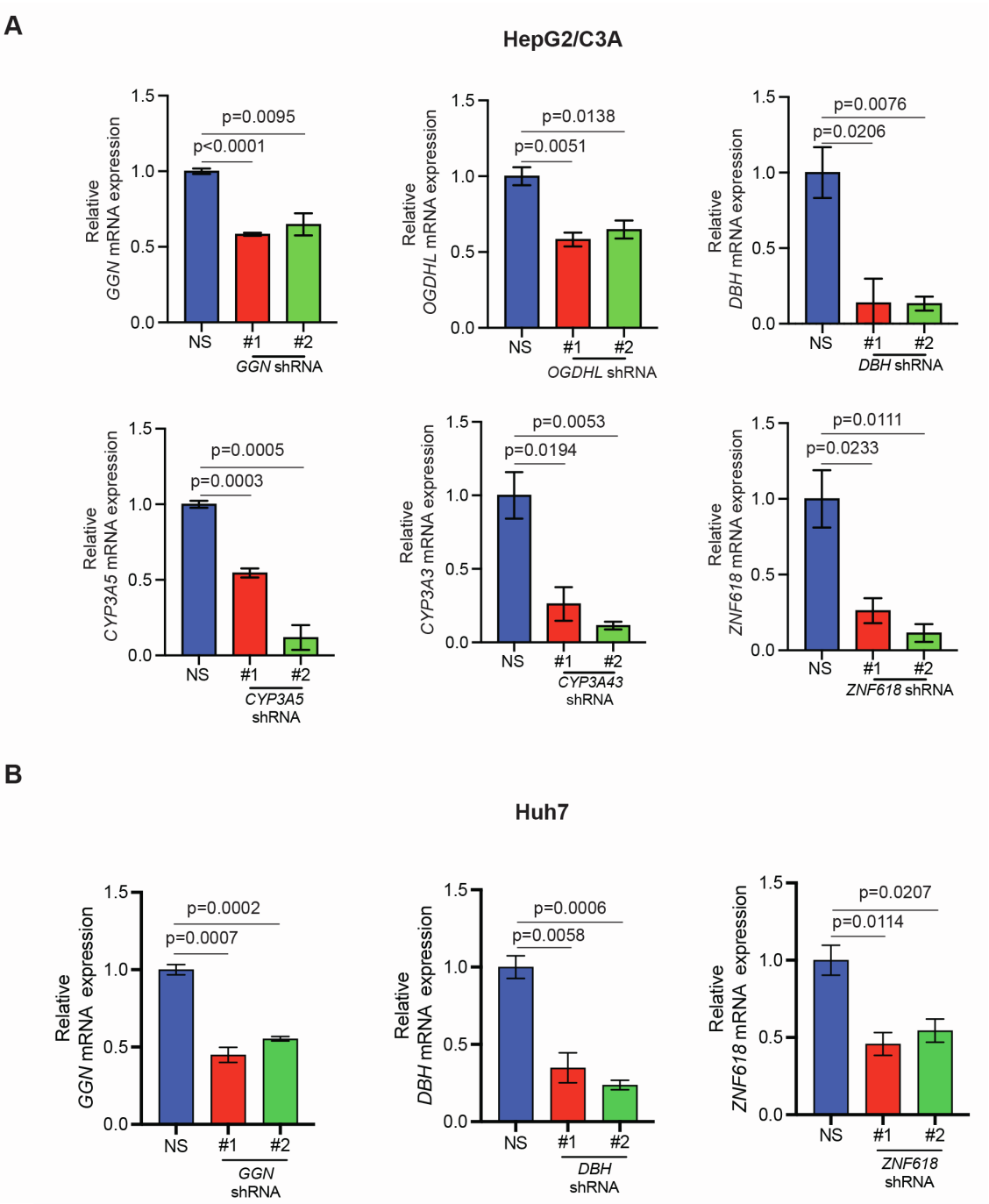

Figure S5. Knockdown validation in HCC cells. Related to Figure 7. A. HepG2C3/A cells expressing indicated shRNAs were analyzed for the expression of indicated genes by RT-qPCR. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n=3 each) for indicated genes in comparison to non-specific (NS) shRNA-expressing cells is shown. For HepG2/C3A cells expressing NS shRNA versus *GGN* shRNA#1 P<0.0001, t=19.70, df=4, and NS shRNA versus *GGN* shRNA#2 P=0.0095, t=4.667, df=4. For HepG2/C3A cells expressing NS shRNA versus *OGDHL* shRNA#1 P=0.0051, t=5.553, df=4, and NS shRNA versus *OGDHL* shRNA#2 P=0.0138, t=4.191, df=4. For HepG2/C3A cells expressing NS shRNA versus *DBH* shRNA#1 P=0.0206, t=3.712, df=4, and NS shRNA versus *DBH* shRNA#2 P=0.0076, t=4.980, df=4. For HepG2/C3A cells expressing NS shRNA

versus *CYP3A5* shRNA#1 P=0.0003, t=11.0, df=4, and NS shRNA versus *CYP3A5* shRNA#2 P=0.0005, t=10.36, df=4. For HepG2/C3A cells expressing NS shRNA versus *CYP3A43* shRNA#1 P=0.0194, t=3.781, df=4, and NS shRNA versus *CYP3A43* shRNA#2 P=0.0053, t=5.515, df=4. For HepG2/C3A cells expressing NS shRNA versus *ZNF618* shRNA#1 P=0.0233, t=3.572, df=4, and NS shRNA versus *ZNF618* shRNA#2 P=0.0111, t=4.466, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. **B**. Huh7 cells expressing indicated shRNAs were analyzed for the expression of indicated genes by RT-qPCR. ACTINB mRNA expression was used as an internal normalization control. Relative mRNA expression (n=3 each) for indicated genes in comparison to non-specific (NS) shRNA-expressing cells is shown. For Huh7 cells expressing NS shRNA versus *GGN* shRNA#1 P=0.0007, t=9.335, df=4, and NS shRNA versus *GGN* shRNA#2 P=0.0002, t=12.42, df=4. For Huh7 cells expressing NS shRNA versus *DBH* shRNA#1 P=0.0058, t=5.383, df=4, and NS shRNA versus *DBH* shRNA#2 P=0.0006, t=9.672, df=4. For Huh7 cells expressing NS shRNA versus *ZNF618* shRNA#1 P=0.0114, t=4.430, df=4, and NS shRNA versus *ZNF618* shRNA#2 P=0.0207, t=3.710, df=4. P-values were calculated using two-tailed, unpaired Student's t-test. Data is presented as mean ± SEM.

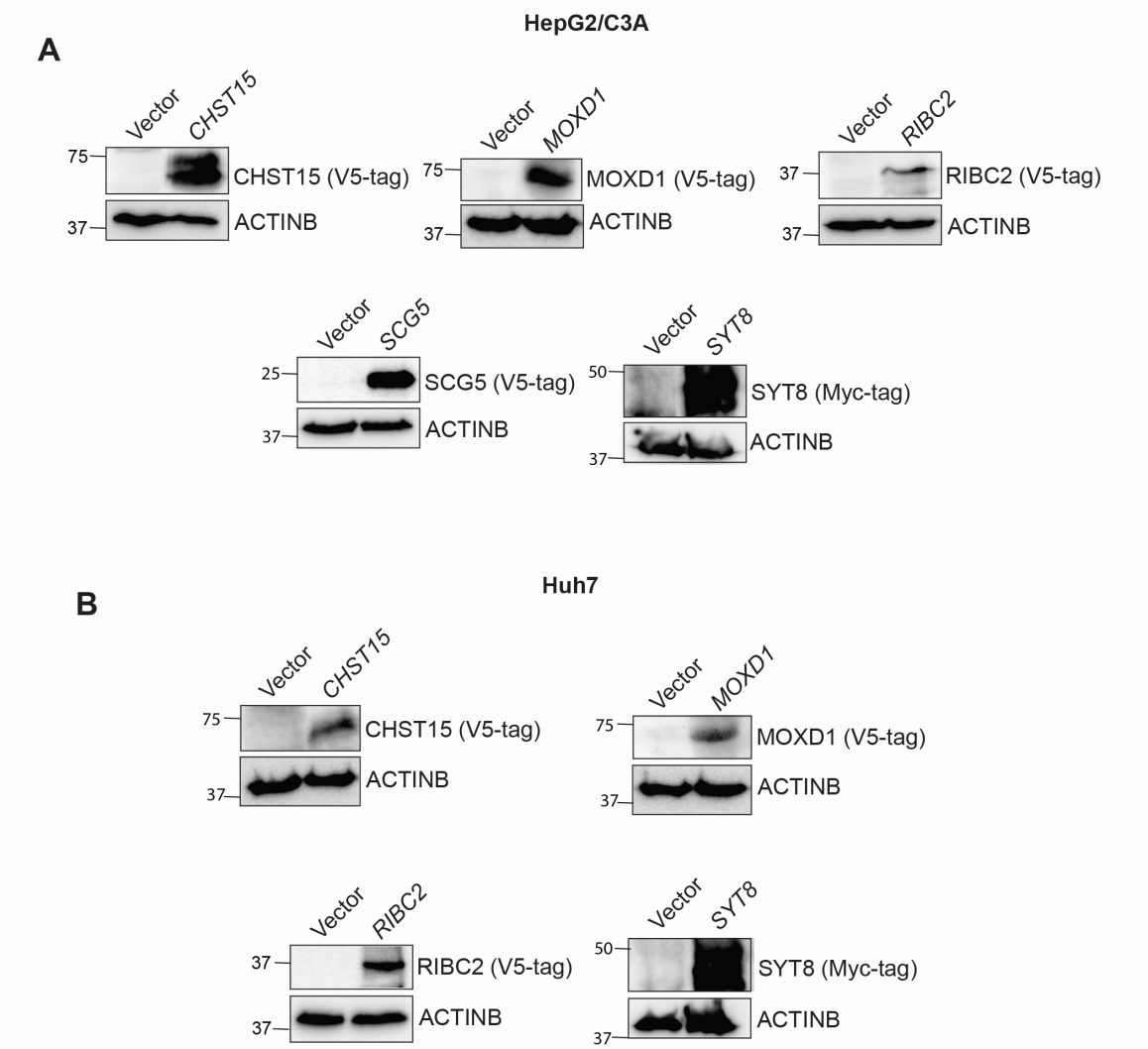

Figure S6. Ectopic expression validation in HCC cells. Related to Figure 7. A. HepG2/C3A cells expressing indicated ORFs were analyzed for the expression of indicated proteins by immunoblotting. Protein expression for indicated proteins in comparison to vector expressing cells are shown. ACTINB was used as a loading control. **B.** Huh7 cells expressing indicated ORFs were analyzed for the expression of indicated proteins by immunoblotting. Protein expression for the indicated proteins in comparison to vector-expressing cells is shown. ACTINB was used as a loading control.

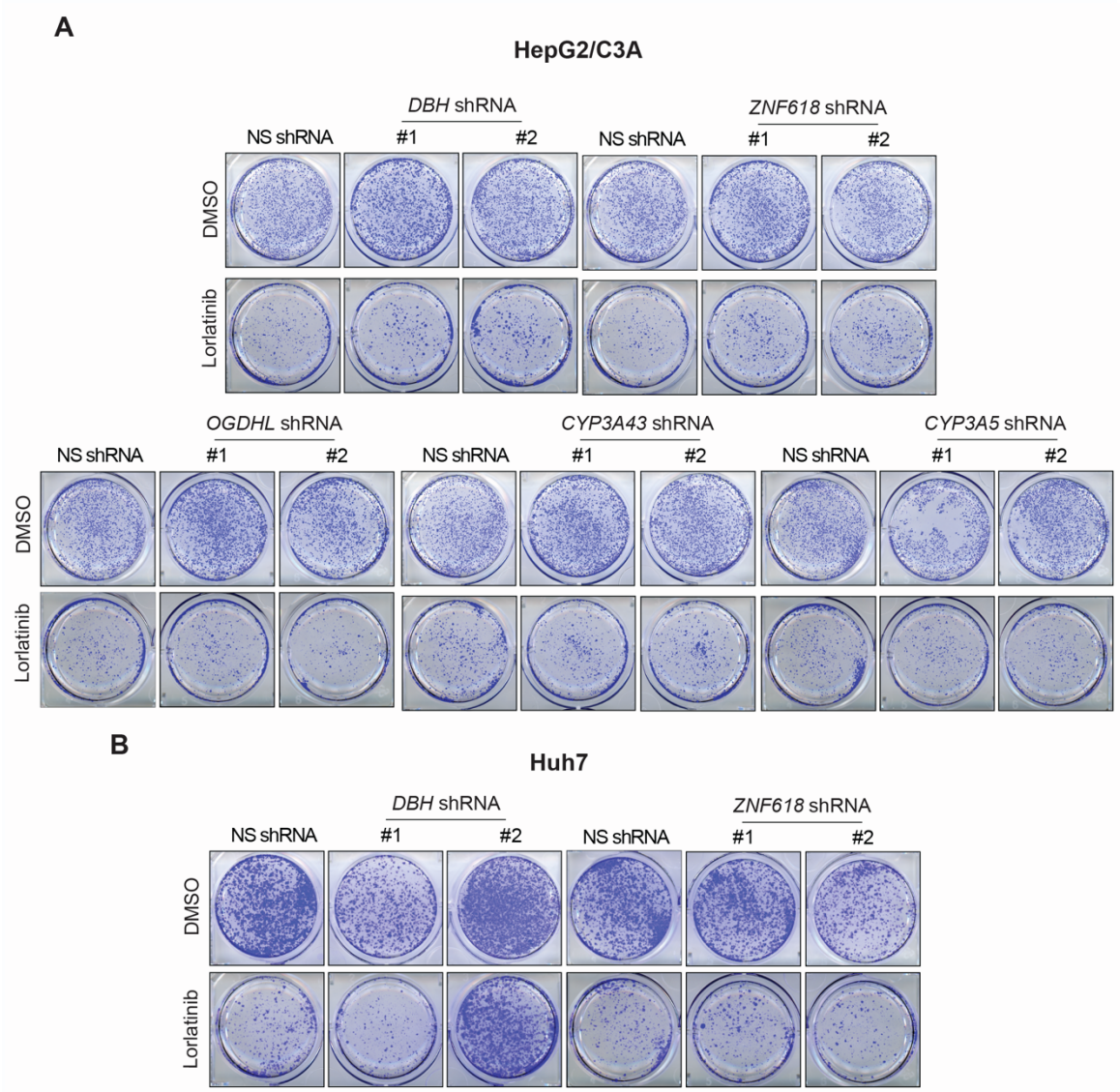

Figure S7. Clonogenic assays to identify potential mediators of ALK response in HCC. Related to Figure 7. A. HepG2/C3A cells expressing either NS shRNA or indicated gene-specific shRNAs were treated with lorlatinib (10 μM) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown. **B.** Huh7 cells expressing either NS shRNA or indicated gene-specific shRNAs were treated with lorlatinib (10 μM) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown.

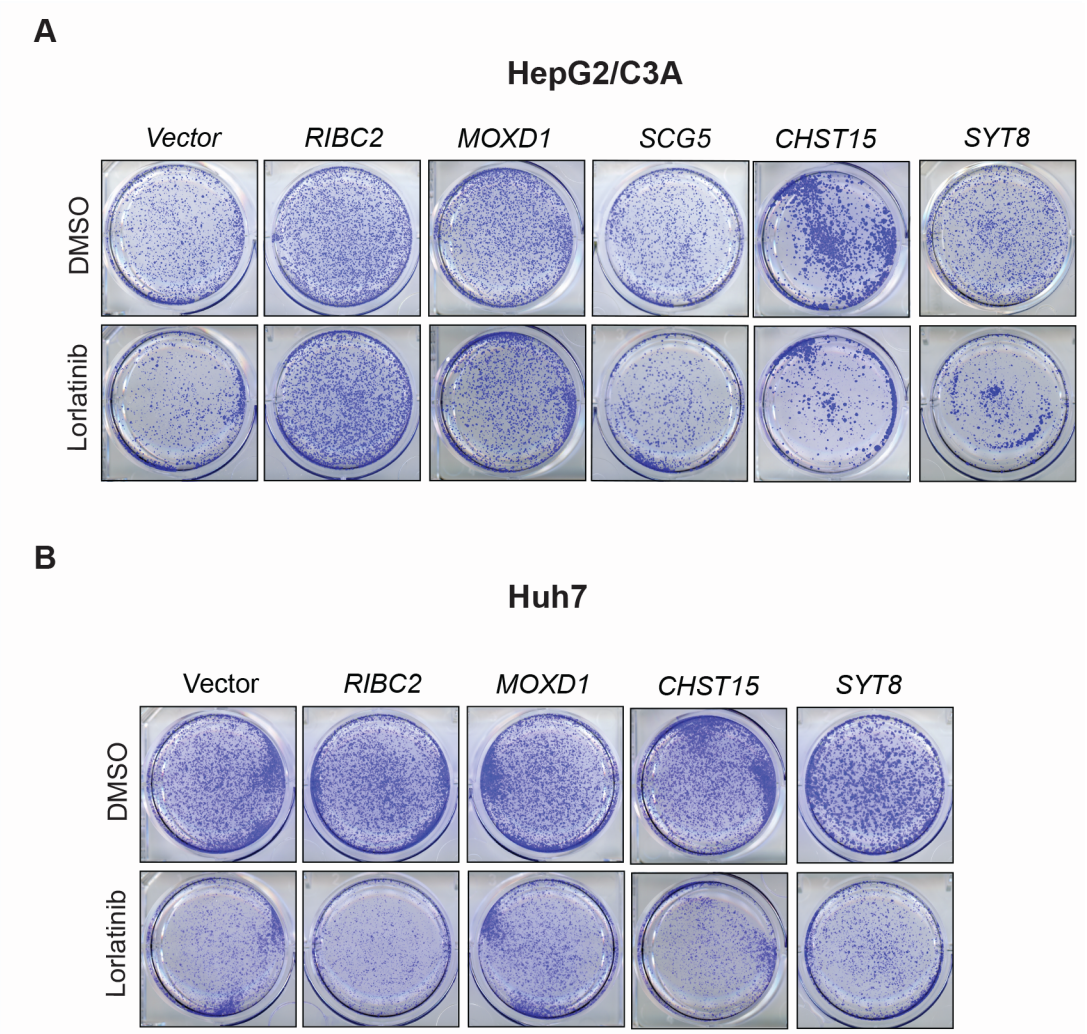

Figure S8. Clonogenic assays to identify potential mediators of ALK response in HCC. Related to Figure 7. A. HepG2/C3A cells expressing empty vector or indicated ORFs were treated with lorlatinib (10 μM) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown. **B.** Huh7 cells expressing empty vector or indicated ORFs were treated with lorlatinib (10 μM) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown.

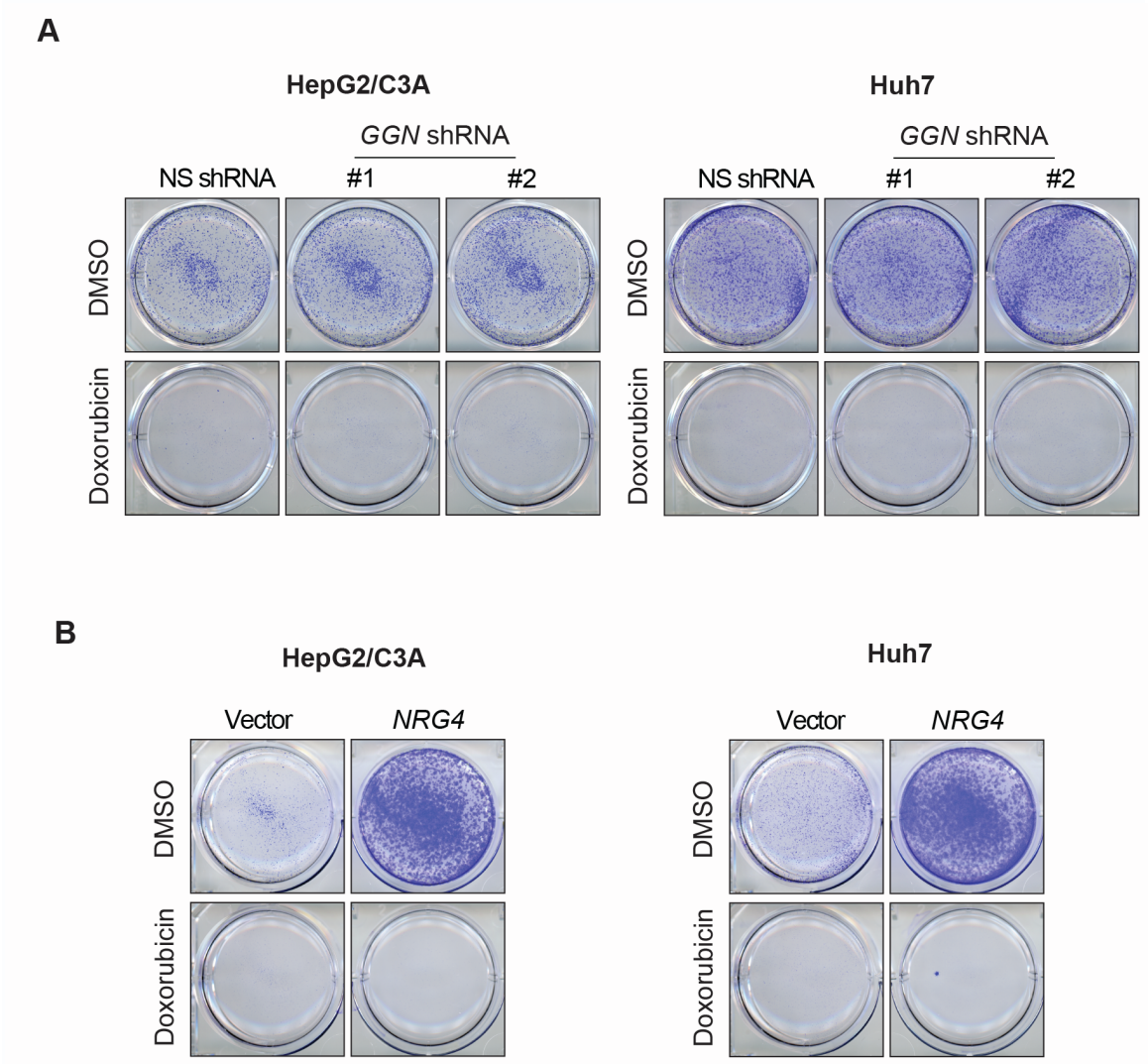

Figure S9. *GGN* knockdown or *NRG4* overexpression does not cause resistance to doxorubicin. Related to Figure 7. A. HepG2/C3A and Huh7 cells expressing either NS shRNA or indicated *GGN*-specific shRNAs were treated with doxorubicin (1 μM) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown. **B.** HepG2/C3A and Huh7 cells expressing either empty vector or *NRG4* ORF were treated with doxorubicin (1 μM) or DMSO, and survival was measured in clonogenic assays. Representative wells for cells grown under the indicated conditions are shown.

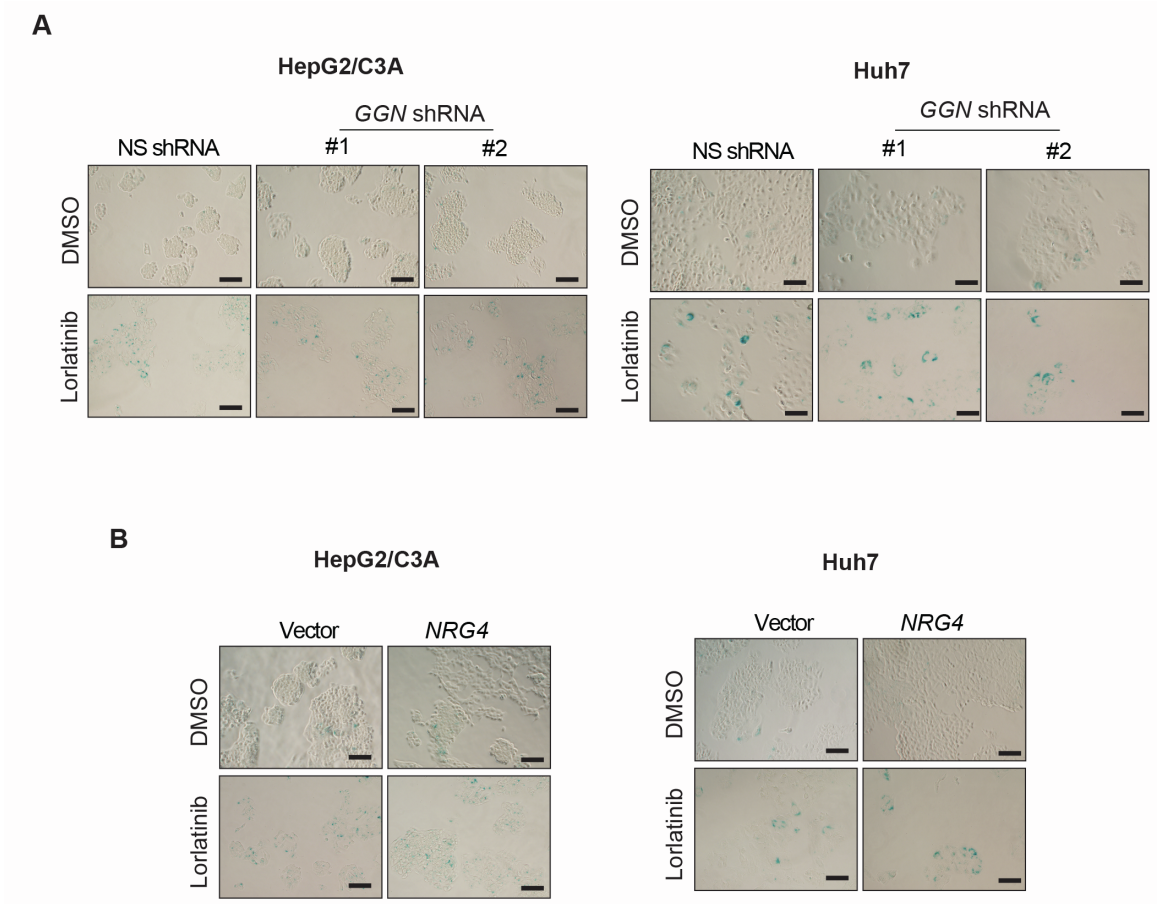

Figure S10. *GGN* knockdown or *NRG4* overexpression does not prevent Lorlatinib-induced senescence in HCC. Related to Figure 7. A. HepG2/C3A and Huh7 cells expressing NS shRNA or *GGN* shRNAs were treated with lorlatinib or DMSO for 96 h, and then analyzed for the senescence-associated β-galactosidase assay (SA β-gal). Representative images are shown. Scale bar; 100 μm. **B.** HepG2/C3A and Huh7 cells expressing empty vector or *NRG4* ORF were treated with lorlatinib or DMSO for 96 h, and then analyzed for the senescence-associated β-galactosidase assay (SA β-gal). Representative images are shown. Scale bar; 100 μm.