Supplemental Data

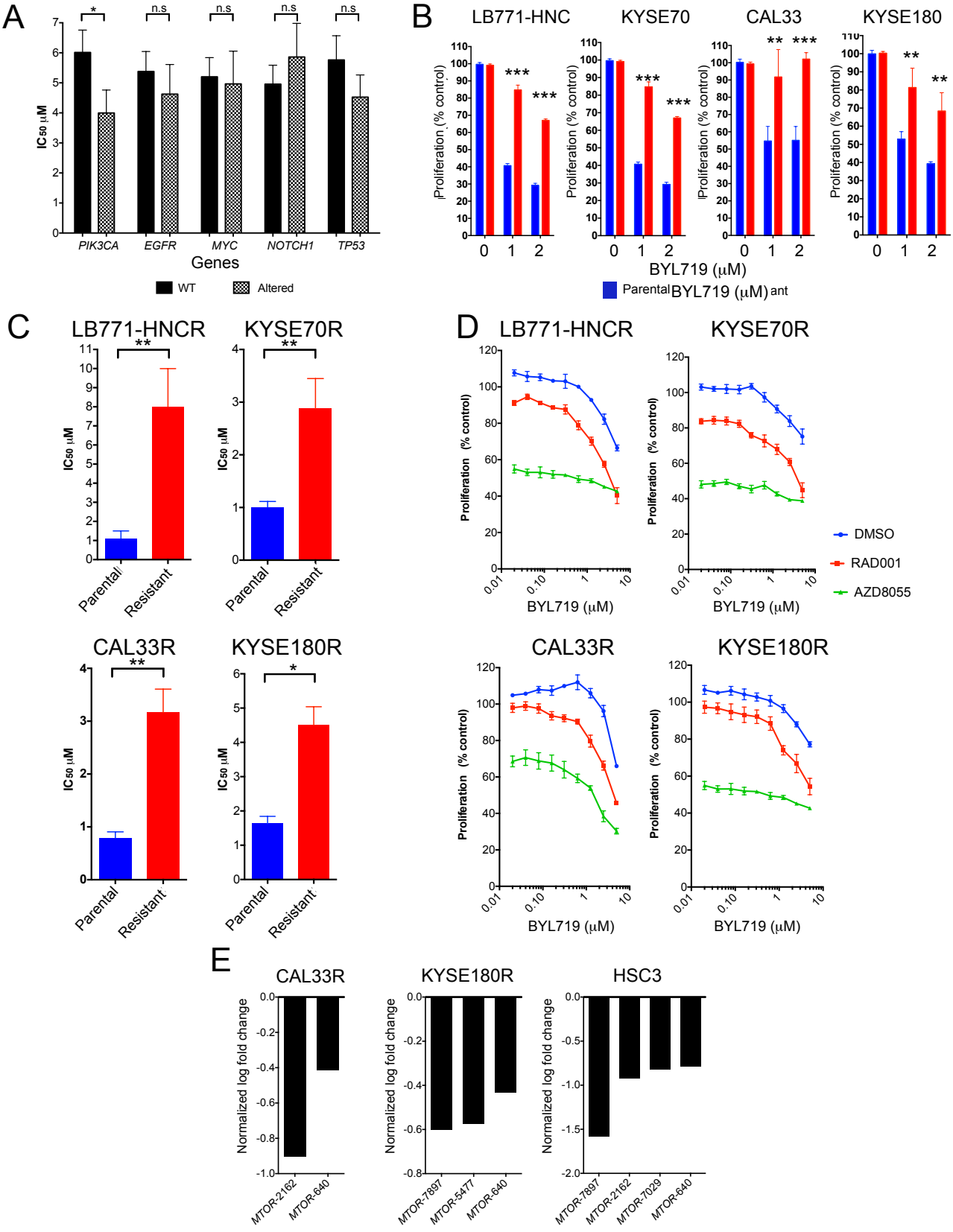


Figure S1 (related to Figure 1): *Establishment of BYL719-resistant cells and their sensitivity to mTOR inhibition.* **(A)** IC_{50} of cell lines classified by the genomic alteration. **(B)** Five days proliferation of parental and resistant cell lines treated with BYL719 as indicated. **(C)** IC_{50} for BYL719 of parental and resistant cell lines. **(D)** Proliferation of cells treated with 1 μM BYL719 with or without RAD001 or AZD8055. **(E)** Reduction in the number or reads by individual *FRAP1* shRNAs transfected into cells with acquired resistance to BYL719 treated 7 days with 1 μM BYL719 (normalized to DMSO-treated controls). Data are presented as means ± SEM. p values were calculated using two-sided Student's t test. *p<0.05, **p<0.01, ***p<0.001.

Table S1, related to Figure 1. shRNA screen. Provided as an Excel file.

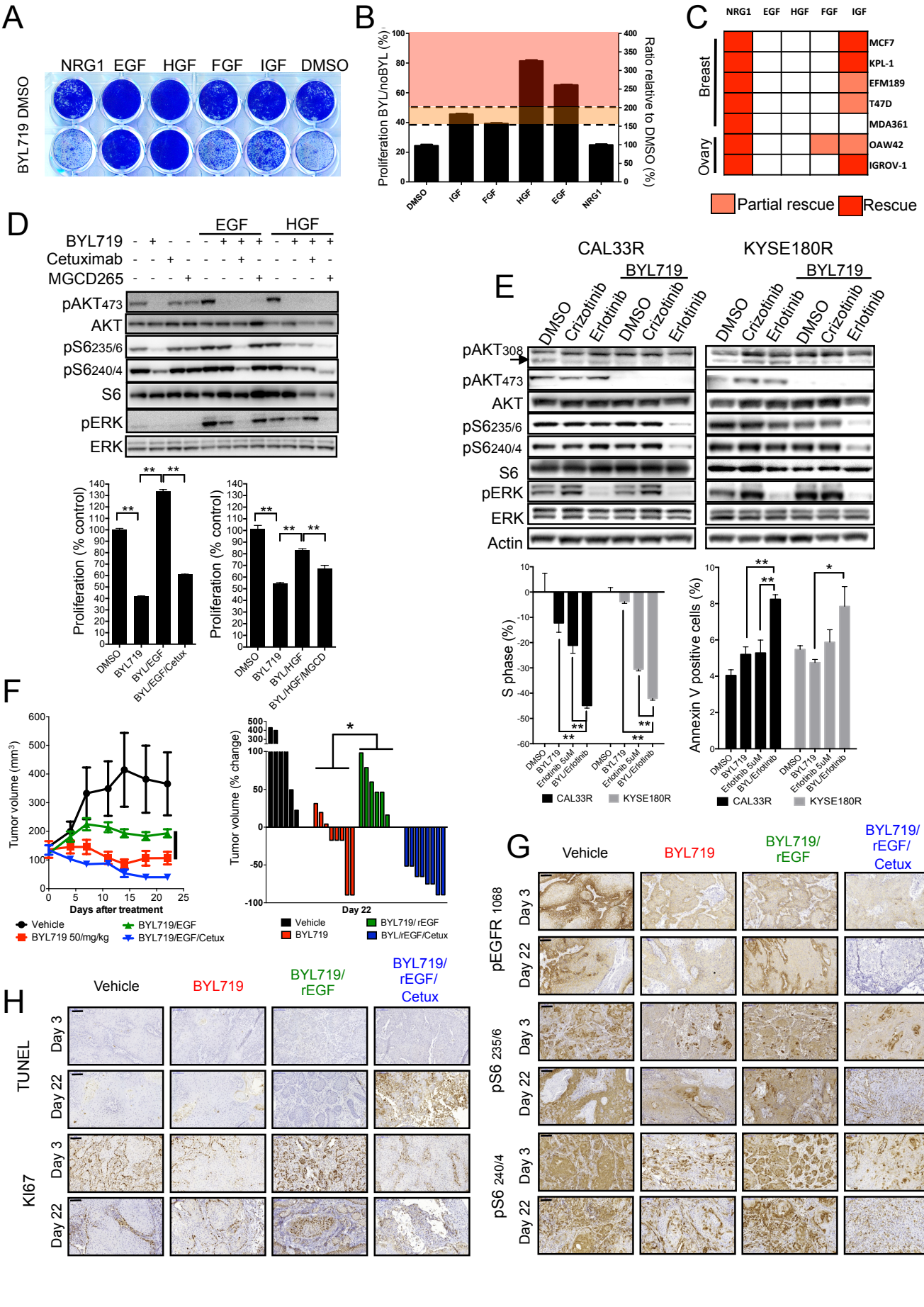

A
NRG1 EGF HGF FGF IGF DMSO
BYL719 DMSO
B
Proliferation BYL/noBYL (%)
Ratio relative to DMSO (%)
DMSO IGF FGF HGF EGF NRG1
C
NRG1 EGF HGF FGF IGF
Breast
Ovary
MCF7
KPL-1
EFM189
T47D
MDA361
OAW42
IGROV-1
Partial rescue
Rescue
D
EGF
HGF
BYL719
Cetuximab
MGCD265
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
ERK
Proliferation (% control)
DMSO
BYL719
BYL/EGF
BYL/EGF/Cetux
BYL/HGF
BYL/HGF/MGCD
E
CAL33R
KYSE180R
BYL719
DMSO
Crizotinib
Erlotinib
pAKT308
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
ERK
Actin
S phase (%)
Annexin V positive cells (%)
Erlotinib 5uM
BYL/Erlotinib
CAL33R
KYSE180R
F
Tumor volume (mm3)
Days after treatment
Vehicle
BYL719 50/mg/kg
BYL719/EGF
BYL719/EGF/Cetux
Tumor volume (% change)
Day 22
Vehicle
BYL719
BYL719/ rEGF
BYL/rEGF/Cetux
G
Vehicle
BYL719
BYL719/ rEGF
BYL719/ rEGF/ Cetux
pEGFR 1068
pS6 235/6
pS6 240/4
Day 3
Day 22
H
Vehicle
BYL719
BYL719/ rEGF
BYL719/ rEGF/ Cetux
TUNEL
KI67
Day 3
Day 22

Figure S2 (related to Figure 2): *Role of EGFR and cMET in limiting the sensitivity to BYL719.* **(A)** Proliferation of cells treated with 1 μM BYL719 for 6 days with or without recombinant IGF1, EGF, FGF, HGF and NRG1. **(B)** Definition of rescue and partial rescue of cells treated as indicated. **(C)** Proliferation of breast and ovary cell lines treated for 6 days with 1 μM BYL719 with or without recombinant ligands. **(D) (top)** Biochemical analysis of LB771-HNC cell line treated for 4 hr with 1 μM BYL719, 10 μg/ml cetuximab and 1 μM MGCD235 in the presence of 50 ng/ml of EGF or HGF as indicated. **(bottom)** Proliferation of LB771-HNC cell line for 6 days treated as indicated with 1 μM BYL719, 10 μg/ml cetuximab (anti-EGFR Ab) and 1 μM MGCD235 (cMET inhibitor) in the presence of 50 ng/ml of EGF or HGF. **(E) (top)** Western blot of resistant cells treated for 4 hr with 5 μM erlotinib or 1 μM crizotinib with and without 1 μM of BYL719. **(bottom)** S-phase arrest and cell death (annexin V) in BYL719-resistant cells after 48 hr of treatment with 1 μM BYL719, 5 μM erlotinib or their combination. Mean of two independent experiments performed in duplicate per cell line. **(F) (left)** Tumor growth of xenografts derived from CAL33 (BYL719-sensitive) cell lines treated with BYL719, in the absence and the presence of EGF (pump released), and neutralization of EGF by cetuximab (n = 6 per arm). **(right)** Relative change in tumor mass compared to baseline. **(G)** Pharmacodynamics of EGFR, AKT and mTOR pathways in tumors. **(H)** IHC for proliferation (KI67) and cell death (TUNEL). Scale bar = 100 μm. Data are presented as means ± SEM. p values were calculated using two-sided Student's t test. **p<0.01

Table S2, related to Figure 2. Secretome screen. Provided as an Excel file.

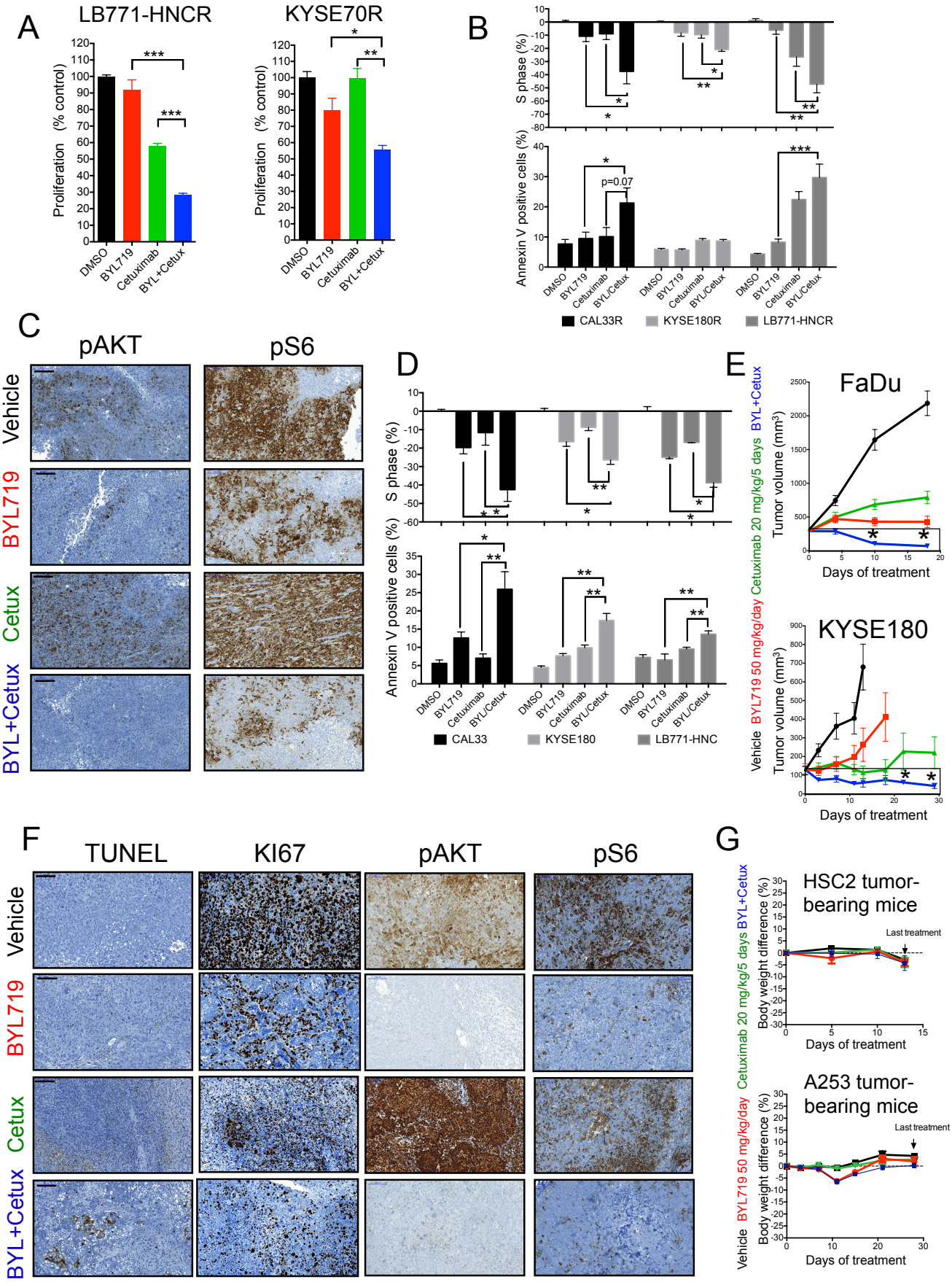
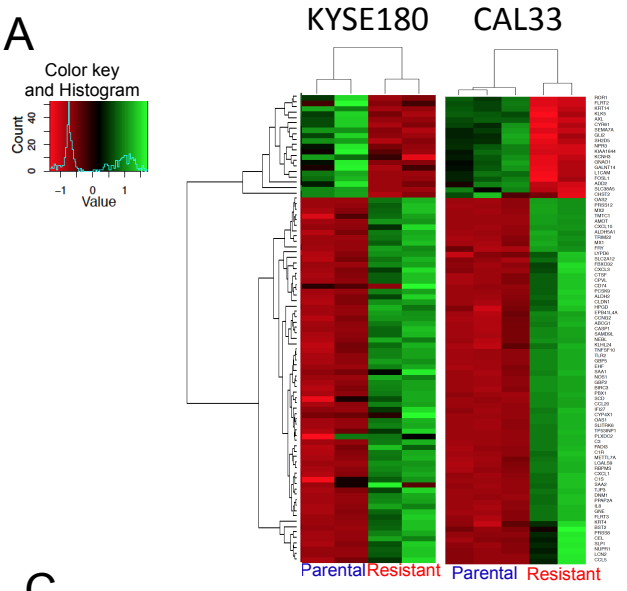

A
LB771-HNCR
KYSE70R
Proliferation (% control)
DMSO
BYL719
Cetuximab
BYL+Cetux
B
S phase (%)
Annexin V positive cells (%)
p=0.07
CAL33R
KYSE180R
LB771-HNCR
C
pAKT
pS6
Vehicle
BYL719
Cetux
BYL+Cetux
D
S phase (%)
Annexin V positive cells (%)
CAL33
KYSE180
LB771-HNC
E
FaDu
KYSE180
Tumor volume (mm³)
Days of treatment
Vehicle
BYL719 50 mg/kg/day
Cetuximab 20 mg/kg/5 days
BYL+Cetux
F
TUNEL
KI67
pAKT
pS6
G
HSC2 tumor-bearing mice
A253 tumor-bearing mice
Body weight difference (%)
Last treatment
Days of treatment

Figure S3 (related to Figure 3): *Efficacy of BYL719 in combination with cetuximab.* **(A)** Proliferation of resistant cells treated with 1 μM BYL719, 5 μg/ml cetuximab or the combination for 6 days. **(B)** S-phase arrest and cell death (annexin V) in BYL719-resistant cells after 48 hr of treatment with 1 μM BYL719, 5 μg cetuximab or their combination. Mean of two independent experiments performed in duplicate per cell line. **(C)** Pharmacodynamics of AKT and mTOR pathways in KYSE180R tumors. **(D)** S-phase arrest and cell death (annexin V) in BYL719-sensitive cells after 48 hr of treatment with 1 μM BYL719, 5 μg cetuximab or their combination. Mean of two independent experiments performed in duplicate per cell line. **(E)** Tumor growth of xenografts derived from FaDu and KYSE180 (BYL719-sensitive) cell lines treated with BYL719, cetuximab or the combination (n = 8-10 per arm). For FaDu significance of combination versus BYL719 is displayed. For KYSE180 significance of combination versus cetuximab is displayed. **(F)** Pharmacodynamics of AKT and mTOR pathways and TUNEL staining in KYSE180 tumors treated as indicated. **(G)** Body weight of mice treated as indicated.
Scale bar = 100 μm Data are shown. Data are presented as means ± SEM. p values were calculated using two-sided Student's t test. *p<0.05, **p<0.01, ***p<0.001

Table S3, related to Figure 3. BYL719+cetuximab efficacy and synergy. Provided as an Excel file.

A



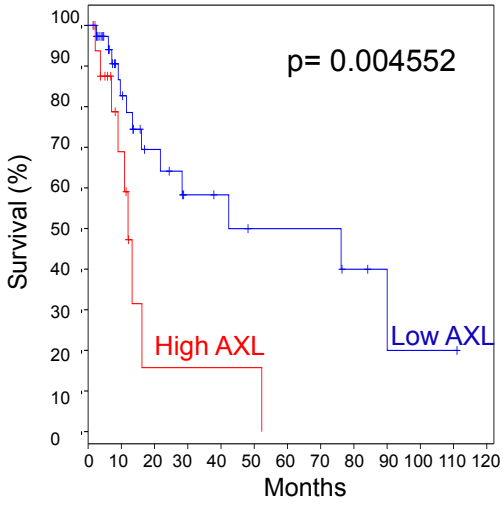
B

Gene	log2Fold Change	padj
AXL	2.687	1.76E-73
DKK1	1.466	6.83E-61
KRT14	2.449	1.61E-45
L1CAM	2.532	2.19E-42
KRT6A	1.246	1.10E-38
KLK5	1.734	9.83E-34
CYR61	1.736	1.63E-27
ROR1	1.904	6.03E-26
SFN	1.389	3.41E-24
FLRT2	1.72	6.60E-24

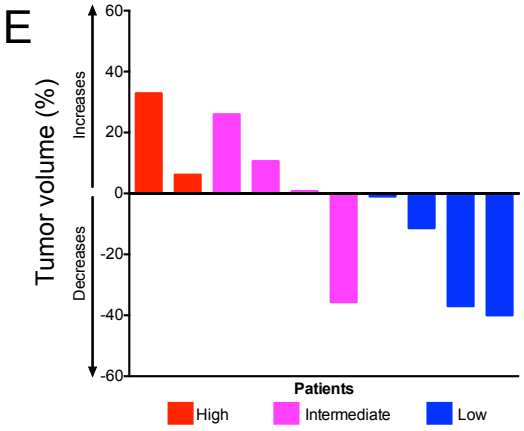
C

Gene	Survival *PIK3CA* mut/amp (p Value)	Survival *PIK3CA* WT (p Value)
AXL	0.00787	0.472
DKK1	0.524	0.353
KRT14	0.486	0.963
L1CAM	0.997	0.366
KRT6A	0.824	0.30
KLK5	0.167	0.166
CYR61	0.310	0.437
ROR1	0.68	0.831
SFN	0.576	0.979
FLRT2	0.954	0.799

D



E



F

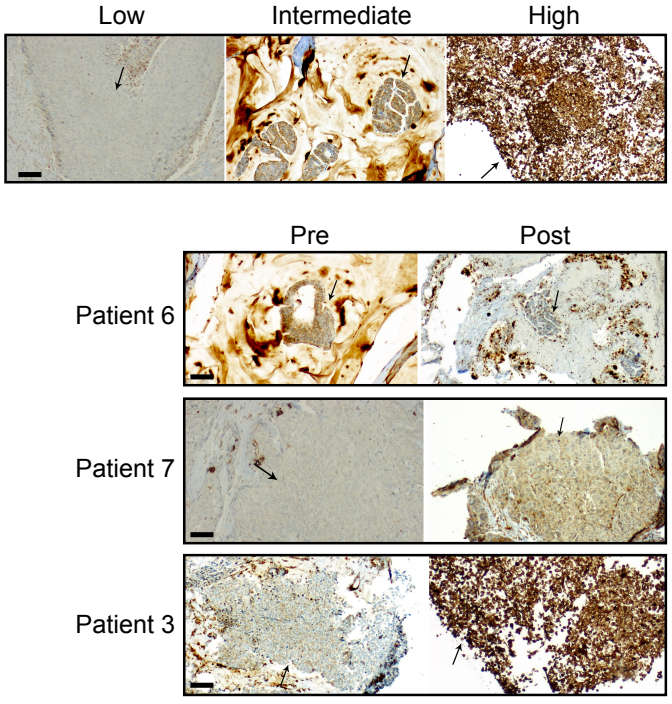


Figure S4 (related to Figure 4): *AXL up-regulated in BYL719 acquired resistant cells.* **(A)** Heat map images showing the genes grouped by the K-means clustering method. Cell lines are indicated above each column. Gene expression is shown in rows. The quantitative changes in gene expression are represented in color: green indicates up-regulation whereas red indicates down-regulation. **(B)** List of up-regulated gene in resistant cells compared to their sensitive counterparts. **(C)** p value for survival for each up-regulated gene in *PIK3CA* altered and *PIK3CA* WT head and neck patients (TCGA) (Z score >0). **(D)** Survival of patients with high AXL protein levels (Z score >0) in *PIK3CA* altered head and neck patients. **(E)** Waterfall plot of the 10 SCC patients treated with BYL719 showing response to therapy based on AXL expression. **(F)** Upper panel: representative images of tumors with low, intermediate and high levels of AXL. Lower panel: AXL levels in pre-treatment samples (pre) and in samples collected at disease progression (post) of three patients treated with BYL719. Arrows indicate tumor cells. Scale bar = 100 μm.

Table S4, related to Figure 4. Gene expression analysis and AXL expression and IC_{50}. Provided as an Excel file.

Table S5, related to Figure 4. Clinical information and AXL expression in SCC patients treated with BYL719.

Patient No.	Site	*PIK3CA*	Treatment	AXL expression (H score)	H score nterpretation	Best response	RECIST (% best response)
Patient 1	H&N	E545K	BYL719 (400 mg)	0	Low	SD	-1.0
Patient 2	H&N	WT	BYL719 (200 mg) MEK162 (45 mg)	0	Low	PR	-40.0
Patient 3	H&N	E545K	BYL719 (180 mg)	20	Low	SD	-11.4
Patient 4	Cervix	E545K	BYL719 400 mg	60	Low	PR	-37.0
Patient 5	H&N	E545K	BYL719 (400 mg)	105	Intermediate	SD	-35.7
Patient 6	H&N	H1047R	BYL719 (270 mg)	120	Intermediate	SD	0.8
Patient 7	H&N	E545K	BYL719 (400 mg)	120	Intermediate	SD	10.6
Patient 8	H&N	E545K	BYL719 (300 mg)	150	Intermediate	PD	26
Patient 9	H&N	E545K	BYL719 (270 mg)	300	High	PD	32.9
Patient 10	H&N	E545K	BYL (400 mg)	300	High	SD	6.2

Best tumor response was defined as maximum tumor volume change compared to pretreatment control and was determined per RECIST criteria. SD=stable disease; PR=partial response; PD=progressive disease. AXL level was measured by H-score.

Table S6, related to Figure 4. AXL expression in SCC patients treated with BYL719 with available paired biopsies.

Patient No.	AXL expression H score	H score interpretation
Patient 3-Pre	20	Low
Patient 3-Post	300	High
Patient 6-Pre	120	Intermediate
Patient 6-Post	120	Intermediate
Patient 7-Pre	120	Intermediate
Patient 7-Post	400	High

Pre: sample before starting BYL719; Post: sample at disease progression

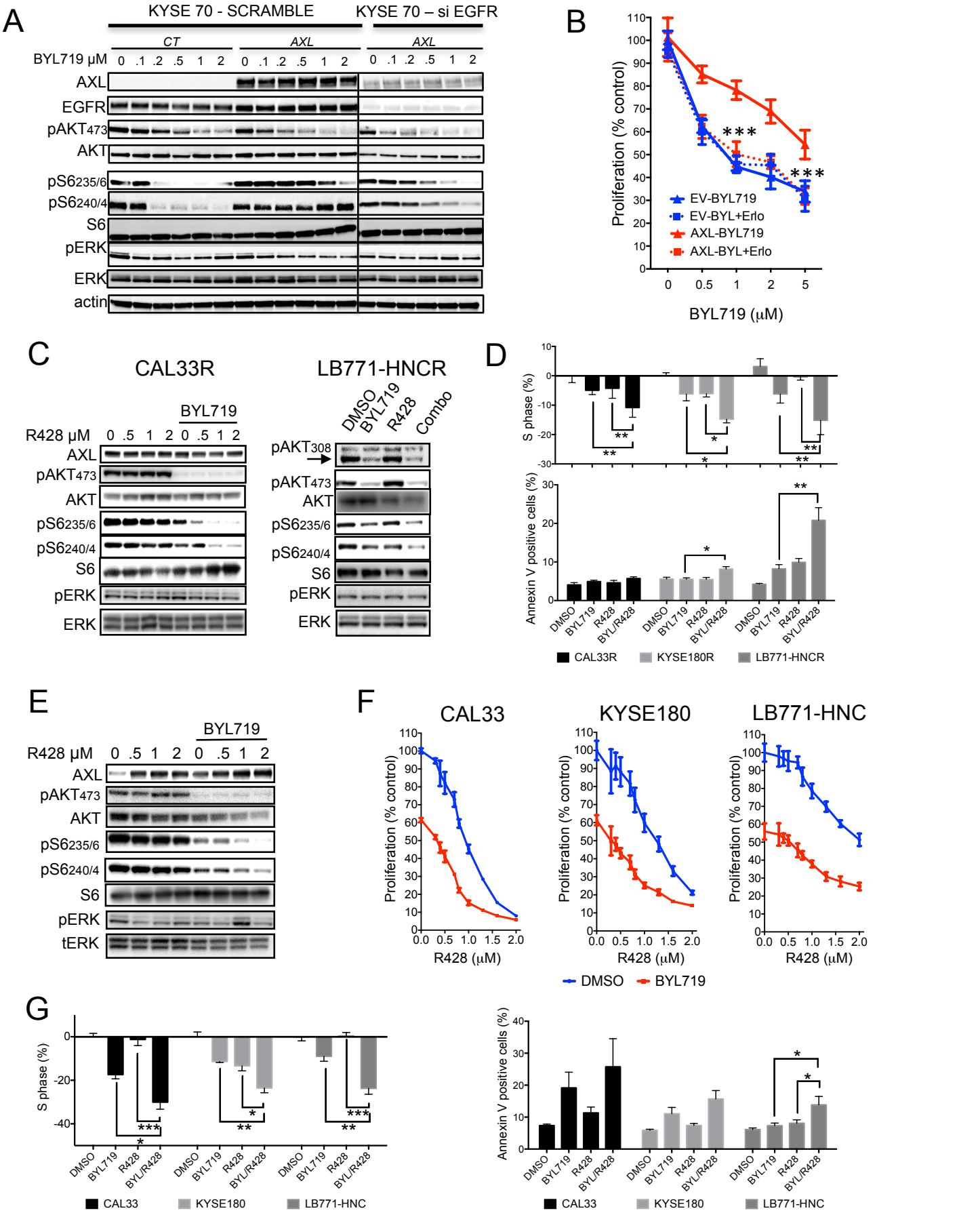

A
KYSE 70 - SCRAMBLE
KYSE 70 – si EGFR
CT
AXL
AXL
BYL719 μM 0 .1 .2 .5 1 2 0 .1 .2 .5 1 2 0 .1 .2 .5 1 2
AXL
EGFR
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
ERK
actin
B
Proliferation (% control)
EV-BYL719
EV-BYL+Erlo
AXL-BYL719
AXL-BYL+Erlo
***
***
BYL719 (μM)
C
CAL33R
LB771-HNCR
BYL719
R428 μM 0 .5 1 2 0 .5 1 2
DMSO
BYL719
R428
Combo
AXL
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
ERK
pAKT308
D
S phase (%)
Annexin V positive cells (%)
DMSO
BYL719
R428
BYL/R428
CAL33R
KYSE180R
LB771-HNCR
E
BYL719
R428 μM 0 .5 1 2 0 .5 1 2
AXL
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
tERK
F
CAL33
KYSE180
LB771-HNC
Proliferation (% control)
R428 (μM)
DMSO
BYL719
G
S phase (%)
Annexin V positive cells (%)
CAL33
KYSE180
LB771-HNC

Figure S5 (related to Figure 5): *AXL inhibition sensitizes cells to BYL719.* **(A)** Western blot with the indicated antibodies of KYSE70 cells overexpressing AXL with or without EGFR knock-down treated with BYL719 at different concentrations. KYSE70-siEGFR was run on a separate gel. **(B)** Cell viability of KYSE70 over expressing cells treated with 1 μM BYL719, 5 μM erlotinib or the combination. **(C)** Western blot with the indicated antibodies of resistant cells treated for 4 hr with an AXL inhibitor (R428, as indicated) with or without 1 μM BYL719. **(D)** S-phase arrest and cell death (annexin V) in BYL719-resistant cells after 48 hr of treatment with 1 μM BYL719, 1 μM R428 or their combination. Mean of two independent experiments performed in duplicate per cell line are shown. **(E)** Western blot with the indicated antibodies of KYSE180 cells treated as indicated for 4 hr. **(F)** Proliferation of BYL719-sensitive cell lines for 4 days treated with R428 with or without 1 μM BYL719. Data are presented as means ± SEM. **(G)** S-phase arrest and cell death (annexin V) in BYL719-sensitive cells after 48 hr of treatment with 1 μM BYL719, 1 μM R428 or their combination. Mean of two independent experiments performed in duplicate per cell line. Data are presented as means ± SEM. p values were calculated using two-sided Student's t test. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

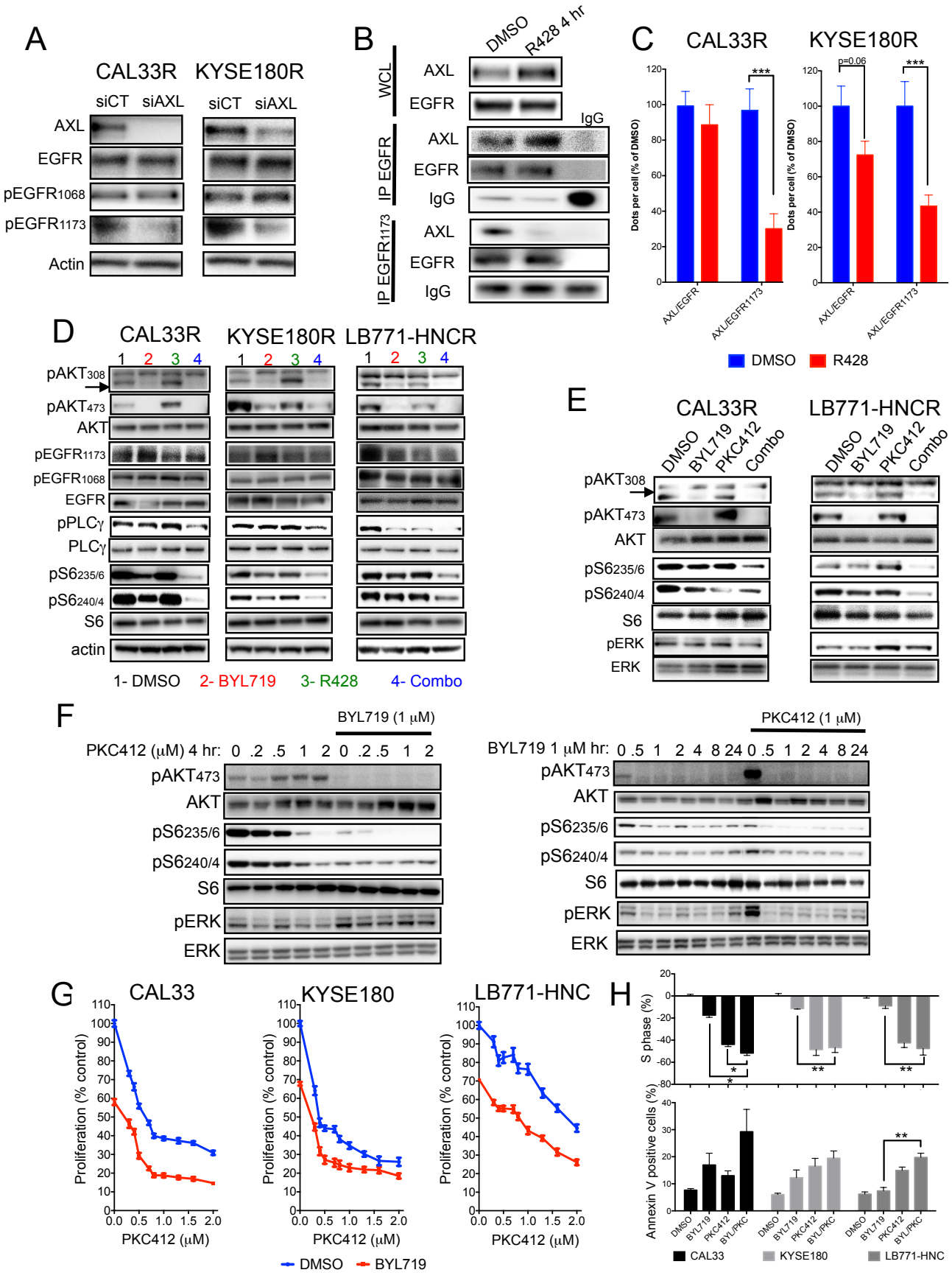

A
CAL33R
KYSE180R
siCT
siAXL
AXL
EGFR
pEGFR1068
pEGFR1173
Actin
B
DMSO
R428 4 hr
WCL
IP EGFR
IP EGFR1173
IgG
C
CAL33R
KYSE180R
p=0.06
***
Dots per cell (% of DMSO)
AXL/EGFR
AXL/EGFR1173
DMSO
R428
D
CAL33R
KYSE180R
LB771-HNCR
pAKT308
pAKT473
AKT
pEGFR1173
pEGFR1068
EGFR
pPLCγ
PLCγ
pS6235/6
pS6240/4
S6
actin
1- DMSO
2- BYL719
3- R428
4- Combo
E
CAL33R
LB771-HNCR
DMSO
BYL719
PKC412
Combo
pAKT308
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
ERK
F
BYL719 (1 μM)
PKC412 (μM) 4 hr: 0 .2 .5 1 2 0 .2 .5 1 2
PKC412 (1 μM)
BYL719 1 μM hr: 0 .5 1 2 4 8 24 0 .5 1 2 4 8 24
pAKT473
AKT
pS6235/6
pS6240/4
S6
pERK
ERK
G
CAL33
KYSE180
LB771-HNC
Proliferation (% control)
PKC412 (μM)
DMSO
BYL719
H
S phase (%)
Annexin V positive cells (%)
DMSO
BYL719
PKC412
BYL/PKC
CAL33
KYSE180
LB771-HNC

Figure S6 (related to Figure 6): *AXL/EGFR interaction activates the PLCγ-PKC pathway to sustain mTOR activity.* **(A)** Western blot with the indicated antibodies in resistant cells with or without AXL knock-down. **(B)** Immuno-precipitation of EGFR1173 and EGFR in KYSE180R treated with 1 μM of R428 **(C)** In-situ proximity assay (PLA) with AXL and EGFR or EGFR1173 on resistant cells treated with 1 μM of R428 for 4 hr. Quantification of AXL/EGFR complex was carried out using Image J. **(D)** Western blot with the indicated antibodies of resistant cells treated for 4 hr with 1 μM R428, 1 μM BYL719 or the combination. **(E)** Western blot with the indicated antibodies of resistant cells treated with 1 μM BYL719, 1 μM PKC412 or combination for 4 hr. **(F)** Western blot with the indicated antibodies of KYSE180 cells treated with 1 μM BYL719, PKC412 or combination as indicated. **(G)** Proliferation of sensitive cell line for 4 days treated with PKC412 with or without 1 μM BYL719. **(H)** S-phase arrest and cell death (annexin V) in BYL719-sensitive cells after 48 hr of treatment with 1 μM BYL719, 1 μM R428 or their combination. Mean of two independent experiments performed in duplicate per cell line.
Data are presented as means ± SEM. p values were calculated using two-sided Student's t test. **p<0.01.

Supplemental Experimental Procedures

Determination of IC_{50}

To calculate IC_{50}, 5 x 10^5 cells were seeded in 96-well plates and treated with escalating concentration of BYL719. After 5 days of treatment, cell proliferation was analyzed with CellTiter-Glo Luminescent Cell Viability Assay (Promega) as described by the manufacturer. IC_{50} values were calculated with GraphPad Prism (GraphPad Software).

RNAi knockdown

Knockdown of AXL was performed using specific pooled siRNAs purchased from Origene. Si-control non-target served as negative controls. siRNA was introduced to cells with Lipofectamin 2000 reagent according to the manufacturer's instructions.

Whole transcriptome sequencing (RNAseq) on Proton.

Cells were treated with 1 μM of BYL719 or DMSO for 24 hr. RNA was extracted by Rneasy mini kit (Qiagen). After ribogreen quantification and quality control of Agilent BioAnalyzer(RIN>7), poly(A) RNA was isolated using Dynabeads® mRNA DIRECT™ Micro Kit(Life Technologies)from 1 μg of total RNA. mRNA was then fragmented using RNaseIII and purified. The fragmented samples quality and yield were evaluated using Agilent BioAnalyzer. Subsequently, the fragmented material underwent whole transcriptome library preparation according to the Ion Total RNA-Seq Kit v2 protocol (Life Technologies), with 16 cycles of PCR. Samples were barcoded and template-positive Ion PI™ Ion Sphere™ Particles (ISPs) were prepared using the ion one touch system II and Ion PI™Template OT2 200kit v2 Kit (Life Technologies). Enriched particles were sequenced on a Proton sequencing system using 200bp version 2 chemistry. Averages of 70 to 80 million reads per sample were generated.

Gene expression analysis

We performed differential expression analysis looking for genes that varied significantly between parental and resistant cell lines: CAL33 and KYSE180. The analysis was done jointly resulting in genes that largely agreed on fold change in both cell types.

For gene expression quantifications, first we converted the BAM files to FASTQ format. Reads were then quality trimmed using the fastx toolkit to remove bases that had a base quality score less than 10 and then reads shorter than 50 bp were discarded. We did a two pass mapping procedure that first mapped with RNAStar (Dobin et al., 2013) that maps reads genomically and resolves reads across splice junctions. Any reads that did not map in this pass were re-mapped using BWA MEM method. We merged the output BAM files from these two steps and then counted gene level expression with htseq-count (-s yes -m intersection-strict) and exon level expression was quantitated with DEXSeq. For the analysis we considered only coding genes.

After quantification of gene expression we assessed replicate quality in the different cell types and conditions and removed replicates that showed considerable amount of variation in gene expression ('CAL33_2', 'KYSE180B_1', 'KYSE180_1').

For the differential analysis we used the DESeq2 package in R. DESeq2 performs differential analysis using raw read counts, under a Negative Binomial noise model. Our linear model explained gene expression by cell-line type and whether it is parental or resistant off-drug. For every gene G we fitted the model $G \sim \beta_0 + \beta_{parental} + \beta_{cell-line}$ and tested whether $\beta_{parental}$ was statistically significant, i.e. if there was a statistically significant change between parental and resistant cell lines. Model fitting was followed by FDR (BH) correction for multiple hypothesis testing.

Our list of up-regulated genes was determined by requiring logFoldChange > 1, baseMean > 200 (parental expression level), p.adj < 0.01, similarly for the down-regulated genes we required logFoldChange < -1, baseMean > 200, p.adj < 0.01.

The clustering plot was done using the standard heatmap.2 function in the gplots package in R. Expression is log transformed and each row was normalized to zero mean and 1 standard deviation.

Cell viability/proliferation

For cell viability analyses, $5X10^5$ or $2X10^6$ cells were seeded in 96 or 24 well plates, respectively, and treated for 3-11 days. Cells were fixed and stained with Crystal Violet. For ligand-induced proliferation experiments, 5×10^5 cells were seeded in 24 well plates treated with BYL719 (1 μM) in the present of 50 ng/ml IGF1, 0.8 ng/ml NRG1, 50 ng/ml HGF, 50 ng/ml EGF and 10 ng/ml FGF for 6 days and then stained with Crystal Violet. The percentage of rescue was normalized for the effect of growth factors on tumor cells proliferation. Ligand-induced rescue from BYL719 inhibition above 50% was detected as positive.

Immunohistochemistry (IHC)

For xenografts samples dissected tissues were fixed immediately after removal in a 10% buffered formalin solution for a maximum of 24h at room temperature before being dehydrated and paraffin embedded under vacuum. The tissue sections were deparaffinized with EZPrep buffer (Ventana Medical Systems), antigen retrieval was performed with CC1 buffer (Ventana Medical Systems) and sections were blocked for 30 minutes with Background Buster solution (Innovex). Anti-Ki67 (Vector, cat# VP-K451, 0.4 μg/ml), anti-pS6 (Cell Signaling, cat# 2211L, 0.12 μg/ml), Anti-AXL (Cell Signaling, cat# 8661, 1 μg/ml), Anti-pEGFR1068 (Cell signaling, Cat#2234 0.1 μg/ml) and anti-pAkt (Ser473) (Cell Signaling, cat#4060, 1 μg/ml) antibodies were applied and sections were incubated for 5 hr, followed by 60 minutes incubation with biotinylated goat anti-rabbit IgG (Vector labs, cat#PK6101) at 1:200 dilution. For pAkt antibodies TSA

amplification was performed by incubation with of Srteptavidin-HRP (Ventana) for 12 min followed by incubation with TSA (PerkinElmer, cat# FP1019, 1:100 in amplification buffer) for 16 min. The detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific).

For TUNEL assay, slides were manually deparaffinized in xylene, re-hydrated in series of alcohol dilutions (100%, 95% and 70%) and tap water, placed in Discovery XT (Ventana Medical Systems) autostainer, treated with Proteinase K (20 µg/ml in PBS) for 12 minutes and then incubated with TdT-biotin-dUTP labeling mix (Roche) for 1 hr. The detection was performed with DAB detection kit (Ventana Medical Systems) according to manufacturer instruction. Slides were counterstained with hematoxylin and coverslipped with Permount (Fisher Scientific). For each IHC random areas from 2 individual stained tumors were captured.

For patient samples, four-micron sections were cut from whole tumor sections of the tumors and staining was performed using an automated staining platform (Benchmark ULTRA; Ventana Medical Systems, Inc. (Ventana)). Antigen retrieval for AXL was carried out with Cell Conditioning 1 for 72 minutes at 95ºC and pre-diluted AXL antibody (NBP1-83073; 1:200) was manually dispensed, and the slides were incubated at 37ºC for 12 minutes. Ventana OptiView DAB kit was used for detection and sections were counterstained with hematoxylin for 4 minutes and post-counterstained with bluing reagent for 4 minutes. In the negative control, the primary antibody was omitted. Human testis was used as positive control. All specimens were scored as previously described (Linger et al., 2013). Briefly, The H-Score was calculated by multiplying the percentage of positive tumor cells (0% to 100%) by the dominant staining intensity (scale 0 to 4). Thus, the possible range of H-Scores was 0 to 400. AXL expression was considered low

when the H-score was 100 or below; high when H-score was 300 or above and intermediate when H-score was between these values.

shRNA screen

The set of shRNA data across all of the reported studies is pre-processed by scaled followed by quantile normalization. Within each study, the shRNA counts data in both treatment and control arms are scale normalized to sum to 5 x 10^6. Quantile normalization is then applied across all arms of all reported studies. Normalized counts are log2-transformed. A fold-change is determined for each shRNA per study by subtracting the log2 count in the control arm from the treatment arm.

A confidence value is calculated for each shRNA based on its percent knockdown of the gene target relative to its peers. In particular, for each gene K, let $\{k_1, k_2, \ldots, k_m\}$ be the set of percent knockdowns for the shRNAs targeting K and let k_{max} denote the maximum percent knockdown in this set. Then the weights for the shRNAs of gene K is given by $\{w_1, w_2, \ldots, w_m\} = \{\frac{k_1}{k_{max}}, \frac{k_2}{k_{max}}, \ldots, \frac{k_m}{k_{max}}\}$. In essence, the weight of a shRNA corresponds to its percent knockdown of the target gene, normalized by the maximum percent knockdown for that target gene by any of its shRNAs.

For each gene, a score is calculated based the following formula:

$$score(gene) = \frac{\sum w_{a_i} \log(FC_{a_i})}{\sum w_{a_i}} - \frac{\sum w_{b_j} \log(FC_{b_j})}{\sum w_{b_j}}$$

where $A = \{a_1, a_2, \ldots, a_m\}$ and $B = \{b_1, b_2, \ldots, b_n\}$ are the indices of shRNAs targeting the gene and its complement, respectively, in the pool and FC_x and w_x denote the fold-change (compound vs. DMSO) and confidence weight of the x^{th} shRNA, as previously described.

In essence, the score for a gene is the difference in the (weighted) log fold-change of shRNAs targeting that gene vs. all other genes. It therefore measures the degree to which targeting a specific gene differentially sensitizes or rescues the cell line to compound treatment against the average.

A permutation test is used to estimate the significance of the gene-level scores. Namely, the mapping of shRNAs to their gene targets is randomly permuted 10,000 times and in each permutation, a score is calculated per gene as previously described. For any particular gene, the distribution of its 10,000 permuted scores approximates a normal distribution. Consequently, a p value can be determined from a two-sided comparison of the actual gene score against the fitted normal distribution derived from the permuted gene scores. The p values of all genes are also corrected for multiple hypotheses testing using the false discovery rate (FDR) method of Benjamini-Hochberg. Genes with an FDR $\leq$ 0.25 are considered to be significantly sensitizing or rescuing the cell line from the compound when compared to the average gene screened in the shRNA pool. We have considered only genes for which at least two hairpins have been detected.

AXL cDNA and overexpression studies

Cells were transfected with the *AXL*-expressing vectors (WT or KD-p.Lys567Arg) or with empty vector as a control using FuGENE HD reagent according to the manufacturer's protocol (Roche Applied Sciences) as previously described (Zhang et al., 2012). We generated stable KYSE180, CAL33 and KYSE70 subclones that were resistant to 500 µg/ml G418 selection. Cells transfected with empty vector were generated as a control.

Implantation of Alzet Miniosmotic Pumps

Six-week-old female athymic NU/NU nude mice purchased from Charles River were injected with $1X10^7$ CAL33 subcutaneously in a volume of 100 µL culture media/Matrigel

(BD Biosciences) 1:5. Animals were randomized to 4 groups, with n=8-10 tumors per group. Animals were orally treated daily with BYL719 (50 mg/kg in 0.5% carboxymethylcellulose sodium salt (CMC) (Sigma) or cetuximab (10 mg/kg/5days) internal peritoneum.

Alzet pumps (Model 1004, 100 µL, 0.11 µL/hr; Alzet Osmostic Pumps) were filled with 100 µl with saline 0.9% (Vehicle) or 6.72 mg of rhEGF (Peprotech). The rate of EGF administration was calculated for 1ng/ml EGF in the plasma (Robinson et al., 1992) 10ng/h=9.6 µg/kg/day. The pumps were incubated in 0.9% saline at 37°C 2h before subcutaneously transplantation on the back of the neck. Mice bearing CAL33 tumors implanted with ALZET pumps containing either vehicle or rEGF. 26 days after implantation (22 days of treatment) tumors were harvested and analyzed.

Tumor xenografts were measured with digital caliper, and tumor volumes were determined with the formula: (length × width^2) × (π/6). At the end of the experiment, animals were euthanized using CO_2 inhalation. Tumor volumes are plotted as means ± SEM.

Mice were maintained and treated in accordance with Institutional Guidelines of Memorial Sloan Kettering Cancer Center. Mice were housed in air-filtered laminar flow cabinets with a 12-hr light cycle and food and water *ad libitum*.

In situ Proximity ligation assay (PLA)

PLA (Duolink in situ PLA™; Olink Bioscience, Uppsala, Sweden) was performed to detect EGFR and AXL as indicated in Table S5. To visualize the bound antibody pairs, the Duolink Detection kit (#DUO92101 -Sigma) with PLA plus and minus probes for mouse and rabbit (Olink Bioscience) was used, according to the manufacturer's description. Specimens were mounted with the Duolink Brightfield Mounting Medium

(Olink Bioscience). Analysis was performed by FIJI and number of dots was normalized to nuclei. 8-15 random fields have been analyzed.

Determination of cell cycle and apoptosis

Cell cycle was quantified by flow cytometry. Briefly, cells were washed with phosphate-buffered saline (PBS), fixed in cold 70% ethanol, and then stained with propidium iodide (PI) while being treated with ribonuclease (Sigma-Aldrich).

Cell death was measured by annexin V–FITC (fluorescein isothiocyanate)/PI staining and evaluated by flow cytometry according to the manufacturer's protocol (BD Pharmingen). Cells (2 × 105) were washed twice with PBS and stained with 5 µl of annexin V–FITC and 10 µl of PI (5 µg/ml) in 1× binding buffer (BD) for 15 min at room temperature in the dark. The apoptotic cells were detected with an LSR flow cytometer. Both apoptotic (annexin V–positive, PI-negative) and necrotic (annexin V–positive and PI-positive) cells were included in cell death determinations. Quantitative analyses were carried out by FlowJo software.

Cell lines

Cell line	Source	Medium	Medium Cat. Number	Supplements
A-253	ATCC	McCoy's 5A	ATCC 30-2007	
BHY	DSMZ	DMEM	ATCC 30-2002	
BICR 16	ECACC	DMEM	ATCC 30-2002	0.4 µg/ml Hydrocortisone
BICR 18	ECACC	DMEM	ATCC 30-2002	0.4 µg/ml Hydrocortisone
BICR 22	ECACC	DMEM	ATCC 30-2002	0.4 µg/ml Hydrocortisone
BICR 31	ECACC	DMEM	ATCC 30-2002	0.4 µg/ml Hydrocortisone
BICR 56	ECACC	DMEM	ATCC 30-2002	0.4 µg/ml Hydrocortisone

BICR 6	ECACC	DMEM	ATCC 30-2002	0.4 µg/ml Hydrocortisone
CAL 27	ATCC	DMEM	ATCC 30-2002	
CAL-33	DSMZ	DMEM	ATCC 30-2002	
Detroit 562	ATCC	EMEM	ATCC 30-2003	
FaDu	ATCC	EMEM	ATCC 30-2003	
Hs 840.T	ATCC	DMEM	Invitrogen 11995-065	
HSC-2	HSRRB	EMEM	ATCC 30-2003	
HSC-3	HSRRB	EMEM	ATCC 30-2003	
HSC-4	HSRRB	EMEM	ATCC 30-2003	
LB771-HNC	MGH	DMEM:HAM's F12 1:1	ATCC 30-2006	
PE/CA-PJ15	ECACC	IMDM	ATCC 30_2005	
PE/CA-PJ34 (clone C12)	ECACC	IMDM	ATCC 30_2005	
PE/CA-PJ41 (clone D2)	ECACC	IMDM	ATCC 30_2005	
PE/CA-PJ49	ECACC	IMDM	ATCC 30_2005	
SCC-15	ATCC	DMEM:HAM's F12 1:1	ATCC 30-2006	0.4 µg/ml Hydrocortisone
SCC-25	ATCC	DMEM:HAM's F12 1:1	ATCC 30-2006	0.4 µg/ml Hydrocortisone
SCC-4	ATCC	DMEM:HAM's F12 1:1	ATCC 30-2006	0.4 µg/ml Hydrocortisone
SCC-9	ATCC	DMEM:HAM's F12 1:1	ATCC 30-2006	0.4 µg/ml Hydrocortisone
SNU-1041	KCLB	RPMI 1640	Invitrogen 11875-093	
SNU-1066	KCLB	RPMI 1640	Invitrogen 11875-093	
SNU-1076	KCLB	RPMI 1640	Invitrogen 11875-093	
SNU-1214	KCLB	RPMI 1640	Invitrogen 11875-093	
SNU-46	KCLB	RPMI 1640	Invitrogen 11875-093	
SNU-899	KCLB	RPMI 1640	Invitrogen 11875-093	
YD-10B	KCLB	RPMI 1640	Invitrogen 11875-093	
YD-15	KCLB	RPMI 1640	Invitrogen 11875-093	

YD-38	KCLB	RPMI 1640	Invitrogen 11875-093	
YD-8	KCLB	RPMI 1640	Invitrogen 11875-093	
COLO-680N	DSMZ	RPMI 1640	ATCC 30-2001	
EC-GI-10	RIKEN	RPMI 1640	ATCC 30-2001	
KYSE-140	DSMZ	HamsF-12K	Invitrogen11765-054	
KYSE-150	DSMZ	RPMI:HamsF-12K(1:1)	Invitrogen 11875-093:11765-054	
KYSE-180	DSMZ	RPMI 1640	Invitrogen 11875-093	
KYSE-270	DSMZ	RPMI:HamsF-12K(1:1)	Invitrogen 11875-093:11765-054	
KYSE-30	DSMZ	RPMI:HamsF-12K(1:1)	Invitrogen 11875-093:11765-054	
KYSE-410	DSMZ	RPMI-1640	ATCC 30-2001	
KYSE-450	DSMZ	RPMI:HamsF-12K(1:1)	Invitrogen 11875-093:11765-054	
KYSE-510	DSMZ	RPMI 1640	ATCC 30-2001	
KYSE-520	DSMZ	RPMI 1640	ATCC 30-2001	
KYSE-70	DSMZ	RPMI 1640	ATCC 30-2001	
OE19	ECACC	RPMI 1640	ATCC 30-2001	
OE21	ECACC	RPMI 1640	Invitrogen 11875-093	
OE33	ECACC	RPMI 1640	ATCC 30-2001	
TE-1	RIKEN	RPMI 1640	Invitrogen 11875-093	
TE-10	RIKEN	RPMI 1640	Invitrogen 11875-093	
TE-11	RIKEN	RPMI 1640	Invitrogen 11875-093	
TE-14	RIKEN	RPMI 1640	ATCC 30-2001	
TE-15	RIKEN	RPMI 1640	ATCC 30-2001	
TE-4	RIKEN	RPMI 1640	Invitrogen 11875-093	
TE-5	RIKEN	RPMI 1640	Invitrogen 11875-093	
TE-6	RIKEN	RPMI 1640	ATCC 30-2001	
TE-8	RIKEN	RPMI 1640	ATCC 30-2001	
TE-9	RIKEN	RPMI 1640	ATCC 30-2001	
TT	ATCC	F-12K	ATCC 30-2004	

Antibodies

Antibody	Company	Catalog number	Dilution	Assay
pAKT (473)	Cell Signaling	4060	1:1000	WB
pAKT (308	Cell Signaling	9275	1:1000	WB
AKT	Cell Signaling	9272S	1:1000	WB
pS6 (240/4)	Cell Signaling	5364	1:1000	WB
pS6 (235/6)	Cell Signaling	4857	1:1000	WB
S6	Cell Signaling	2217	1:1000	WB
phospho-PRAS40 (T246)	Cell Signaling	2997S	1:1000	WB
PRAS40	Cell Signaling	1961	1:1000	WB
pERK	Cell Signaling	4377	1:1000	WB
ERK	Cell Signaling	9742	1:1000	WB
Beta-Actin	Cell Signaling	4970	1:10000	WB
AXL	Cell Signaling	8661	1:1000, 1:300	WB, IHC
EGFR	Cell Signaling	2646	1:1000, 1:100	WB, IP
pEGFR (1173)	Cell Signaling	4407	1:1000, 1:50	WB, IP
pEGFR (1068)	Cell Signaling	2236	1:1000	WB
pPKCa		9375	1:1000	WB
pPKCz	Cell Signaling	2060	1:1000	WB
p-PLCg	Cell Signaling	2821	1:1000	WB

Protein A Magnetic Beads	Cell Signaling	8687		IP
Rabbit IgG Isotype Control (Magnetic Bead Conjugate)	Cell Signaling	8726		IP

Drugs

Drug	**Company**	**Catalog number**
R428 (BGB324)	Selleck	S2841
Cetuximab		
Go6967	Santa Cruz	sc-221684
PKC-412	Santa Cruz	sc-200691
BIM1	Santa Cruz	sc-24003
Rottlerin	Santa Cruz	sc-3350
BIM8	Santa Cruz	sc-24005
Enzastautin	Selleck	s1055
Gö 6983	Santa Cruz	sc-203432
RAD001	Selleck	S1120
AZD8055	Selleck	S1555
Erlotinib	Selleck	S1023
MGCD265	Selleck	S1361

Growth factors

Recombinant Proteins	**Company**	**Catalog Number**
EGF	Peprotech	AF100-15
HGF	Peprotech	100-39
FGF	Peprotech	100-18B
IGF	Peprotech	100-11
NRG1	Peprotech	100-03

Supplemental References

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