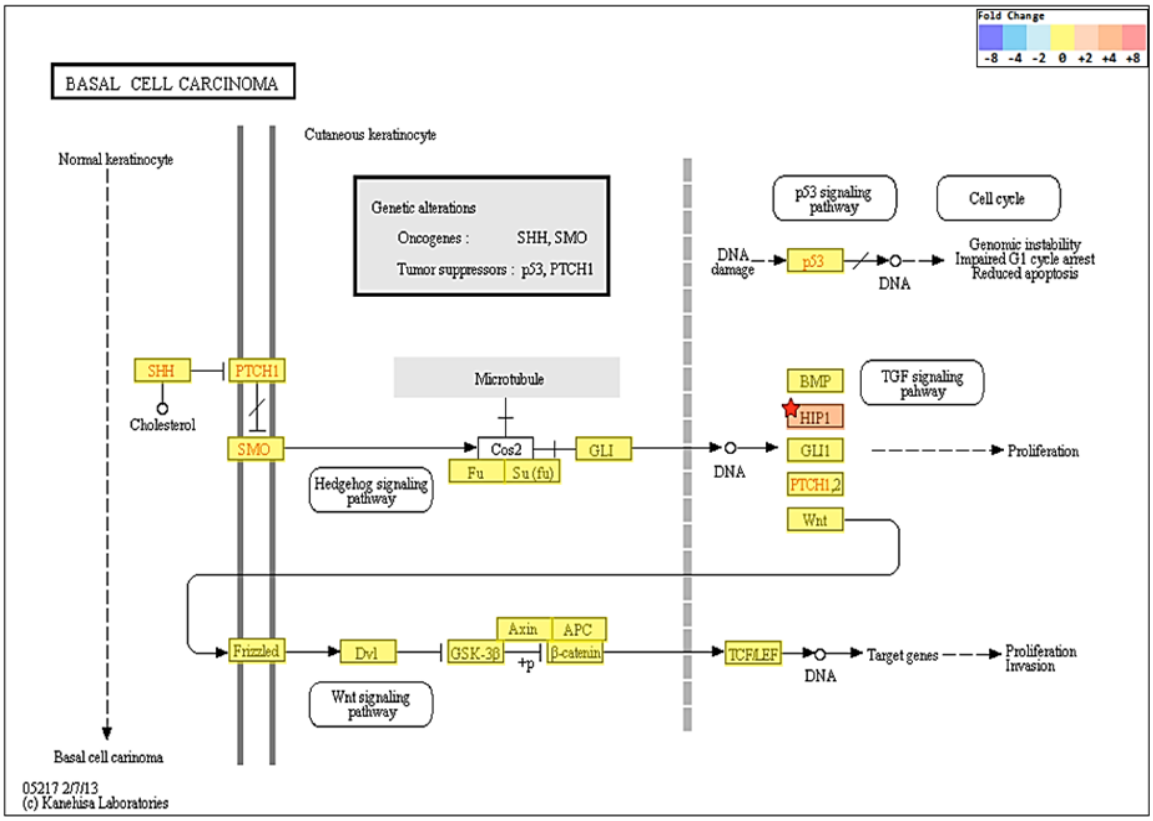
Supplementary File

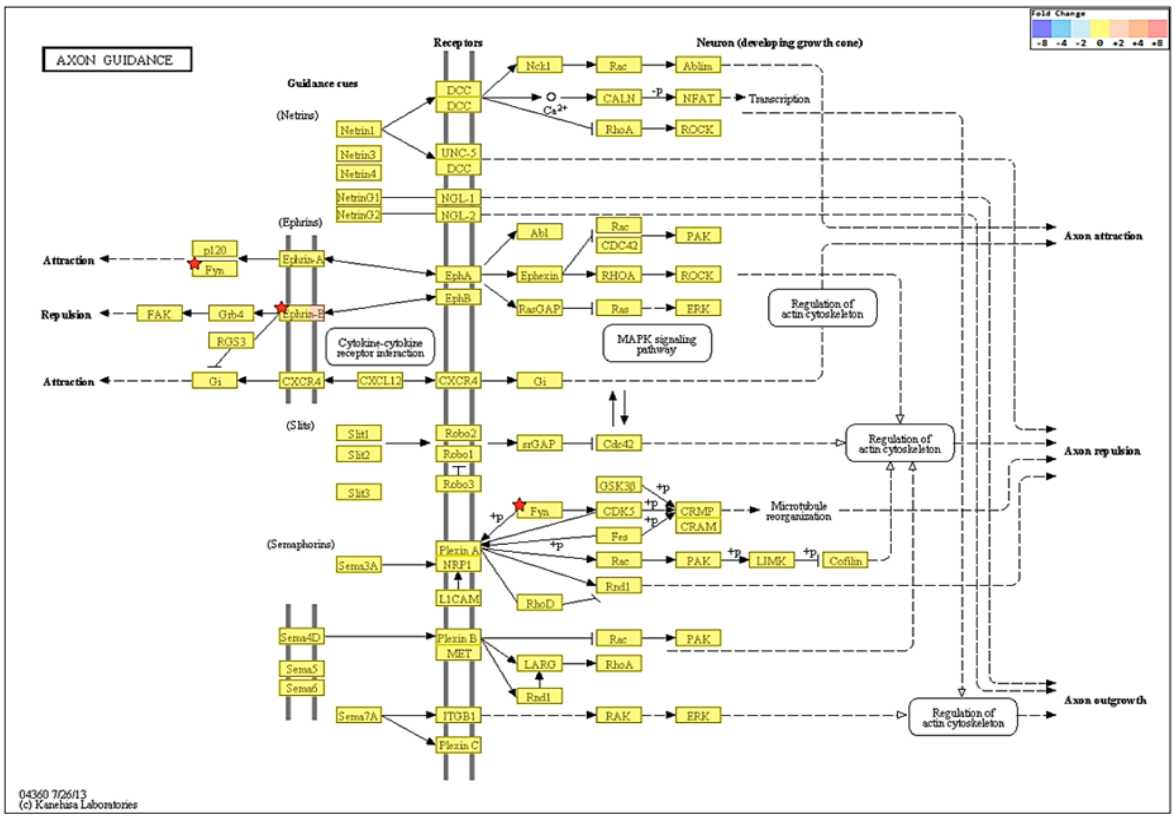
Supplementary Table S1. List of cetuximab resistance target genes.

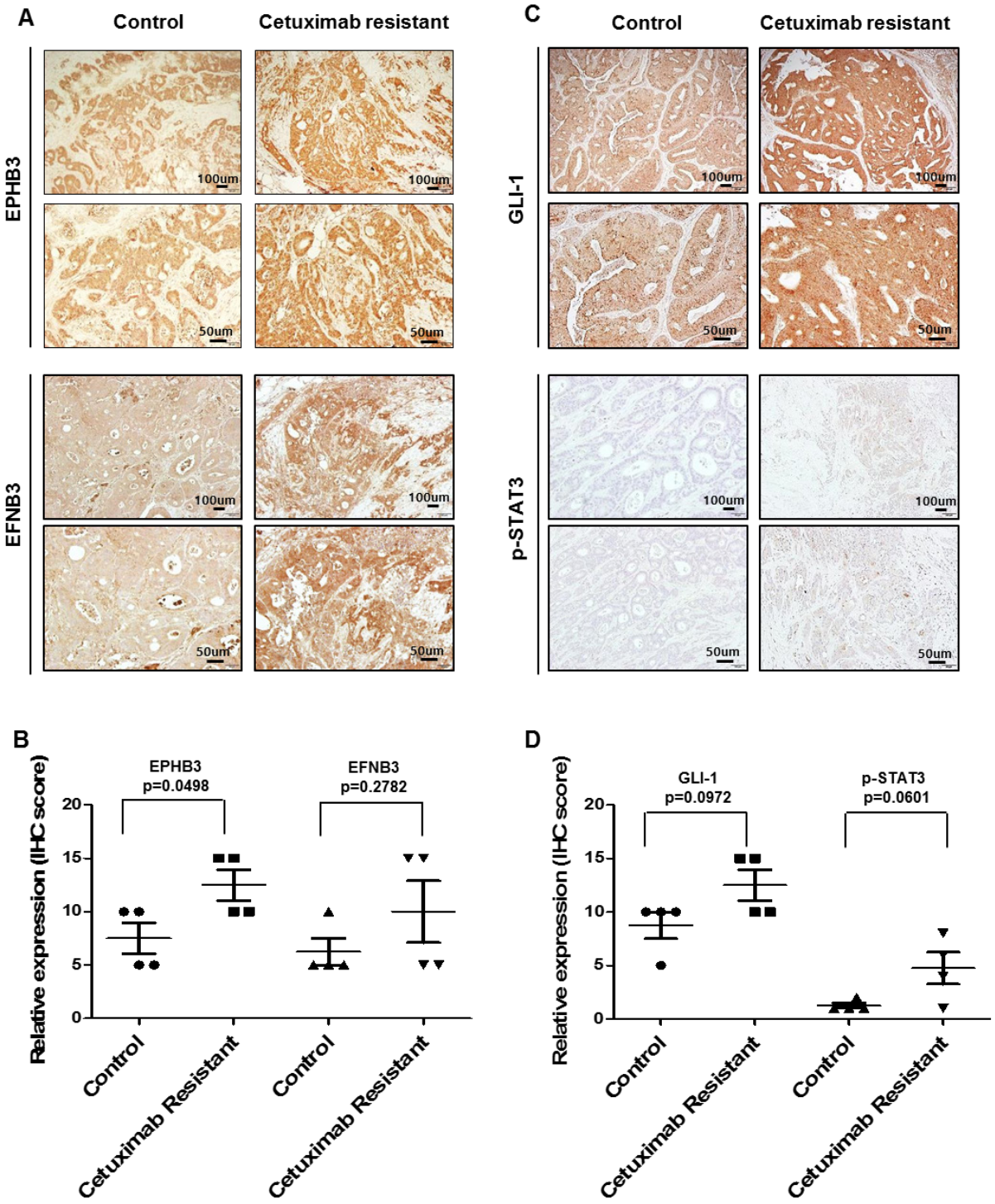
Gene Symbol	Target ID	SW48R/SW48. Fold change of gene expression
SOX2	NM_003106.2	5.267482
HHIP	NM_022475.1	4.802096
S100A4	NM_019554.2	4.262277
TJP3	NM_014428.1	2.081920
BCL11B	NM_138576.2	2.939502
FOXQ1	NM_033260.3	2.037583
FLJ22447	XM_943476.2	2.051179
EFNB3	NM_001406.3	2.604695
TXNIP	NM_006472.2	2.141614
TBX3	NM_005996.3	2.116830
OSR1	NM_145260.2	2.553626
MYEOV	NM_138768.2	2.913401
AKAP12	NM_005100.2	2.207059
LOC400578	XR_017543.1	2.706147
SCNN1A	NM_001038.4	2.928251
ASCL2	NM_005170.2	2.513275
KRT19	NM_002276.3	2.914128
SIPA1L2	NM_020808.3	2.543186
MGC102966	XR_015970.1	2.704920
ZNF217	NM_006526.2	2.178871
ELF3	NM_004433.3	2.282792
HAS2	NM_005328.1	2.383841
PPARG	NM_015869.4	2.502287
IGF2BP3	NM_006547.2	2.061941
FLJ40504	NM_173624.1	2.143779
CD24	NM_013230.2	2.115076
HES2	NM_019089.3	2.016409
SEZ6L2	NM_201575.1	2.077040
KRT18P28	XR_017689.1	2.036644
KRT18P17	XR_037953.1	2.146687
KRT18P13	XM_001726959.1	2.238622
LOC149501	XR_017100.2	2.143014
SPNS2	NM_001124758.1	2.105924
HIF1A	NM_181054.1	1.884402
GLI-1	NM_005269.1	1.039062
SHH	NM_000193.2	1.095282
EPHB3	NM_004443.3	1.055114
SMO	NM_005631.3	1.001898
PTCH2	NM_003738.3	1.091162

Gene Symbol	Target ID	SW48R/SW48. Fold change of gene expression
PMEPA1	NM_199169.1	-4.399733
TSPAN8	NM_004616.2	-3.133551
PROM2	NM_144707.1	-3.203582
CPNE8	NM_153634.2	-3.032428
EBI2	NM_004951.3	-3.114587
CLCF1	NM_013246.2	-2.051573
FOS	NM_005252.2	-2.366714
TNFRSF12A	NM_016639.1	-2.003347
LPHN2	NM_012302.2	-2.114441
DCLK1	NM_004734.2	-2.054700
EBI2	NM_004951.3	-2.536306
CYR61	NM_001554.3	-2.185408
PMEPA1	NM_199169.1	-2.861657
HEPH	NM_138737.1	-2.473364
SORBS2	NM_003603.4	-2.459048
CPNE8	NM_153634.2	-2.514729
EGR3	NM_004430.2	-2.474281
SORBS2	NM_003603.4	-2.075738
PTCH1	NM_001083605.1	-1.010164
STAT3	NM_213662.1	-1.066877

Supplementary Table S2. The pathway of cetuximab resistance (microarray result analysis).





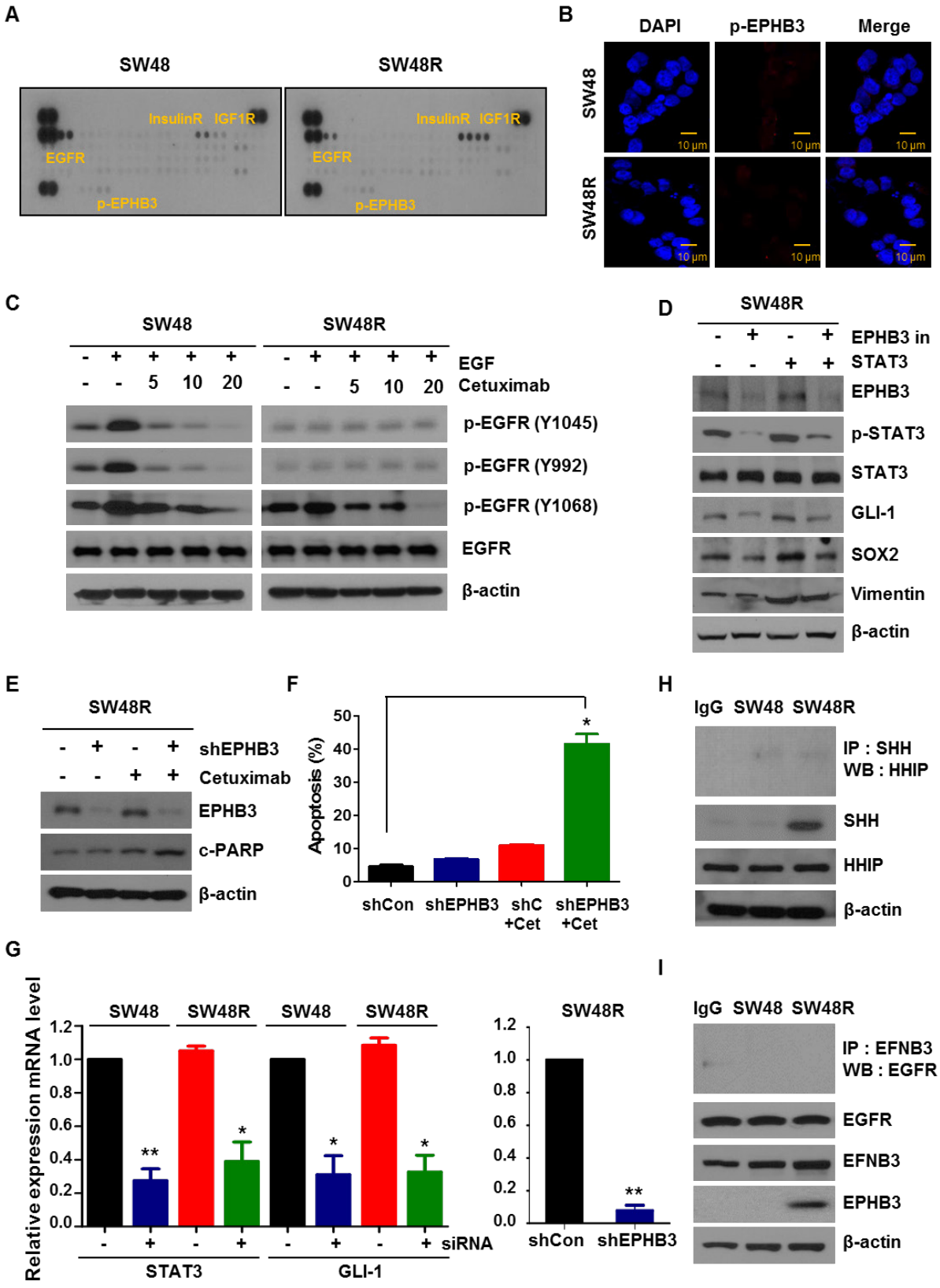

A
Control
Cetuximab resistant
EPHB3
EFNB3
100um
50um
C
Control
Cetuximab resistant
GLI-1
p-STAT3
100um
50um
B
EPHB3
p=0.0498
EFNB3
p=0.2782
Relative expression (IHC score)
20
15
10
5
0
Control
Cetuximab Resistant
Control
Cetuximab Resistant
D
GLI-1
p=0.0972
p-STAT3
p=0.0601
Relative expression (IHC score)
20
15
10
5
0
Control
Cetuximab Resistant
Control
Cetuximab Resistant

Supplementary Figure S1. Differences in gene expression between pre-cetuximab-treatment and cetuximab-resistant patients in clinical colon cancer specimens. (A) Representative picture of immunohistochemistry on human colon cancer specimens stained for EPHB3 and EFNB3. Scale bars: 100 μm and 50 μm. (B) Box plots indicate the percentage of EPHB3- and EFNB3-positive tissues in pre-cetuximab treated patient and cetuximab-resistant patient samples. Pre-cetuximab treated patients: n=4, cetuximab-resistant patients: n=4. (C) Representative picture of immunohistochemistry of human colon cancer specimens stained for GLI-1 and p-STAT3. Scale bars: 100 μm and 50 μm. (D) Box plots indicate the percentage of GLI-1 and p-STAT3 positive tissues in pre-cetuximab treated and cetuximab-resistant patient samples. Pre-cetuximab treated patients: n=4, cetuximab-resistant patients: n=4

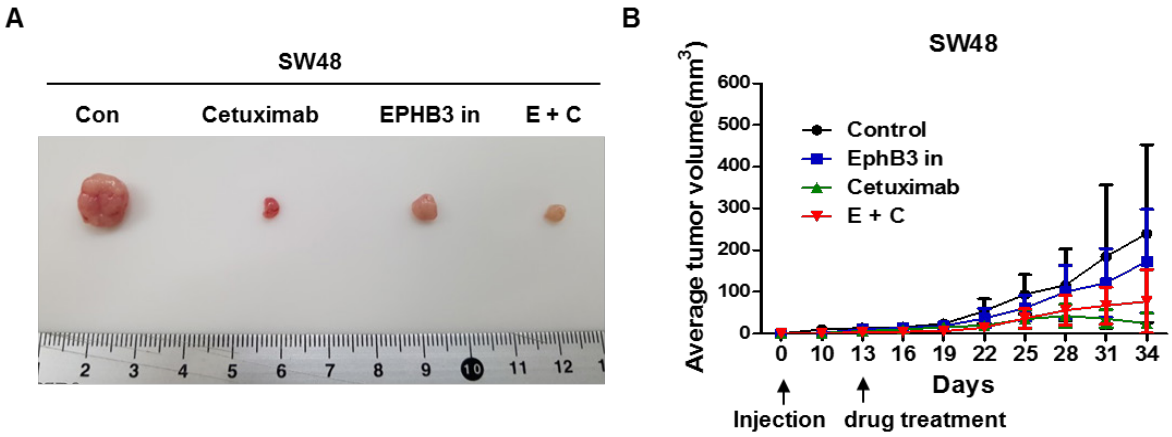
A
SW48
SW48R
100um
B
SW48R Con
EPHB3 in
Cetuximab
E + C
C
SW48 SW48R
p-ERK
ERK
p-mTOR
mTOR
p-AKT
AKT
p-JNK
JNK
β-actin
D
SW48R
- 1 2 3 4 (h)
p-ERK
GLI-1
SHH
SMO
β-actin
E
SW48 SW48R
VEGFR2
HER2
EPHB3
β-actin
F
SW48
1 2 3 4 5 24h
EPHB3
β-actin
1. Con
2. FGFR in
3. PDGFR in
4. VEGFR in
5. Cetuximab
G
HT29
HT29R
- + + + + - + + + +
EGF
- - 5 10 20 - - 5 10 20
Cetuximab
EGFR
EPHB3
β-actin
H
SW48
SW48R
- + - + - + - +
GANT61
- - + + - - + +
Cetuximab
GLI-1
GLI-2
c-PARP
β-actin

Supplementary Figure S2. Effects of cetuximab on human colorectal cancer cell lines with acquired resistance to cetuximab, such as EGFR activation and EPHB3 signaling.

(A) SW48 and SW48R Cell morphology was observed under a light microscope (Olympus CKX53). Scale bar: 100 μm. (B) Cells were treated with an EPHB3 inhibitor (20 μM), cetuximab alone, or an EPHB3 inhibitor in combination with cetuximab for 24 h. Cell morphology was observed under a light microscope. Scale bar: 100 μm. (C) Western blotting was performed to detect levels of EGFR signaling markers ERK, m-TOR, AKT and JNK in SW48 and SW48R cells. (D) The levels of SHH signaling was examined using western blotting after treatment with an ERK inhibitor (PD98059) for the indicated times in SW48R cells. (E) Western blotting was performed to detect levels of Receptor markers VEGFR, HER2 and EPHB3 in SW48 and SW48R cells. (F) The levels of EPHB3 were examined using western blotting after treatment with the indicated inhibitors for 24 h in SW48 cells. (G) HT29 and HT29 cells were treated with increasing concentrations of cetuximab (5, 10 and 20 μg/mL) for 24 h with EGF after overnight 2% FBS starvation. The level of EPHB3 expression was confirmed by western blot. (H) Combinatorial treatment with cetuximab and the GANT61 (GLI-1 and GLI-2 inhibitor, 10 μM) for 24 h led to loss of GLI-1 expression in SW48 and SW48R cells. The levels of c-PARP, GLI-1 and GLI-2 were detected by western blotting.


A
SW48
SW48R
InsulinR
IGF1R
EGFR
p-EPHB3
B
DAPI
p-EPHB3
Merge
SW48
SW48R
10 μm
C
SW48
SW48R
EGF
Cetuximab
p-EGFR (Y1045)
p-EGFR (Y992)
p-EGFR (Y1068)
EGFR
β-actin
D
SW48R
EPHB3 in
STAT3
EPHB3
p-STAT3
STAT3
GLI-1
SOX2
Vimentin
β-actin
E
SW48R
shEPHB3
Cetuximab
EPHB3
c-PARP
β-actin
F
Apoptosis (%)
shCon
shEPHB3
shC +Cet
shEPHB3 +Cet
H
IgG
SW48
SW48R
IP : SHH
WB : HHIP
SHH
HHIP
β-actin
G
Relative expression mRNA level
siRNA
STAT3
GLI-1
shCon
shEPHB3
I
IgG
SW48
SW48R
IP : EFNB3
WB : EGFR
EGFR
EFNB3
EPHB3
β-actin

Supplementary Figure S3. Regulation of phospho-receptor tyrosine kinases (RTKs) by EPHB3. (A) Whole blot of human phospho-RTK array in the indicated samples. (B) The immunofluorescence of p-EPHB3 was detected by confocal laser-scanning microscopy (original magnification: 40X). Bar: 10 μm. (C) SW48 and SW48R cells were treated with increasing concentrations of cetuximab (5, 10 and 20 μg/mL) for 24 h with EGF (10 ng/mL) after overnight 2% FBS starvation. Western blotting was performed to detect levels of indicated p-EGFR and EGFR in SW48 and SW48R cells. (D) EPHB3 regulates STAT3 and SHH signaling. The levels of EPHB3, p-STAT3, GLI-1, SOX2, Vimentin were detected by western blotting. (E) Knockdown of EPHB3 cells treated with cetuximab increased the level of cleaved PARP expression in SW48R cells. (F) Cetuximab-treated EPHB3-knockdown SW48R cells were stained with Annexin V and PI, and then evaluated by FACS analysis. (G) STAT3 and GLI-1 were silenced in cells by STAT3 and GLI-1 siRNA. EPHB3 was silenced in SW48R cells with EPHB3 shRNA. The level of mRNA expression of STAT3, GLI-1, EPHB3 and GAPDH were determined by real-time-PCR. (H) SHH did not display increased association with HHIP in SW48R cells. Cells were harvested and SHH or HHIP were immunoprecipitated with anti-goat SHH or anti-rabbit HHIP antibodies. The immunoprecipitated complexes were fractionated on SDS followed by immunoblotting for the indicated proteins. (I) EGFR did not display increased association with EFNB3 in SW48R cells. Cells were harvested and EFNB3 or EGFR were immunoprecipitated with anti- rabbit EFNB3 or anti-rabbit EGFR antibodies. The immunoprecipitated complexes were fractionated on SDS followed by immunoblotting for the indicated proteins. $^{**}P < 0.01$, $^{*}P < 0.05$.



Supplementary Figure S4. Effects of the combination of cetuximab and an EPHB3 inhibitor in the treatment of SW48 tumor cells *in vivo*. (A) SW48 cells were implanted subcutaneously into nude mice (left), and then tumor growth was evaluated by measuring the tumor volume after 3 weeks of treatment with cetuximab (10 mg/kg), the EPHB3 inhibitor (0.1 mg/kg), or the combination of cetuximab and the EPHB3 inhibitor (every 2 days; n=7). (B) Line graph illustrating the tumor volume (mm^3) in SW48 tumor-bearing mice treated with PBS alone, cetuximab alone, the EPHB3 inhibitor alone, or the combination of cetuximab and the EPHB3 inhibitor, from 0 to day 34. Error bars represent the mean ± SD from five mice. For statistical analysis, Student's *t*-test (two-sided, paired) was used.