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Supplemental information

Targeting EphA2 suppresses hepatocellular carcinoma initiation and progression by dual inhibition of JAK1/STAT3 and AKT signaling

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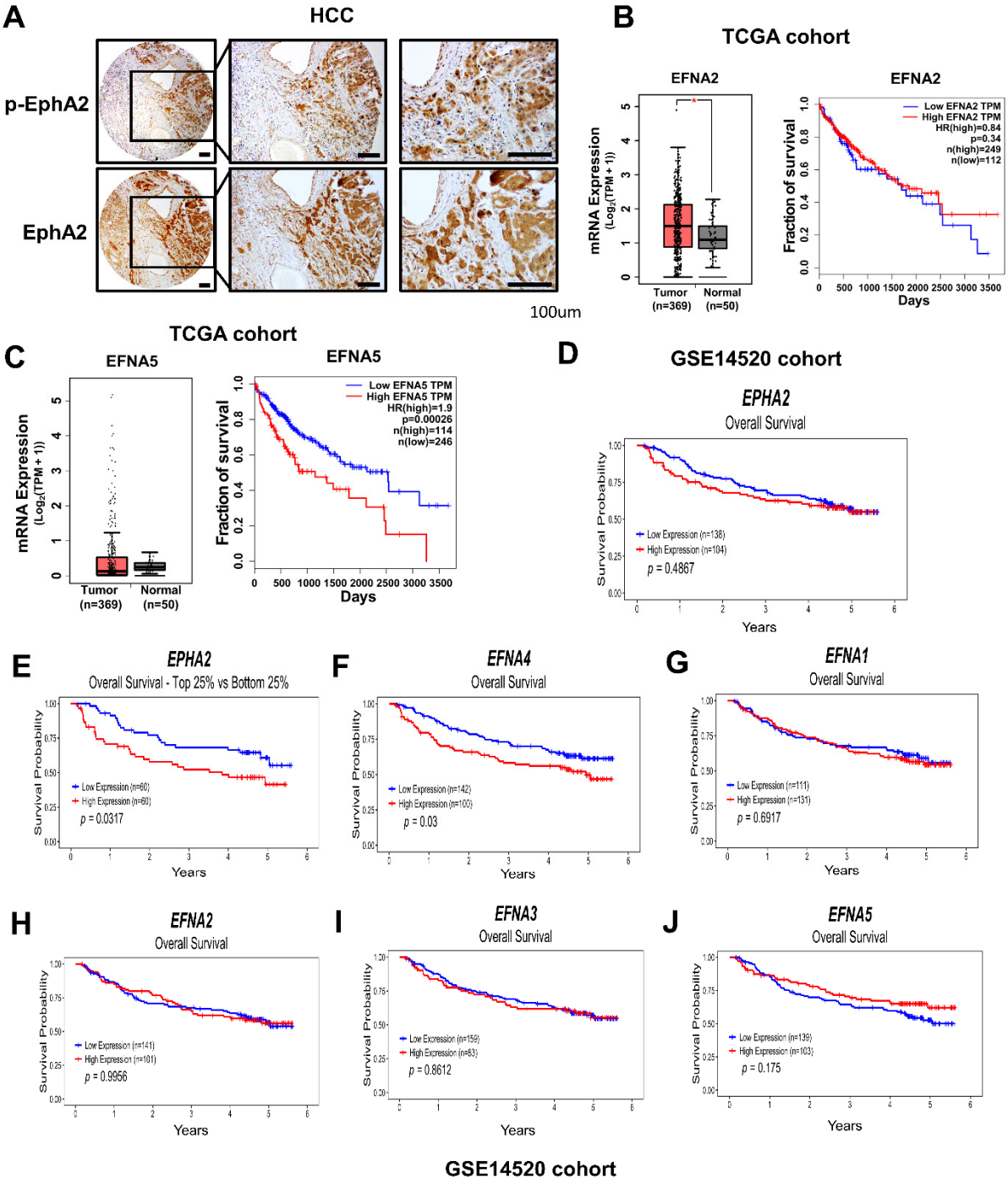

A
HCC
p-EphA2
EphA2
100um
B
TCGA cohort
EFNA2
mRNA Expression
Tumor (n=369)
Normal (n=50)
EFNA2
Low EFNA2 TPM
High EFNA2 TPM
HR(high)=0.84
p=0.34
n(high)=249
n(low)=112
Fraction of survival
Days
C
TCGA cohort
EFNA5
mRNA Expression
Tumor (n=369)
Normal (n=50)
EFNA5
Low EFNA5 TPM
High EFNA5 TPM
HR(high)=1.9
p=0.00026
n(high)=114
n(low)=246
Fraction of survival
Days
D
GSE14520 cohort
EPHA2
Overall Survival
Survival Probability
Low Expression (n=138)
High Expression (n=104)
p = 0.4867
Years
E
EPHA2
Overall Survival - Top 25% vs Bottom 25%
Survival Probability
Low Expression (n=60)
High Expression (n=60)
p = 0.0317
Years
F
EFNA4
Overall Survival
Survival Probability
Low Expression (n=142)
High Expression (n=100)
p = 0.03
Years
G
EFNA1
Overall Survival
Survival Probability
Low Expression (n=111)
High Expression (n=131)
p = 0.6917
Years
H
EFNA2
Overall Survival
Survival Probability
Low Expression (n=141)
High Expression (n=101)
p = 0.9956
Years
I
EFNA3
Overall Survival
Survival Probability
Low Expression (n=159)
High Expression (n=83)
p = 0.8612
Years
J
EFNA5
Overall Survival
Survival Probability
Low Expression (n=139)
High Expression (n=103)
p = 0.175
Years
GSE14520 cohort

Figure S1. EphA2 and its ligands expression in HCC and their correlation with overall survival. Related to Figure 1.

(A) Representative immunohistochemistry (IHC) images of Y588 p-EphA2 and total EphA2 in HCC tissue microarray. Scale bars, 100 μm.
(B) Left, a boxplot of relative mRNA expression levels of EFNA2 comparing normal vs. HCC tissue. Right, Kaplan-Meier plot of overall survival of HCC patients stratified by EFNA2 expression levels. Data from TCGA (GEPIA).
(C) Left, a boxplot of relative mRNA expression levels of EFNA5 comparing normal vs. HCC tissue. Right, Kaplan-Meier plot of overall survival of HCC patients stratified by EFNA5 expression levels. Data from TCGA (GEPIA).
(D-J) Kaplan-Meier plot of overall survival of HCC patients stratified by EphA2 and its ligands expression levels. Data from NCBI GEO (GSE14520). Statistical significance was determined by log-rank test for Kaplan-Meier plot.

Statistical significance was determined by two-tailed Student's t-test *$p < 0.0001$ for boxplots and log-rank test for Kaplan-Meier plot.

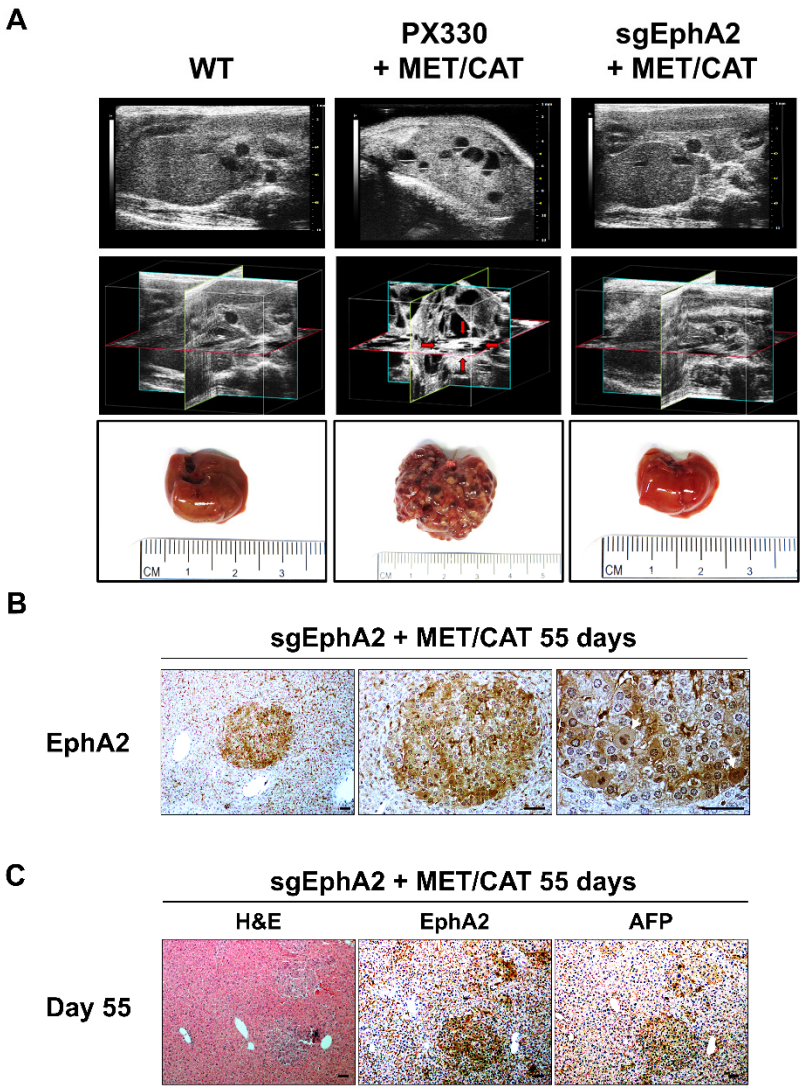


Figure S2. EphA2 knockout delays MET/CAT-induced HCC, and escape from EphA2 deletion reinitiate tumor growth in MET/CAT induced HCC. Related to Figure 3.

(A) Top, representative 2D liver ultrasound images of wild type mice (untreated), PX330, and sgEphA2 injected MET/CAT mice 55 days after injection. Middle, 3D reconstruction mice liver. Prominent hyperechoic tumor mass (red arrow) surrounded by necrotic and hemorrhagic cysts. Bottom, gross representation of the mice livers.

(B) The liver of sgEphA2 MET/CAT mice was extracted 55 days after injection and immunohistochemically assessed for total EphA2 expression. There are multiple microscopic foci of dysplastic hepatocyte nodules stained positive for EphA2. On the other hand, the non-dysplastic background liver hepatocytes are negative for EphA2. Notably, the dysplastic foci are associated with strong EphA2 expression characterized by nuclear crowding, enlarged hyperchromatic nucleus, and frequent mitotic bodies (white arrows) consistent with early HCC. Scale bar, 50 μm.

(C) The liver of sgEphA2 MET/CAT mice described in (A) was collected for H&E and immunohistochemistry for EphA2 and AFP (HCC marker). Scale bar, 50 μm.

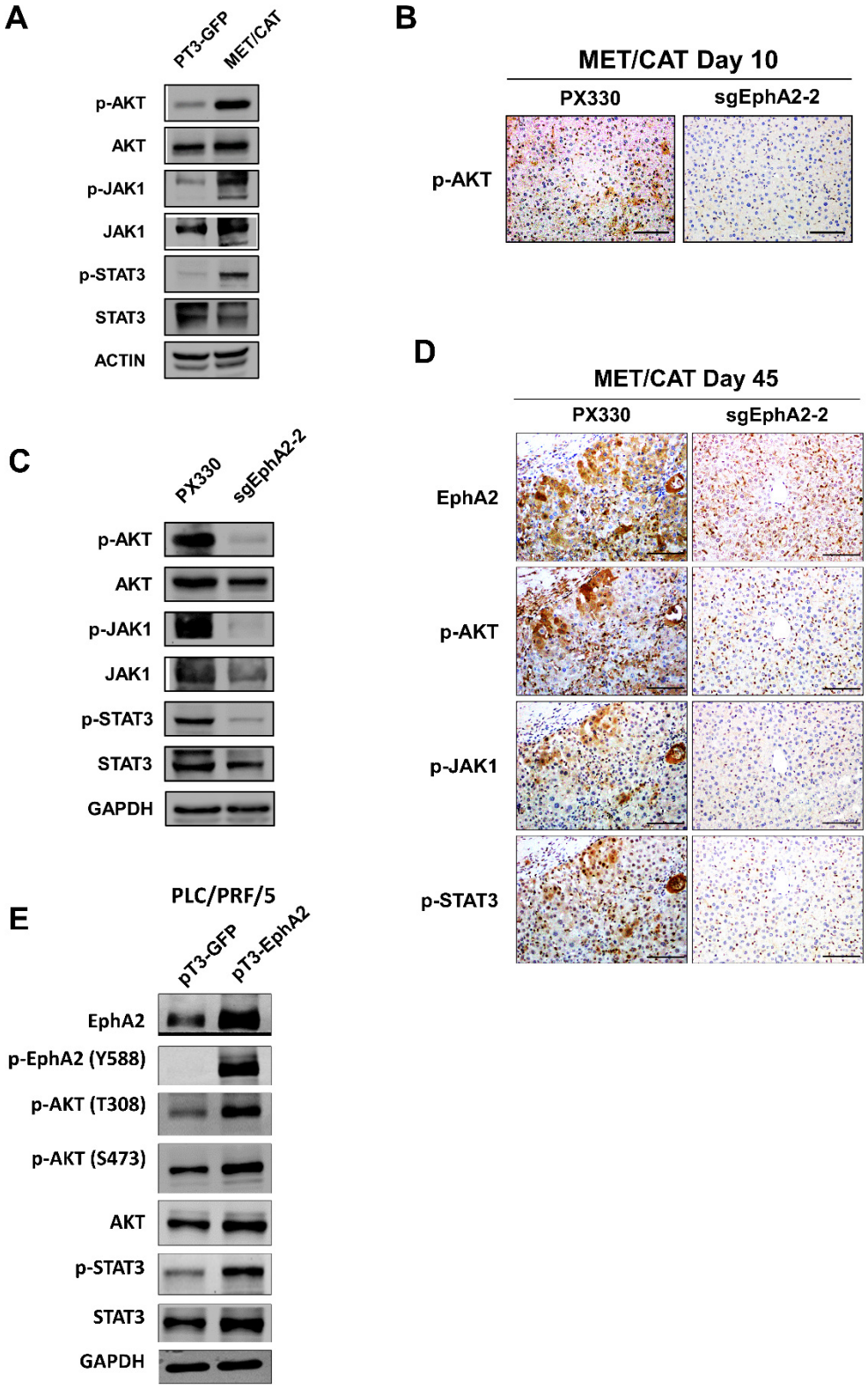

A
PT3-GFP
MET/CAT
p-AKT
AKT
p-JAK1
JAK1
p-STAT3
STAT3
ACTIN
B
MET/CAT Day 10
PX330
sgEphA2-2
p-AKT
C
PX330
sgEphA2-2
p-AKT
AKT
p-JAK1
JAK1
p-STAT3
STAT3
GAPDH
D
MET/CAT Day 45
PX330
sgEphA2-2
EphA2
p-AKT
p-JAK1
p-STAT3
E
PLC/PRF/5
pT3-GFP
pT3-EphA2
EphA2
p-EphA2 (Y588)
p-AKT (T308)
p-AKT (S473)
AKT
p-STAT3
STAT3
GAPDH

Figure S3. Loss of EphA2 inhibits AKT and JAK1/STAT3 activity in MET/CAT induced HCC, and overexpression of EphA2 in HCC cells increases both AKT and STAT3 signaling. Related to Figures 3, 4, 5, and 6.

(A) Western blot analysis of indicated proteins from GFP (PT3-GFP) or MET/CAT mouse liver seven weeks after injection. ACTIN as a loading control.
(B) Liver of EphA2 knockout MET/CAT mice was extracted and immunohistochemically assessed for p-AKT expression comparing to control (PX330). Scale bar, 100 μm.
(C) Western blot analysis of indicated proteins from MET/CAT liver ten days after injection with empty vector (PX330) or sgEphA2. GAPDH as a loading control.
(D) Livers of MET/CAT mice injected with PX330, or sgEphA2 were collected at day 45 and immunohistochemically assessed for EphA2, p-AKT, p-JAK1, and p-STAT3. Scale bars, 100 μm.
(E) Western blot analysis of indicated proteins in PLC/PRF/5 cells transfected with pT3-GFP or pT3-EphA2 for 48 hours. GAPDH as a loading control.

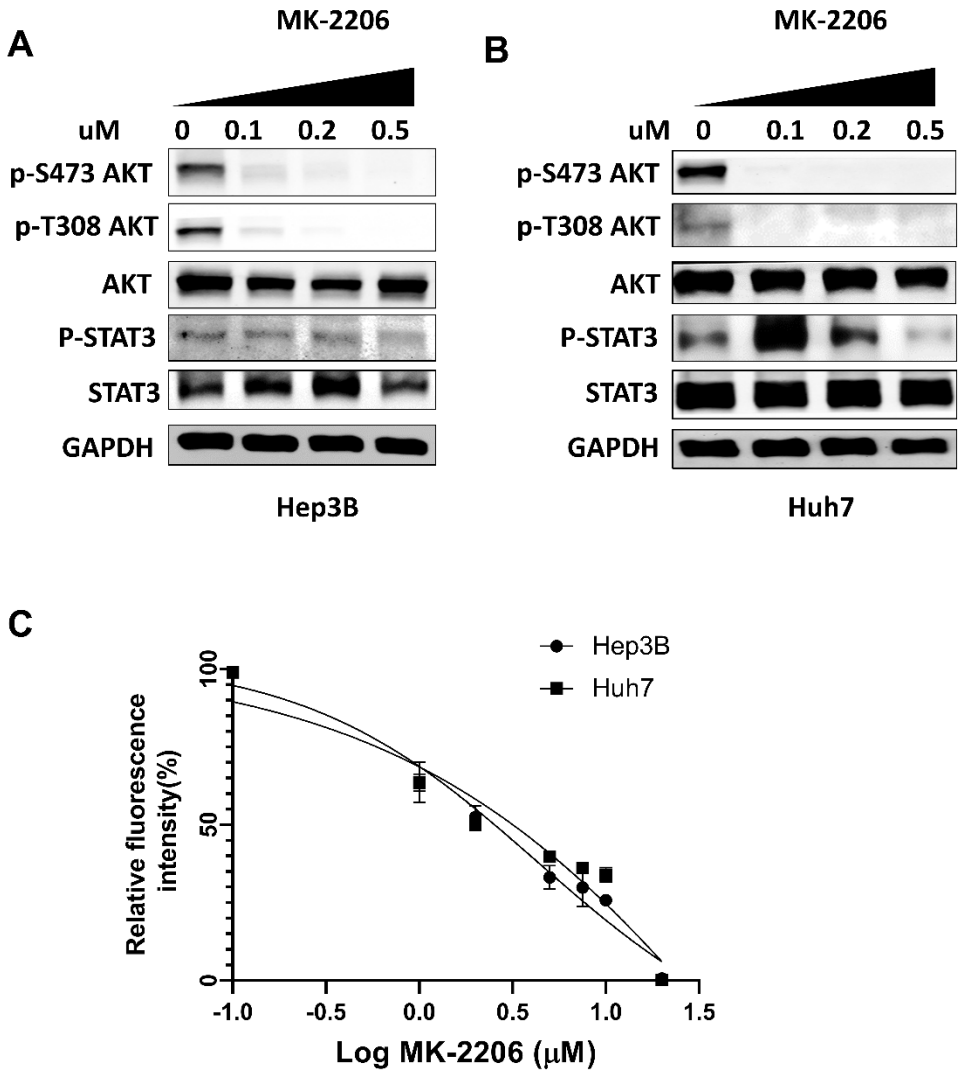


Fig. S4. AKT inhibitor MK2206 suppresses HCC cell growth but not STAT3 activity. Related to Figures 4 and 5.

(A) Western blot analysis of indicated proteins at the selected concentration of MK-2206 for 24 hours in Huh7 cells. GAPDH as a loading control.

(B) Western blot analysis of indicated proteins at the selected concentration of MK-2206 for 24 hours in Hep3B cells. GAPDH as a loading control.

(C) The effect of MK-2206 on Huh7 and Hep3B cells' proliferation was assessed after 48 hours of treatment using CCK-8 assay. Values are mean± SD (n=3).

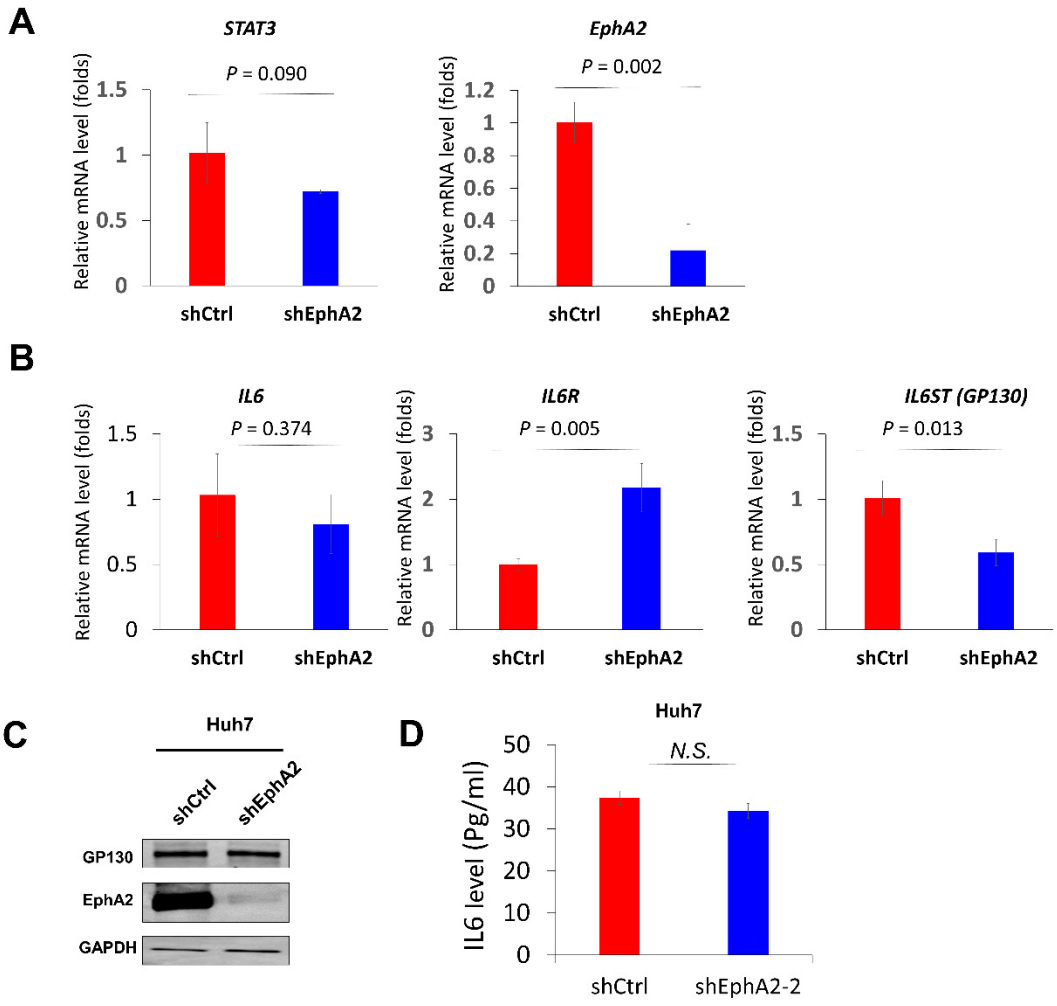


Fig. S5. EphA2 knockdown does not affect the expression and secretion of IL6 in HCC cells. Related to Figures 5.

(A) Real-time PCR analysis of *STAT3* and *EphA2* in scrambled and EphA2 knockdown Huh7 cells. GAPDH was used as an internal control.
(B) Real-time PCR analysis of indicated genes in scrambled and EphA2 knockdown Huh7 cells. GAPDH was used as an internal control.
(C) Cell lysate of scrambled and EphA2 knockdown Huh7 cells was immunoblotted for GP130, EphA2, and GAPDH.
(D) The culture supernatants from the scrambled and EphA2 knockdown Huh7 cells were collected, and IL-6 protein levels were measured using Human IL-6 ELISA Kit.

Statistical significance was determined by a two-tailed Student t-test (A, B,&D). N.S., not significant.

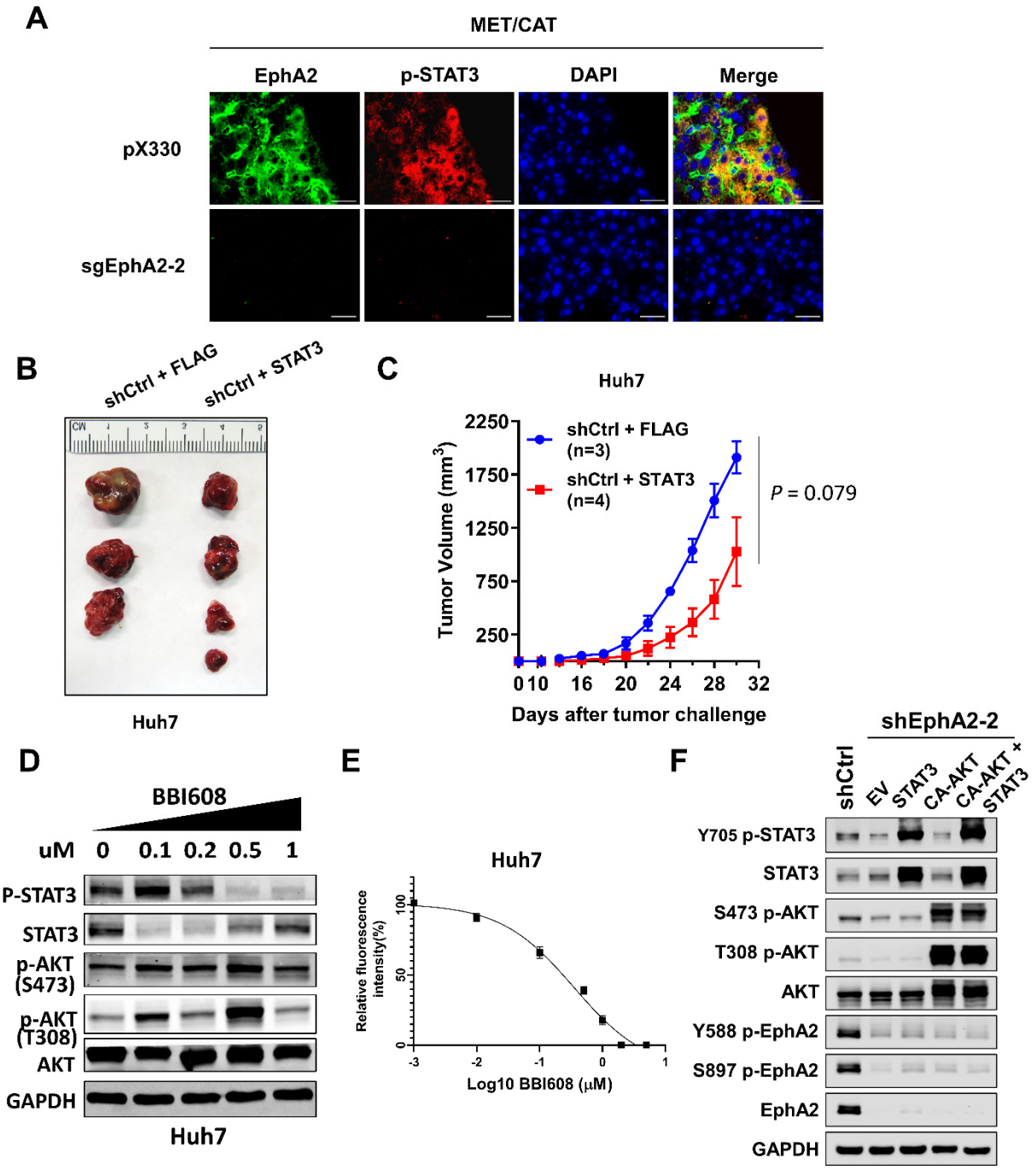

A
MET/CAT
EphA2
p-STAT3
DAPI
Merge
pX330
sgEphA2-2
B
shCtrl + FLAG
shCtrl + STAT3
Huh7
C
Huh7
shCtrl + FLAG (n=3)
shCtrl + STAT3 (n=4)
P = 0.079
Tumor Volume (mm3)
2250
1750
1250
750
250
0 10 16 20 24 28 32
Days after tumor challenge
D
BBI608
uM 0 0.1 0.2 0.5 1
P-STAT3
STAT3
p-AKT (S473)
p-AKT (T308)
AKT
GAPDH
Huh7
E
Huh7
Relative fluorescence intensity(%)
100
50
0
-3 -2 -1 0 1
Log10 BBI608 (μM)
F
shEphA2-2
shCtrl
EV
STAT3
CA-AKT
CA-AKT + STAT3
Y705 p-STAT3
STAT3
S473 p-AKT
T308 p-AKT
AKT
Y588 p-EphA2
S897 p-EphA2
EphA2
GAPDH

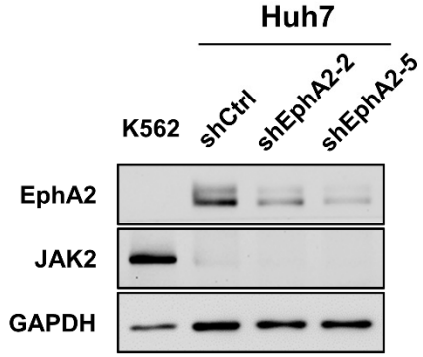
Fig. S6 EphA2 promotes tumor development partially through activation of STAT3 signaling. Related to Figures 5.

(A) Immunofluorescence analysis of EphA2 and p-STAT3 in MET/CAT model 10 days after injection. EphA2 (green), p-STAT3 (red). Nuclei were stained with DAPI (blue). Scale bars, 30 μm
(B) Representative picture of tumors extracted from NSG-A2 mice 30 days after subcutaneous injection with Huh7 cells as described. N=3 or 4 mice in each group.
(C) Primary tumor size from the mice (B) was recorded every two days. Values are mean± SEM.
(D) Western blot analysis of indicated proteins at the selected concentration of BBI608 for 24 hours in Huh7 cells. GAPDH as a loading control.
(E) The effect of BBI608 on cell proliferation of Huh7 cells was assessed after 48 hours of treatment using CCK-8 assay. Values are mean± SD (n=3).
(F) Western blot analysis of indicated proteins in Huh7 scrambled or EphA2 knockdown cells overexpressed with control (EV), AKT, STAT3, or both. GAPDH as a loading control.

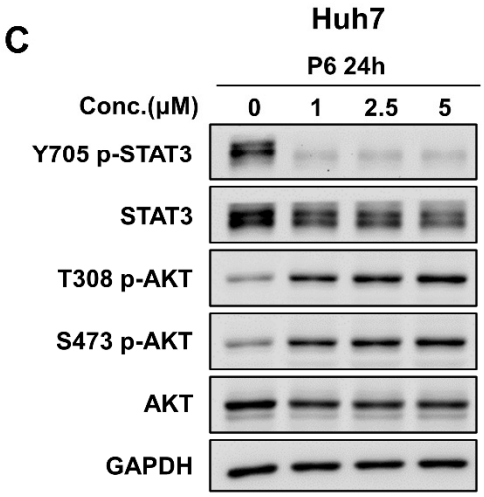
A

	Normalized Expression Levels					
Gene Symbol	shCtrl_1	shCtrl_2	shCtrl_3	shEphA2_2_1	shEphA2_2_2	shEphA2_2_3
JAK1	2508.6316	2547.8344	2461.9476	2319.1578	2471.3773	2430.0674
JAK2	179.371	157.2378	167.5622	101.0915	165.4522	111.7884
JAK3	5.979	7.7648	2.8083	11.8931	5.2029	7.9849

B



C



D

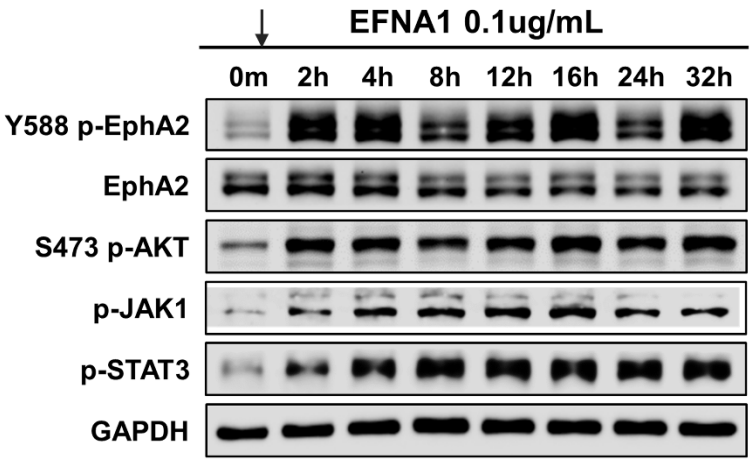


Figure S7. JAK1 is an intermediate of EphA2/STAT3 signaling, and recombinant EFNA1-Fc activates EphA2 signaling and its downstream effectors AKT and JAK1/STAT3 in HCC. Related to Figure 6.

(A) Normalize RNA expression of JAK1, JAK2, and JAK3 in scramble (shCtrl) or *EphA2* knockdown Huh7 cell.
(B) Western blot confirmation of JAK2 expression in Huh7 cells. K562 as a positive control. GAPDH as a loading control.
(C) Western blot analysis of indicated protein from Huh7 cells treated with JAK inhibitor (pyridine 6, P6) for 24 hours. GAPDH as a loading control.
(D) Cell lysates of Huh7 treated with 0.1 μg/mL of EFNA-Fc at indicated times were immunoblotted for Y588 p-EphA2, EphA2, p-JAK1, p-STAT3, and p-AKT. GAPDH as a loading control.

EphA2 IHC Score	0 (n=16)	1 (n=91)	2 (n=32)	3 (n=14)	Total (n=153)	*p* value
Age						0.097
Mean (SD)	62.4 (13.1)	63.9 (13.4)	64.0 (14.0)	53.8 (19.6)	62.8 (14.3)	
Median	64.0	66.0	67.0	62.5	66.0	
Range	32.0 - 79.0	28.0 - 96.0	20.0 - 85.0	23.0 - 77.0	20.0 - 96.0	
Gender						0.614
F	6 (37.5%)	34 (37.8%)	13 (41.9%)	3 (21.4%)	56 (37.1%)	
M	10 (62.5%)	56 (62.2%)	18 (58.1%)	11 (78.6%)	95 (62.9%)	
Race						0.018
Asian	1 (7.7%)	3 (4.2%)	0 (0.0%)	0 (0.0%)	4 (3.2%)	
African American	0 (0.0%)	1 (1.4%)	1 (3.7%)	2 (16.7%)	4 (3.2%)	
Other	0 (0.0%)	6 (8.3%)	0 (0.0%)	0 (0.0%)	6 (4.8%)	
White	12 (92.3%)	62 (86.1%)	26 (96.3%)	9 (75.0%)	109 (87.9%)	
Hepatitis B	1 (6.7%)	13 (15.3%)	2 (7.4%)	4 (28.6%)	20 (14.2%)	0.462
Hepatitis C	2 (13.3%)	20 (23.8%)	7 (25.9%)	2 (14.3%)	31 (22.1%)	0.736
Alcohol Abuse	3 (18.8%)	27 (30.7%)	7 (24.1%)	4 (28.6%)	41 (27.9%)	0.800
Cancer Stage						0.932
T Stage						0.562
N Stage						0.615
M Stage						0.891
No. of Tumors						0.803
One	9 (60.0%)	64 (70.3%)	23 (74.2%)	10 (71.4%)	106 (70.2%)	
Multiple	6 (40.0%)	27 (29.7%)	8 (25.8%)	4 (28.6%)	45 (29.8%)	
Primary Or Metastatic						0.307
metastatic	1 (6.2%)	1 (1.1%)	0 (0.0%)	0 (0.0%)	2 (1.3%)	
primary	15 (93.8%)	89 (98.9%)	31 (100.0%)	14 (100.0%)	149 (98.7%)	

Table S1. TMA Patient characteristics. Related to Figure 1.

Gene	Mean (SD)	Hazard ratio (95% CI) for 1 SD increase	*p*-value
EphA2	4.56 (0.63)	1.31 (1.08-1.57)	0.005
EFNA1	9.50 (0.96)	1.17 (0.92-1.48)	0.20
EFNA2	3.57 (0.22)	1.00 (0.80-1.25)	0.99
EFNA3	4.38 (0.64)	1.13 (0.93-1.38)	0.20
EFNA4	4.29 (0.57)	1.47 (1.21-1.80)	<0.001
EFNA5	3.48 (0.24)	0.84 (0.64-1.11)	0.23

Table S2. Hazard ratios for 3-year survival of the GSE14520 cohort. Related to Figure 1.

A

NAME	NES	*p*-value
MYOGENESIS	-2.0354	0
JAK_STAT3_SIGNALING	-1.9501	0
ERBB2_UP.V1_UP	-1.8854	0
ESTROGEN_RESPONSE-LATE	-1.8029	0
KRAS_SIGNALING_UP	-1.6024	0.0035757
ANGIOGENSIS	-1.572	0.0226897
AKT_UP.V1_UP	-1.5162	0.0095465
CYCLIN_D1_UP.V1_UP	-1.4969	0.0035928
COAGULATION	-1.4911	0.0139417
KRAS.BREAST_UP.V1_UP	-1.4856	0.0227848

B

Species	Gene	FW (5' 3')	RV (5' 3')
human	*EphA2*	TGGCTCACACACCCGTATG	GTCGCCAGACATCACGTTG
human	*NANOG*	TTTGTGGGCCTGAAGAAAACT	AGGGCTGTCCTGAATAAGCAG
human	*SOX2*	GCCGAGTGGAAACTTTTGTCG	GGCAGCGTGTACTTATCCTTCT
human	*KLF4*	CCCACATGAAGCGACTTCCC	CAGGTCCAGGAGATCGTTGAA
human	*EPCAM*	AATCGTCAATGCCAGTGTACTT	TCTCATCGCAGTCAGGATCATAA
human	*CD90*	ATGAAGGTCCTCTACTTATCCGC	GCACTGTGACGTTCTGGGA
human	*HIF1a*	GAACGTCGAAAAGAAAAGTCTCG	CCTTATCAAGATGCGAACTCACA
human	*TGFb1*	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
human	*STAT3*	CAGCAGCTTGACACACGGTA	AAACACCAAAGTGGCATGTGA
human	*IL6*	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
human	*IL6R*	CCCCTCAGCAATGTTGTTTGT	CTCCGGGACTGCTAACTGG
human	*IL6ST*	CGGACAGCTTGAACAGAATGT	ACCATCCCACTCACACCTCA

Table S3. Enriched gene sets affected by EphA2 knockdown in Huh7 cells (A) and primer sequences for RT-qPCR (B). Related to Figures 4, 5, S5, and STAR Methods.