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Supplementary Materials for

mTORC1 Inhibition Is Required for Sensitivity to PI3K p110α Inhibitors in *PIK3CA*-Mutant Breast Cancer

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Other Supplementary Material for this manuscript includes the following:
(available at www.sciencetranslationalmedicine.org/cgi/content/full/5/196/196ra99/DC1)

Table S3. Original data (provided as a separate Excel file).

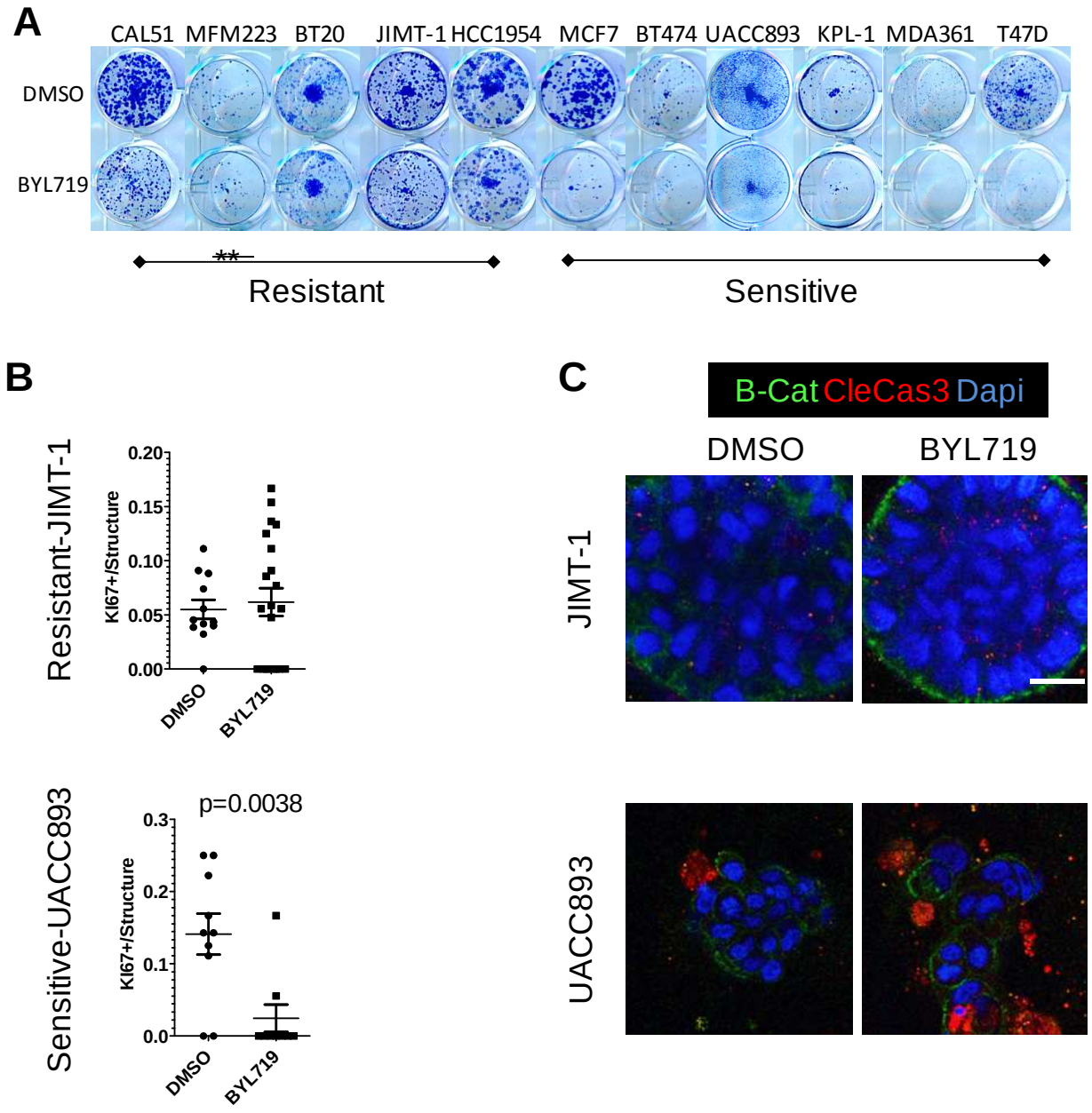

Fig. S1: Cell viability after BYL719 treatment. (A) Crystal Violet staining of breast cancer cells treated with 1 µM BYL719 for 8-9 days. **(B)** Quantification of Ki67 staining per 3D structure of the BYL719 resistant JIMT-1 and the BYL719 sensitive UACC893 spheroids after 48 hours treatment with 1 µM BYL719. **(C)** Representative cleaved caspase 3 (CleCas3, apoptosis, red), beta-catenin (B-cat, cell membranes, green), and nuclei (Dapi, blue) staining of the same spheroids after 48 hours of treatment with 1 µM BYL719. scale bar = 10 µm Data are shown as mean ± SEM. p-value was calculated using two-sided student's t-test.

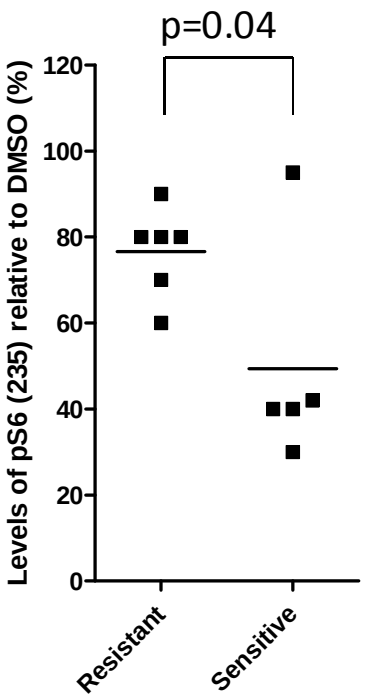

Fig. S2: Inhibition of pS6(235/6) in sensitive and resistant cells. Differences in pS6 (235/6) inhibition between BYL719 resistant and BYL719 sensitive cell lines (total of 11 PIK3CA-mut cell lines) were measured by quantitative immunofluorescence after 2 hours of BYL719 treatment. Mean values are indicated by horizontal bars. p-value was calculated using two-sided student's t-test.

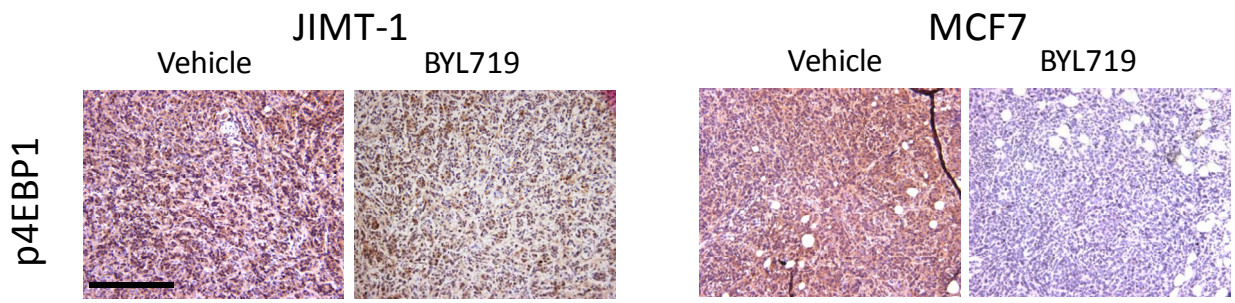

Fig. S3: Immunostaining of p4EBP1 in JIMT-1 and MCF7 xenografts treated with BYL719. IHC staining for p4EBP1 in JIMT-1 and MCF7 xenografts treated with vehicle or 50 mg/kg BYL719 daily. Images were captured at 20X magnification; scale bar = 200 µm.

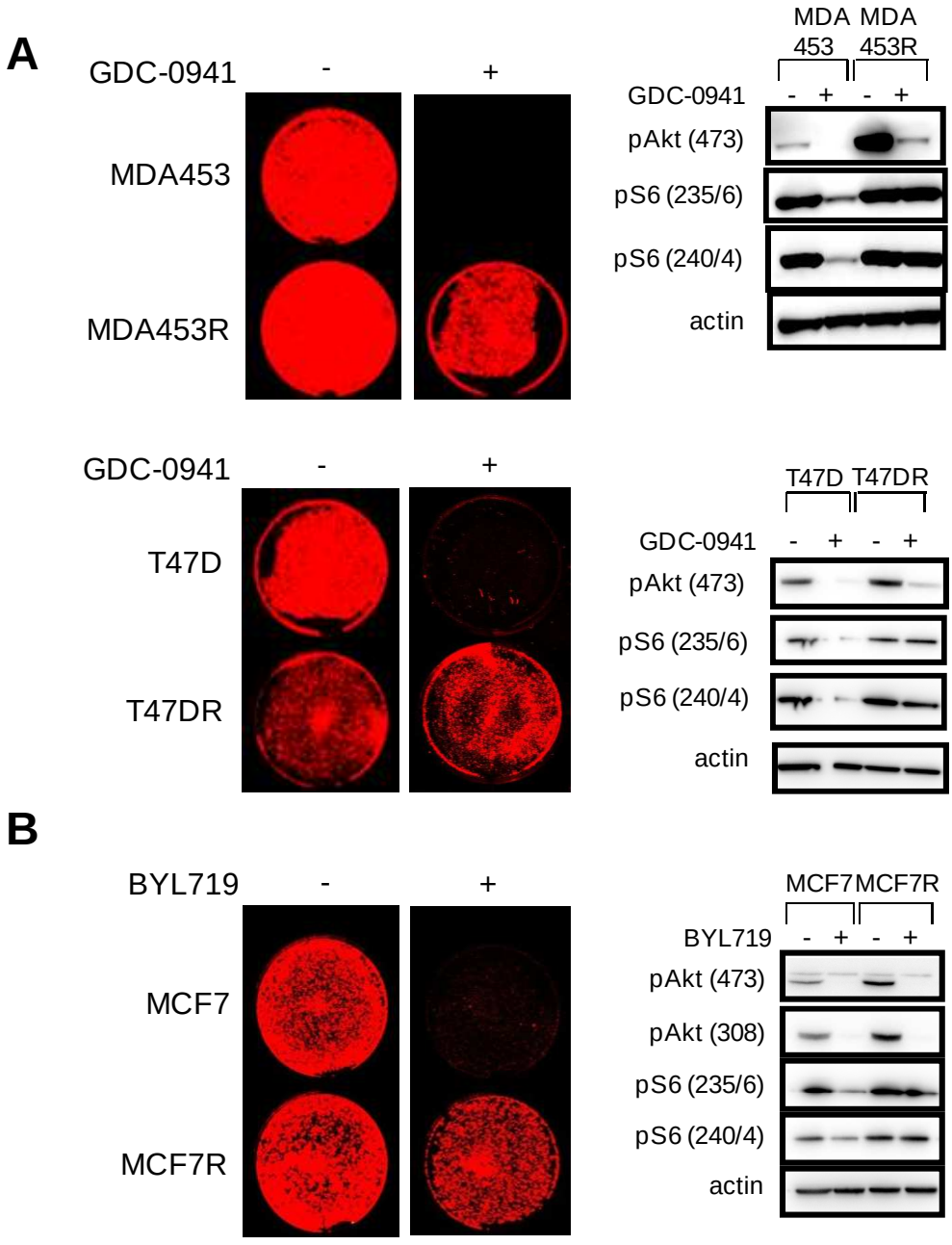

Fig. S4: PI3K/Akt/mTOR pathway inhibition in drug-resistant cells treated with BYL719. **(A)** Sensitivity to GDC-0941 in parental MDA453 and T47D and BYL719-resistant MDA453R and T47DR cells. Protein lysates were isolated after 24 hours of treatment with 1 µM GDC-0941 and probed against the indicated proteins. **(B)** Sensitivity to BYL719 in parental MCF7 and drug-resistant MCF7R cells. Protein lysates were isolated after 24 hours of treatment with 1 µM BYL719 and probed with the indicated antibodies.

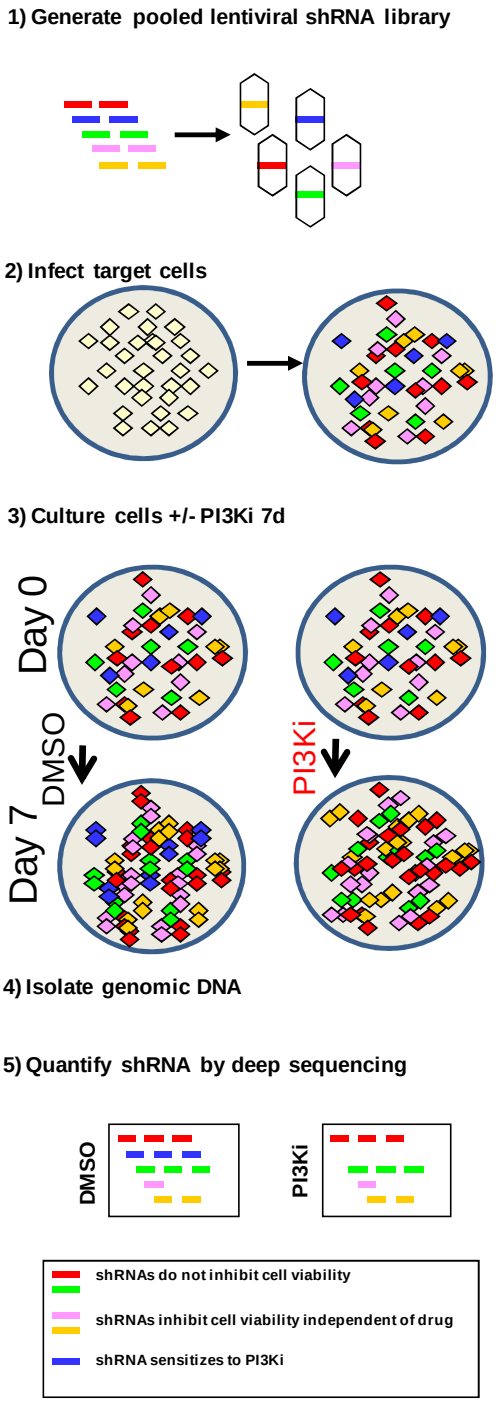

Fig. S5: Scheme of the shRNA screen to identify candidate genes for resistance to PI3K inhibition. Cells were infected with ~600 shRNA then selected by puromycin. Selected cells were re-cultured in the absence or presence of a PI3K inhibitor. After 7 days, genomic DNA was extracted and deep sequenced.

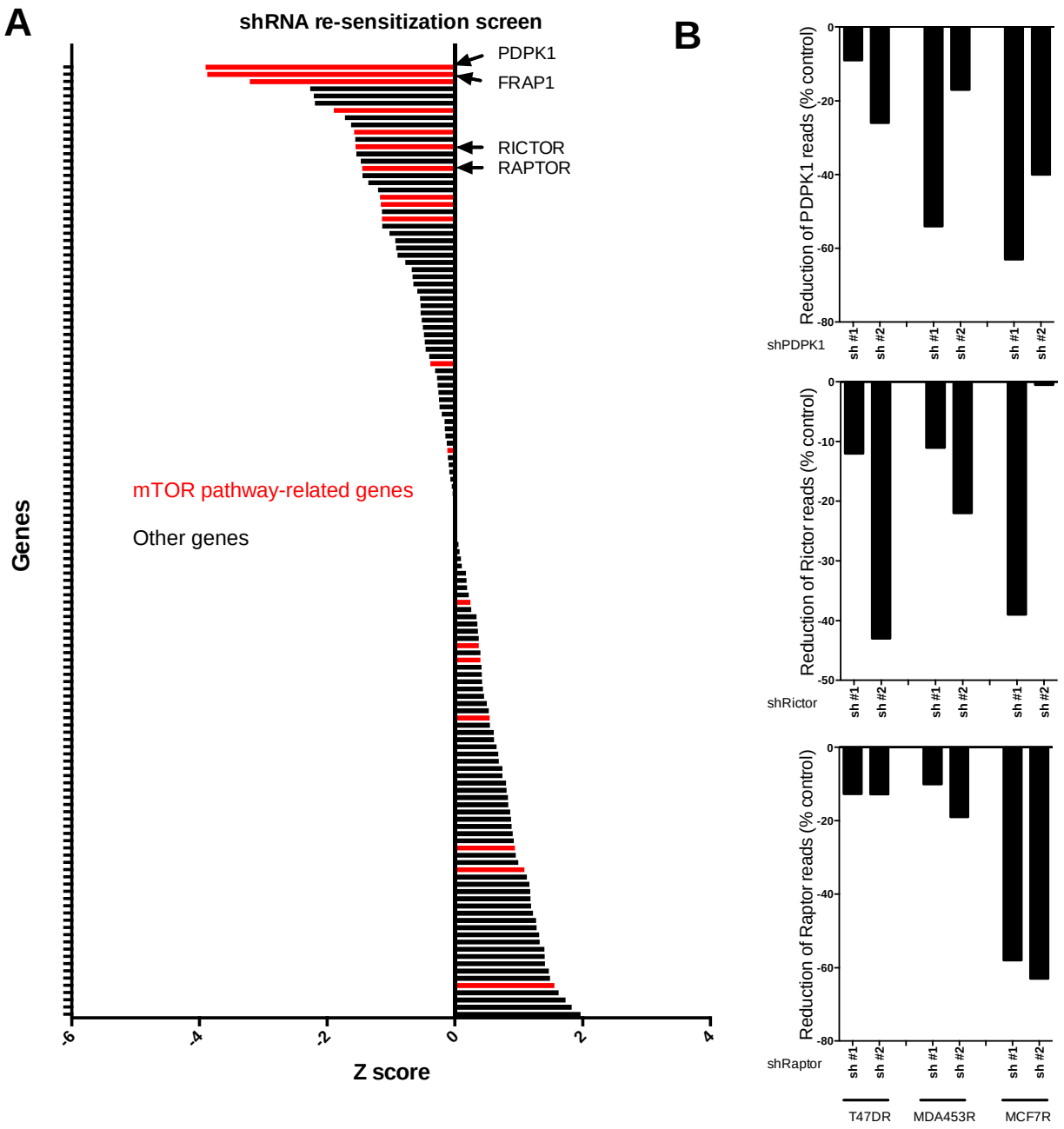

Fig. S6: Results of the shRNA resensitization screen. (A) For each gene, a score was calculated as described in Materials and Methods. 132 genes were ranked based on Z scores, which are presented here. Red bard indicate genes that are part of the mTOR pathway according to the KEGG PATHWAY Database. **(B)** Reduction in the number of reads by the individual PDPK1, Rictor, and Raptor shRNAs transfected into drug-resistant cells after 7 days of treatment with either BYL719 or GDC0941 (normalized to DMSO-treated controls).

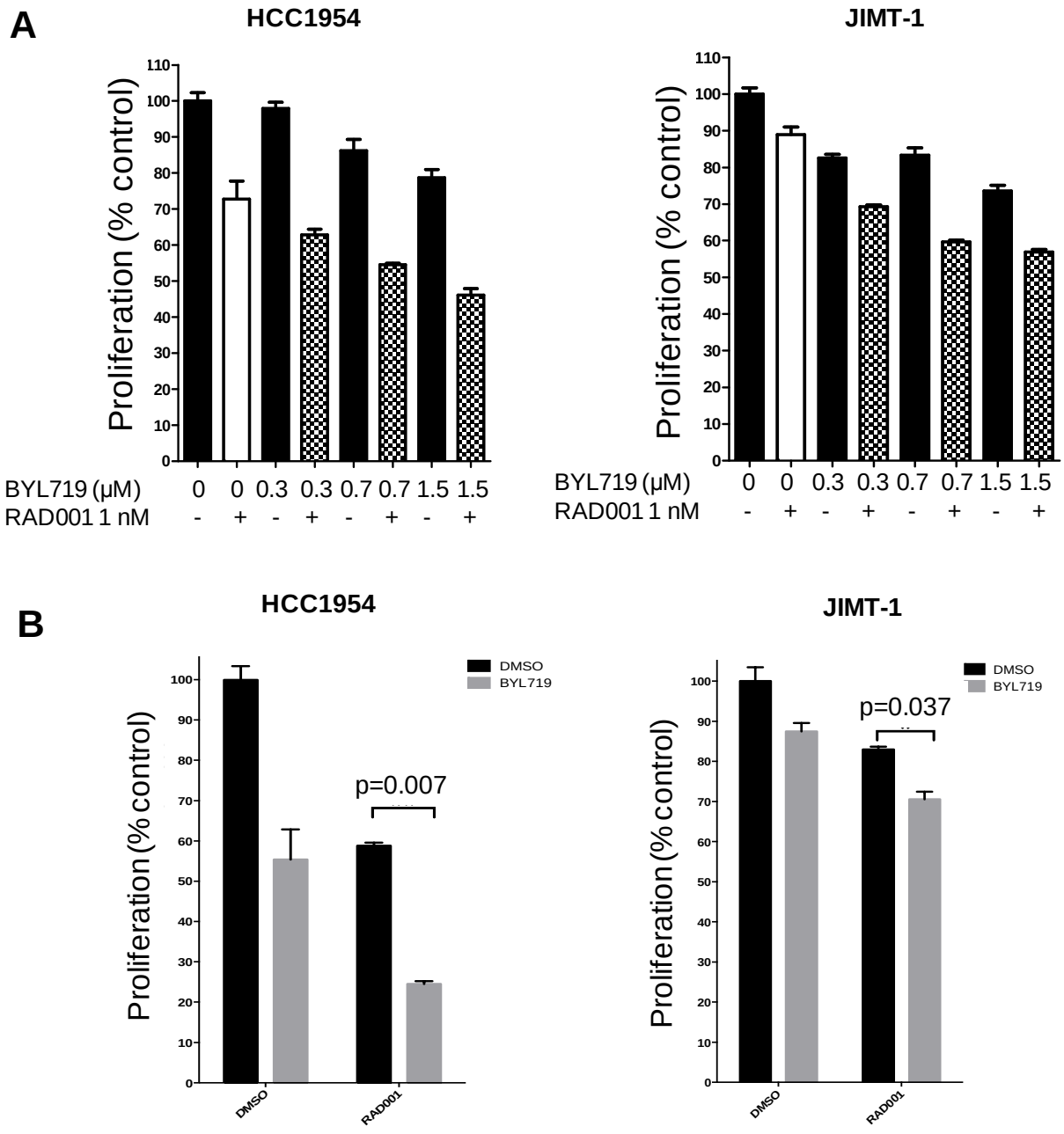

Fig. S7: Proliferation of drug-resistant cells treated with BYL719, RAD001, and the combination. (A) Proliferation assay (3 days) of HCC1954 and JIMT-1 treated with different concentrations of BYL719, with and without 1 nM RAD001. **(B)** Proliferation assay (5 days) of HCC1954 and JIMT-1 treated with different concentrations of RAD001, with and without 1 μM of BYL719. Data are shown as mean ± SEM. p-value was calculated using two-sided student's t-test.

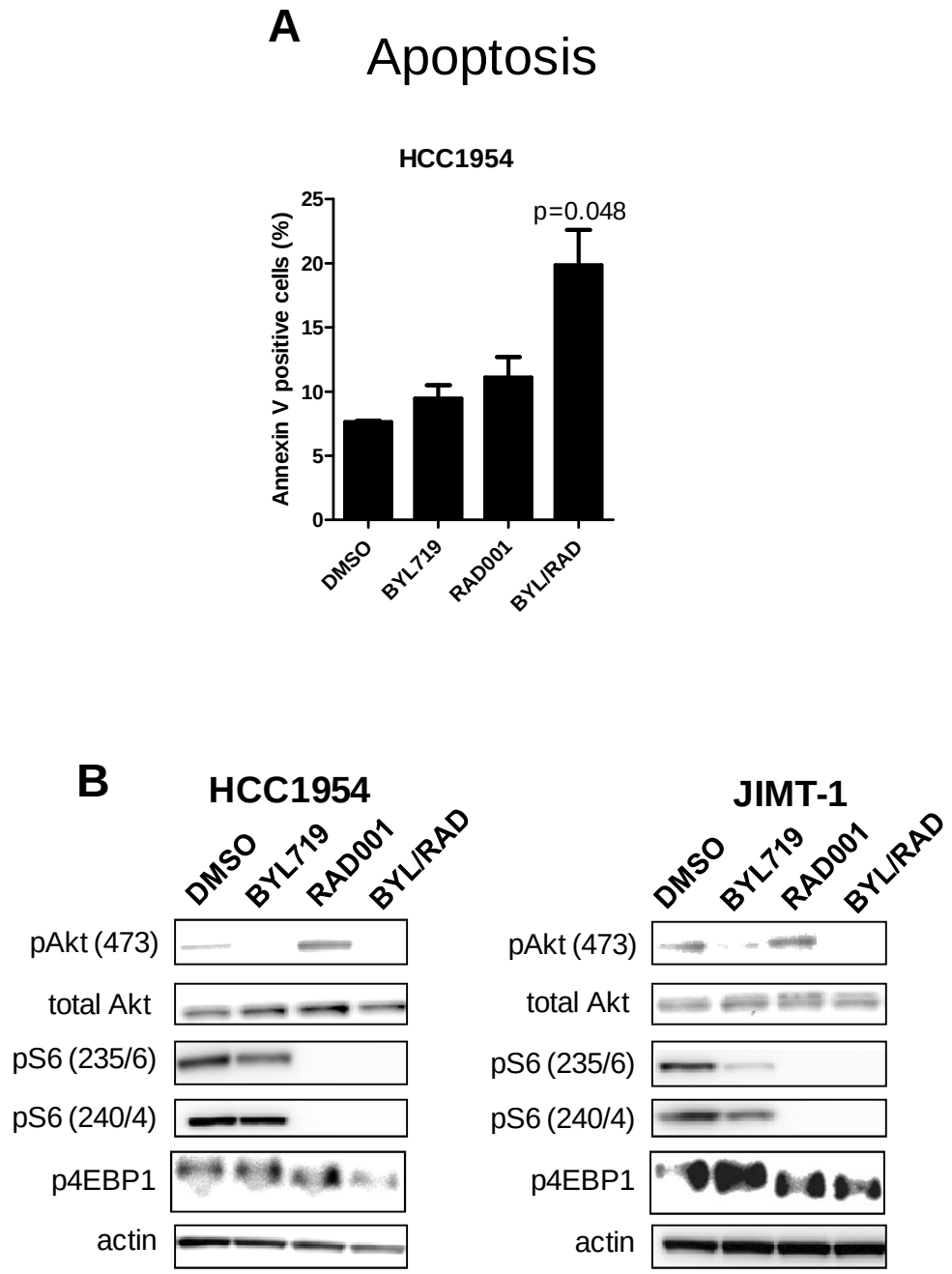

Fig. S8: Apoptosis and PI3K/Akt/mTOR pathway inhibition in cells treated with BYL719, RAD001, or the combination. (A) Annexin V analysis of HCC1954 cells after 72 hours of treatment with 0.5 µM BYL719, 1 nM RAD001, or the combination of both compounds. **(B)** Protein lysates from HCC1954 and JIMT-1 cells treated for 18 hours with 0.5 µM BYL719, 1 nM RAD001 or the combination were analyzed by immunoblotting against the indicated proteins. Data are shown as mean ± SEM. p-value was calculated using two-sided student's t-test.

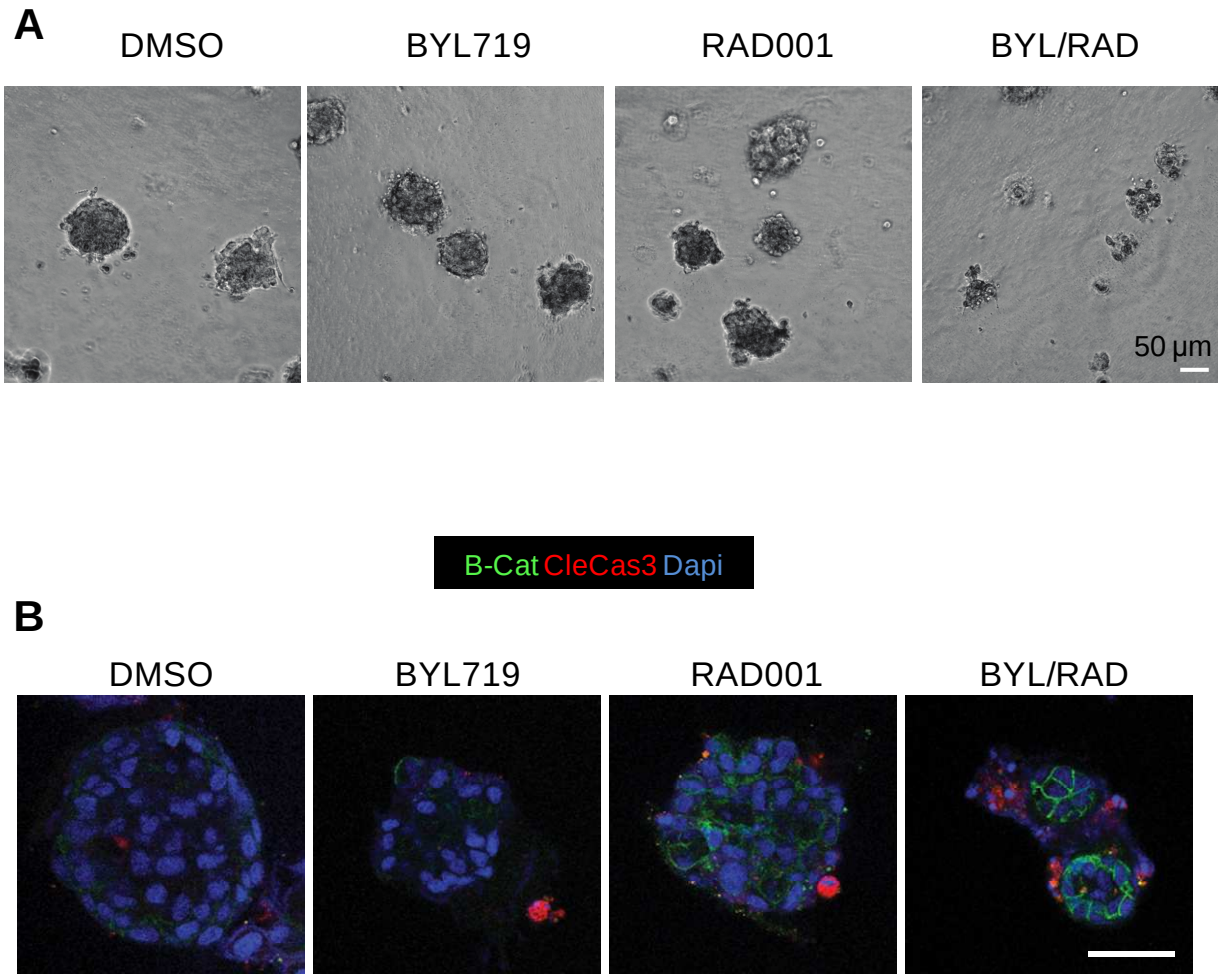

Fig. S9: 3D culture of drug-resistant cells treated with BYL719, RAD001, or the combination. (A) Phase contrast images of HCC1954 spheroids treated with BYL719, RAD001, or the combination for 48 hours. **(B)** Representative cleaved caspase 3 (CleCas3, apoptosis, red), beta-catenin (B-Cat, cell membranes, green), and nuclei (Dapi, blue) of HCC1945 spheroids after 48 hours of treatment with 1 µM BYL719, 10 nM RAD001, or the combination of both compounds. Scale bar = 50 µm.

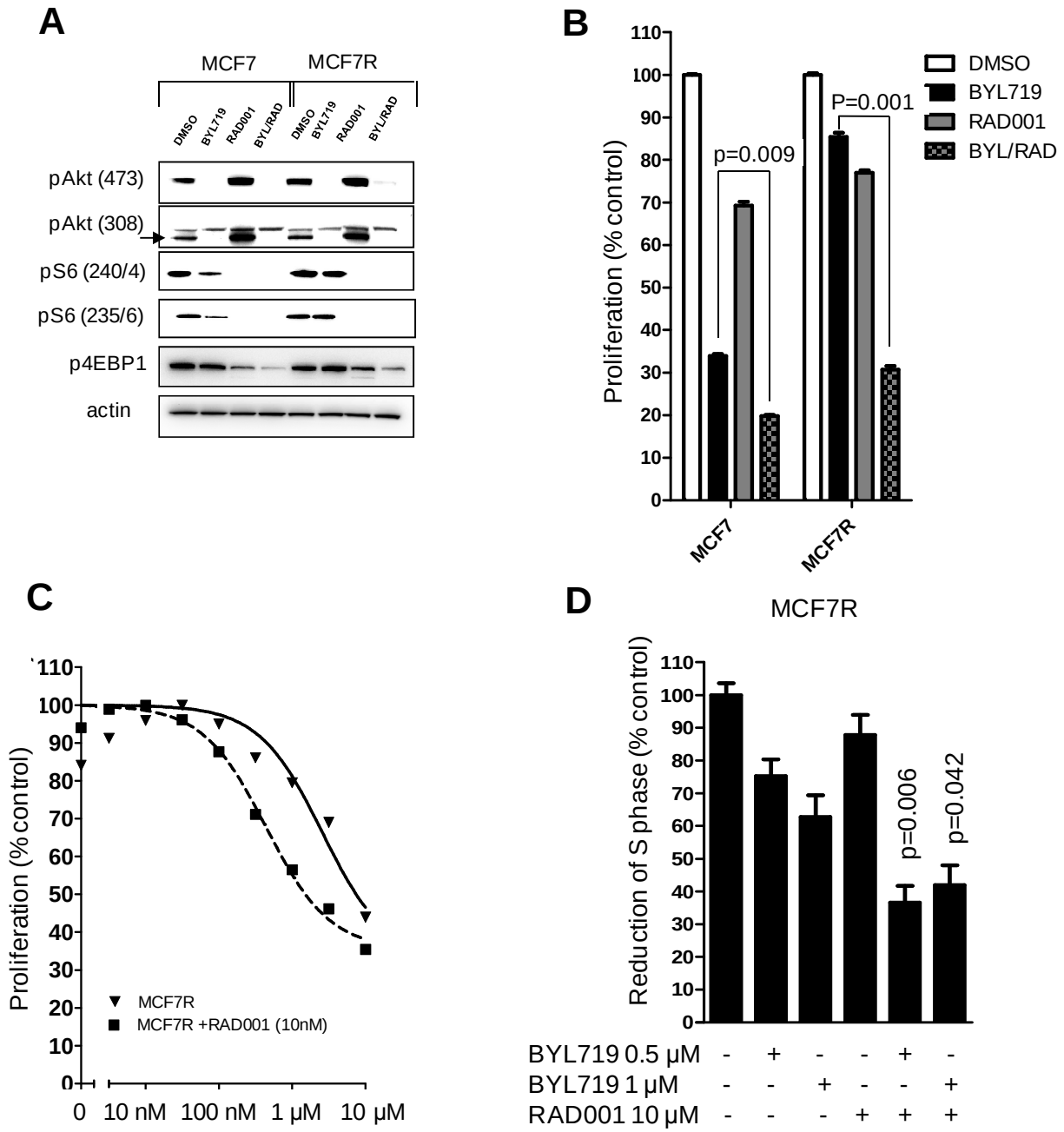

Fig. S10: Biochemical and biological effects of BYL719, RAD001, or the combination on MCF7 parental and drug-resistant cells. (A) Protein lysates from MCF7 and MCF7R cells treated 18 hours with BYL719, RAD001, or the combination were analyzed by immunoblotting against the indicated proteins. Arrow indicates specific bands. (**B)** Proliferation analysis of parental MCF7 and drug-resistant MCF7R after 5 days of treatment with 1 µM BYL719, 10 nM RAD001, or the combination of both compounds. **(C)** Dose-dependent inhibition of proliferation of MCF7R cells treated with BYL719 with or without 10 nM RAD001. **(D)** Cell cycle analysis of MCF7R cells treated as indicated for 48 hours. Data are shown as mean ± SEM. p-values were calculated using two-sided student's t-test.

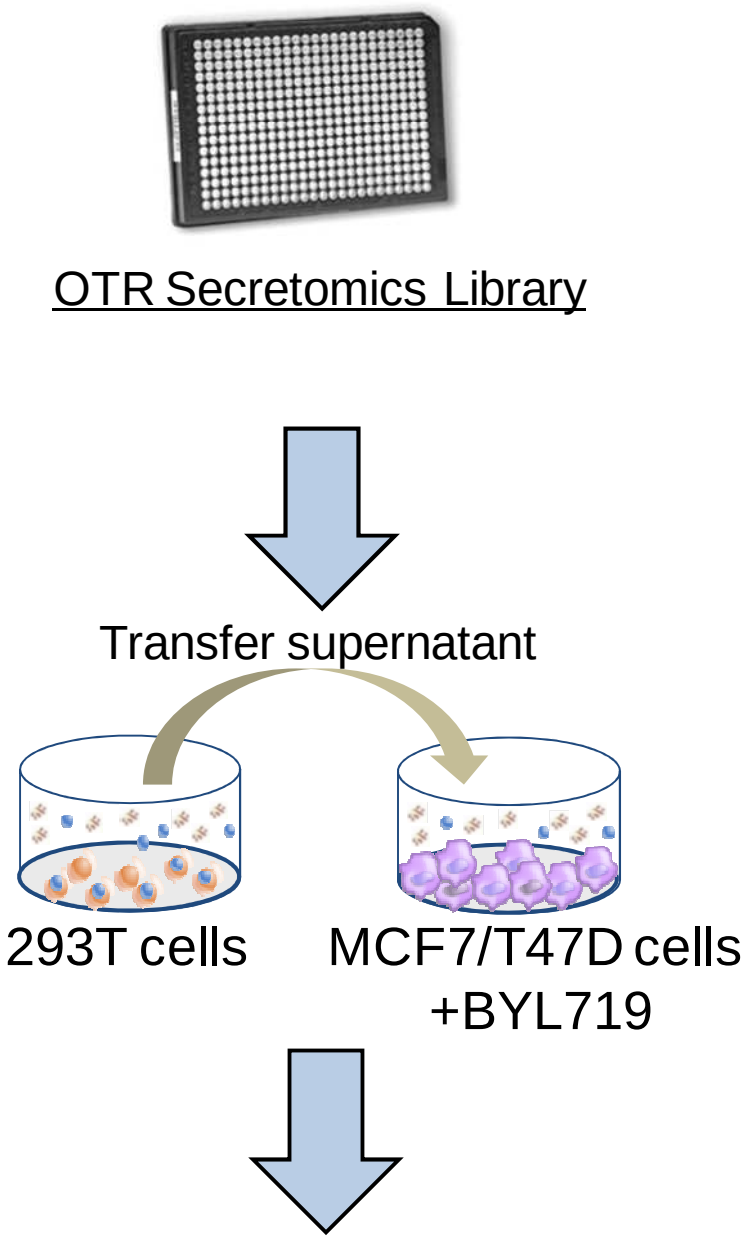

Fig. S11: Scheme of the secreted protein screen. HEK293T cells were transfected with secreted protein library cDNAs and cultured for three days. Supernatants were then transferred to pre-seeded MCF7 and T47D cells that were treated with 1 μM of BYL719 for an additional four days. The viability of MCF7 and T47D was measured by Titer-glo.

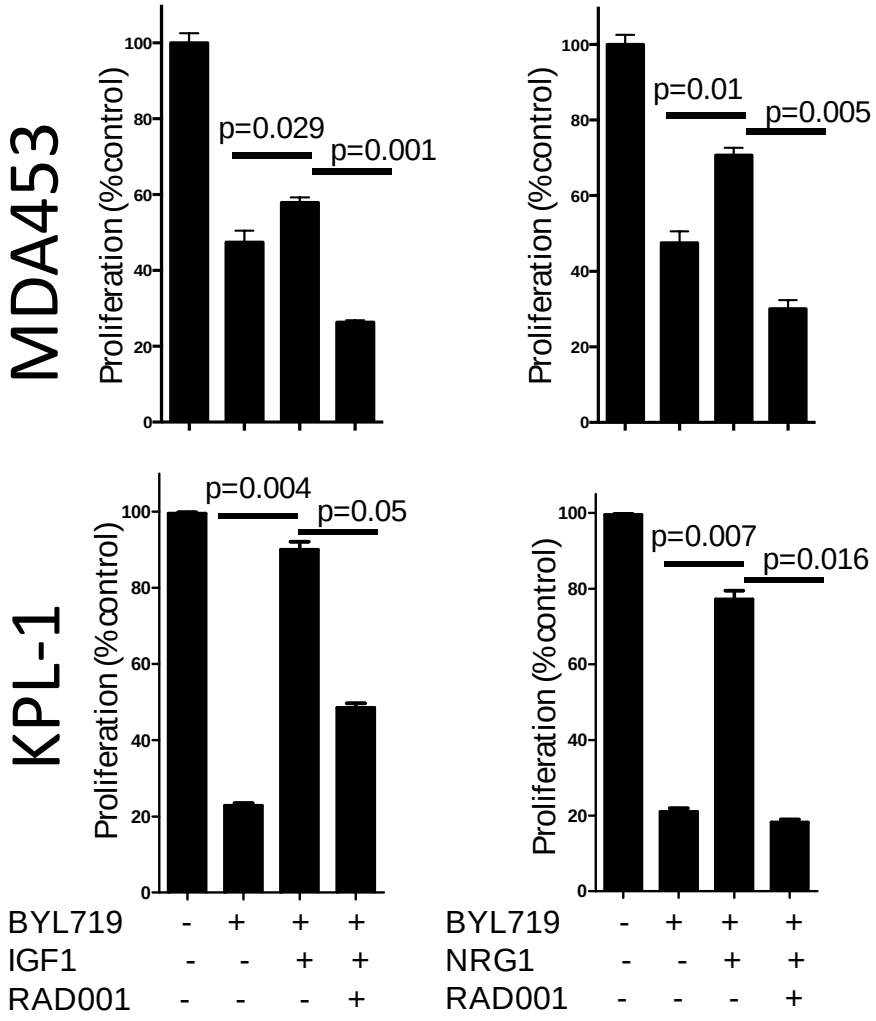

Fig. S12: Effects of IGF1 and NRG1 on BYL719-resistant cells. Proliferation of MDA453 and KPL-1 cells treated with 1 μM BYL719, 1 nM RAD001, or the combination for 6 days, with or without recombinant IGF1 and NRG1. Data are shown as means ± SEM. p-value was calculated using two-sided student's t-test.

ABL1	EGFR	IRAK1	PIK3CB	SGK2
ABL2	EPHB4	IRAK4	PIK3CD	SGK3
Akt1	ERBB2	JAK2	PIK3CG	SMG1
Akt2	ERBB3	KDR	PIK3R5	SMO
Akt3	FDPS	KIF11	PIK4CA	SRC
ALK	FGFR1	KIT	PIM1	SYK
ARAF	FGFR2	KRAS	PIM2	TEK
ATM	FGFR3	LCK	PIM3	TGFBR1
ATR	FGFR4	MAP2K1	PLK4	TNKS
AURKA	FLT3	MAP2K2	POLB	TOP1
AURKB	FRAP1	MAP3K8	PRKCA	TOP2A
AURKC	GOLPH3	MAPK1	PRKCB1	TPMT
BACE1	HDAC1	MAPK14	PRKCD	
BCL2	HDAC10	MAPK3	PRKCE	
BCL2L1	HDAC11	MAPK8	PRKCG	
BIRC2	HDAC2	MCL1	PRKCH	
BIRC3	HDAC3	MDM2	PRKCI	
BIRC4	HDAC4	MELK	PRKCQ	
BMX	HDAC5	MET	PRKCZ	
BRAF	HDAC6	NRAS	PRLR	
CCRK	HDAC7A	NTRK1	PTPN6	
CDK4	HDAC8	NTRK2	RAF1	
CDK6	HDAC9	PAK1	RB1	
CDK9	HIF1A	PAK2	RET	
CSF1R	HRAS	PARP1	RICTOR	
CTNNB1	HSP90AA1	PDGFRA	ROCK1	
DDIT4	HSP90AB1	PDGFRB	ROCK2	
DDR1	IGF1R	PDPK1	RPTOR	
DGAT1	IKBKB	PI4K2B	SCD	
DHFR	ILK	PIK3CA	SGK	

Table S1: List of 132 genes tested in the shRNA screen.

Antibody	Company	Catalog number	Dilution	Assay
pAKT (473)	Cell Signaling	4060	1:100,1:50	WB, IHC
pAKT (308	Cell Signaling	9275	1:100	WB
AKT	Cell Signaling	9272S	1:1000	WB
pS6 (240/4)	Cell Signaling	5364	1:4000,1:400	WB, IHC
pS6 (235/6)	Cell Signaling	4857	1:4000,1:75	WB, IHC
S6	Cell Signaling	2217L	1:1000	WB
p4EBP1(T37/46)	Cell Signaling	2855L	1:500,1:1500	WB, IHC
phospho-PRAS40 (T246)	Cell Signaling	2997S	1:500	WB
Cleaved caspase-3	Cell Signaling	9664	1:200	IF
Beta-Catenin	BD	610153	1:200	IF
KI67	Novocastra	NCL-Ki67-P	1:200	IF
4EBP1	Cell Signaling	9644S	1:1000	WB
eIF4E	Cell Signaling	9742	1:1000	WB
Beta-Actin	Sigma-Aldrich	A2228	1:10000	WB
Alexa-568	Invitrogen	A11061	1:400	IF
Alexa-488	Invitrogen	A12379	1:400	IF

Table S2: Antibody information. WB, western blot; IHC, immunohistochemistry; IF, immunofluorescence.