AURKB as a target in non-small cell lung cancer with acquired resistance to anti-EGFR therapy

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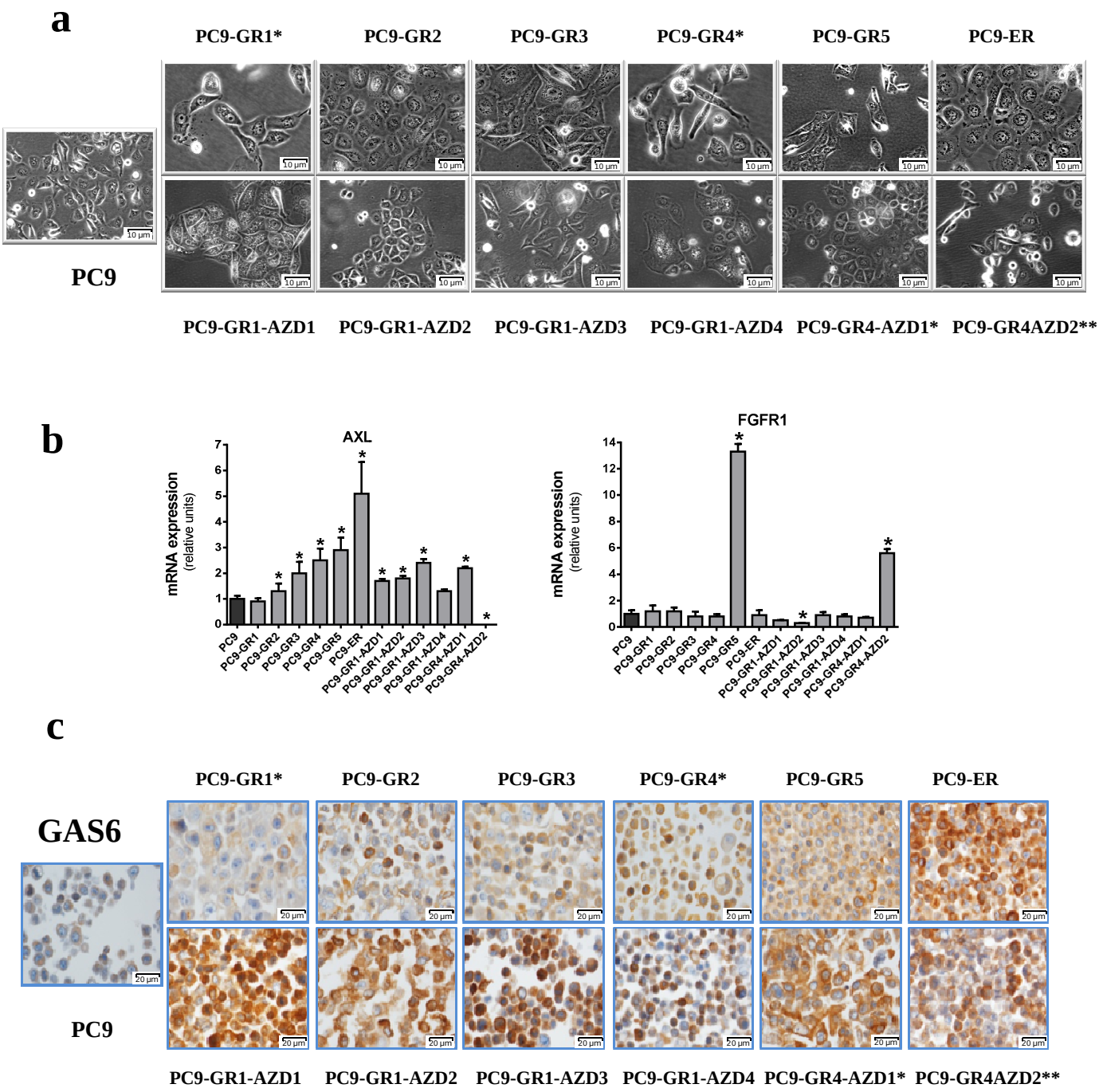
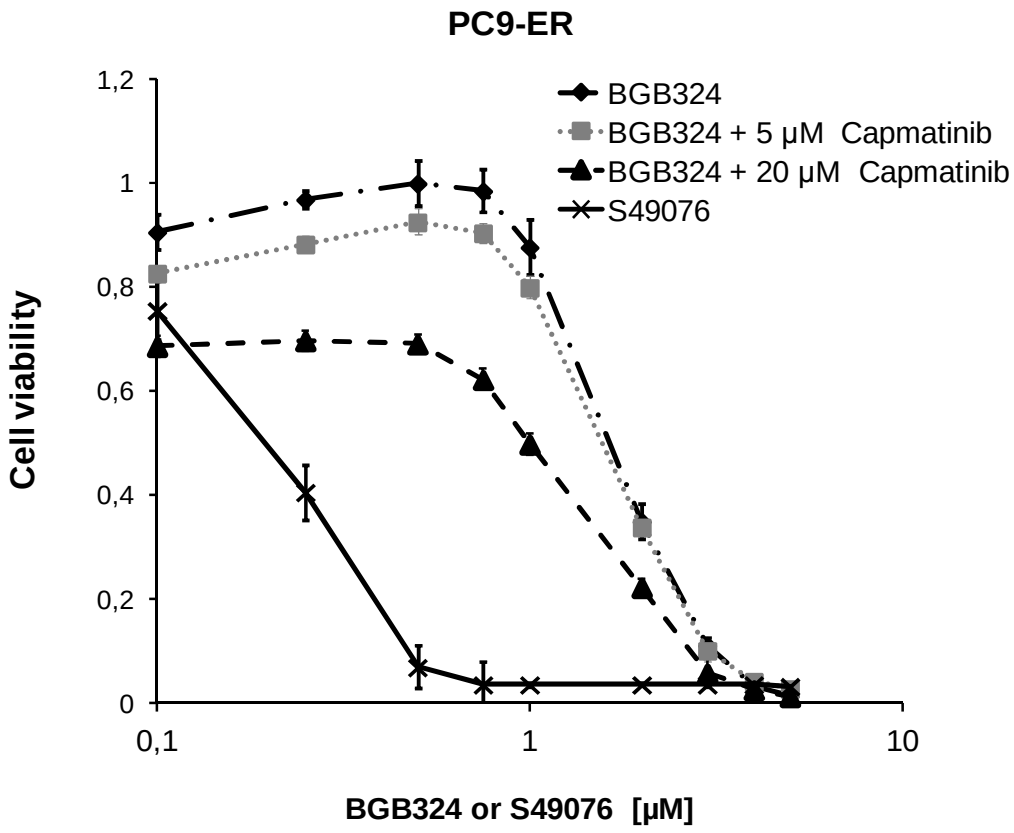
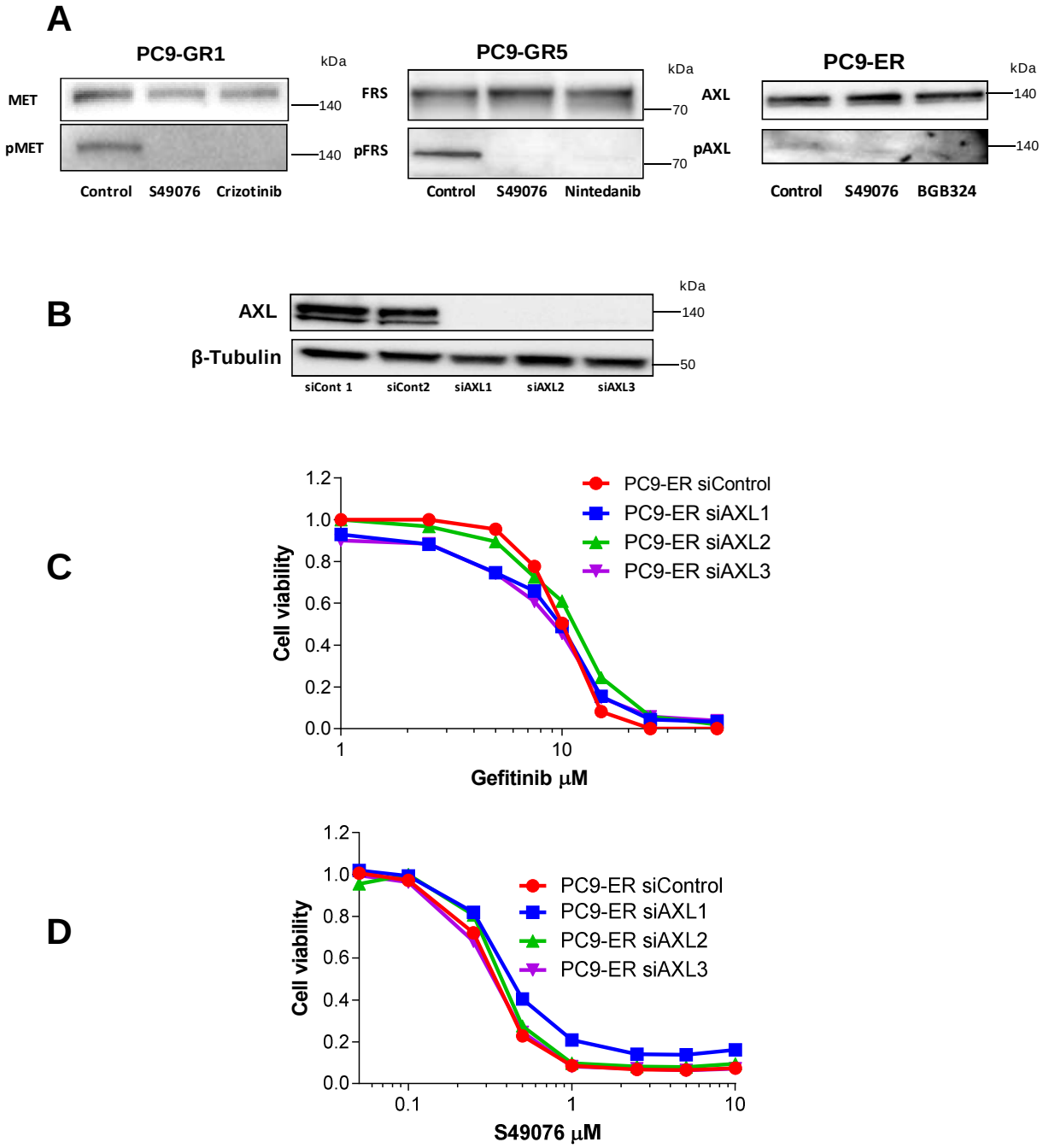
SUPPLEMENTARY FIGURES



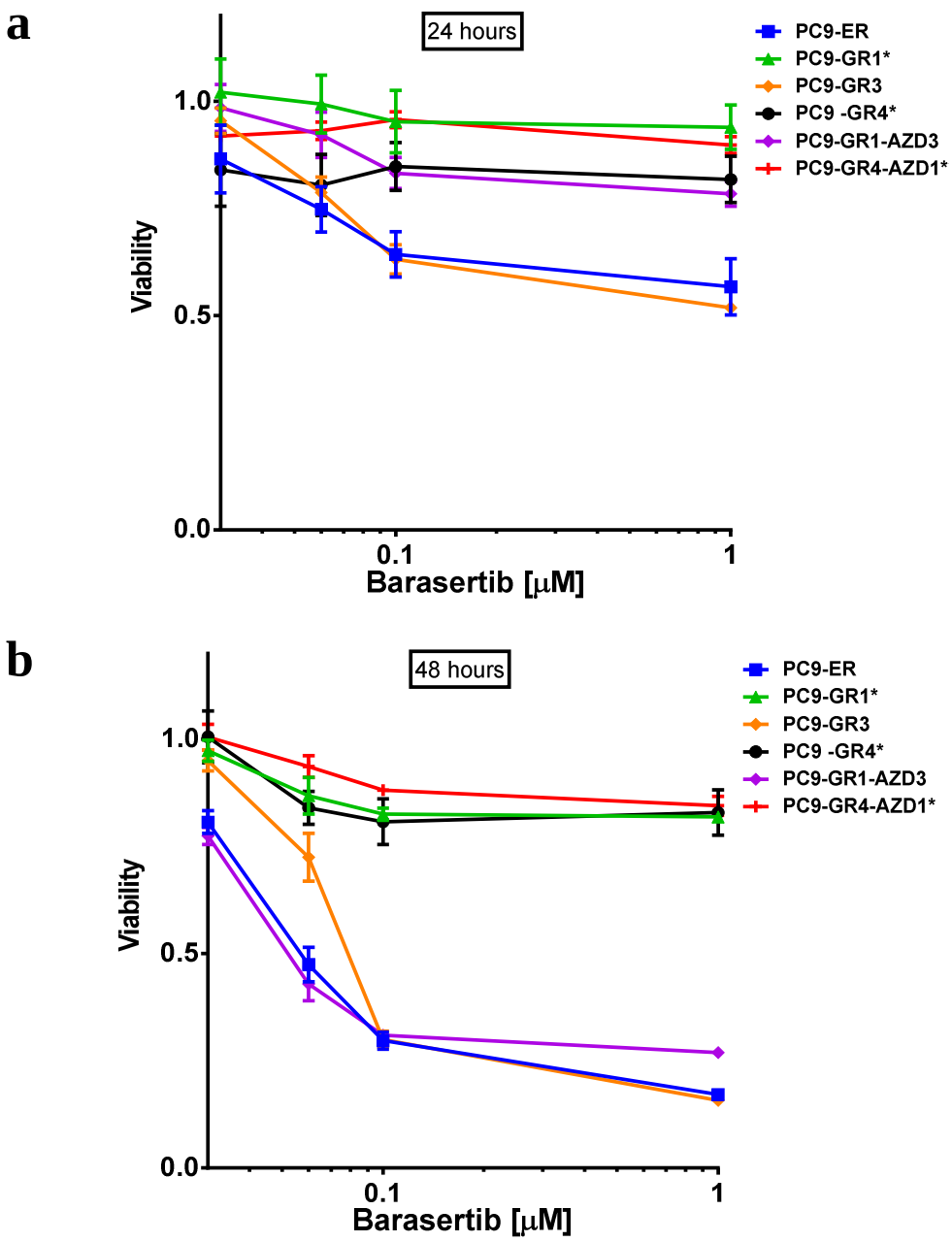
Supplementary Fig. 1: Characterization of the EGFR TKI resistant cell lines used in this study. **a** Microscopic photographs of the parental and resistant PC9 cells. Asterisks indicated cell lines harboring the T790M resistant mutation at >0.1% (*) or <0.1% (**) allelic fraction. Scale bars indicate 10 μm. **b** Relative expression levels of the *AXL* and *FGFR* mRNAs. No significant differences were found on the case of *MER* and *MET*. Experiments were conducted in triplicates, values shown are means ± S.D. and asterisks indicate statistical significance ($P<0.05$ in a Student's t test). **c** IHC staining of GAS6, showing the cytoplasmic localization of the protein in all cell lines of the panel. Images were obtained with an x40 objective. Scale bars indicate 20 μm.



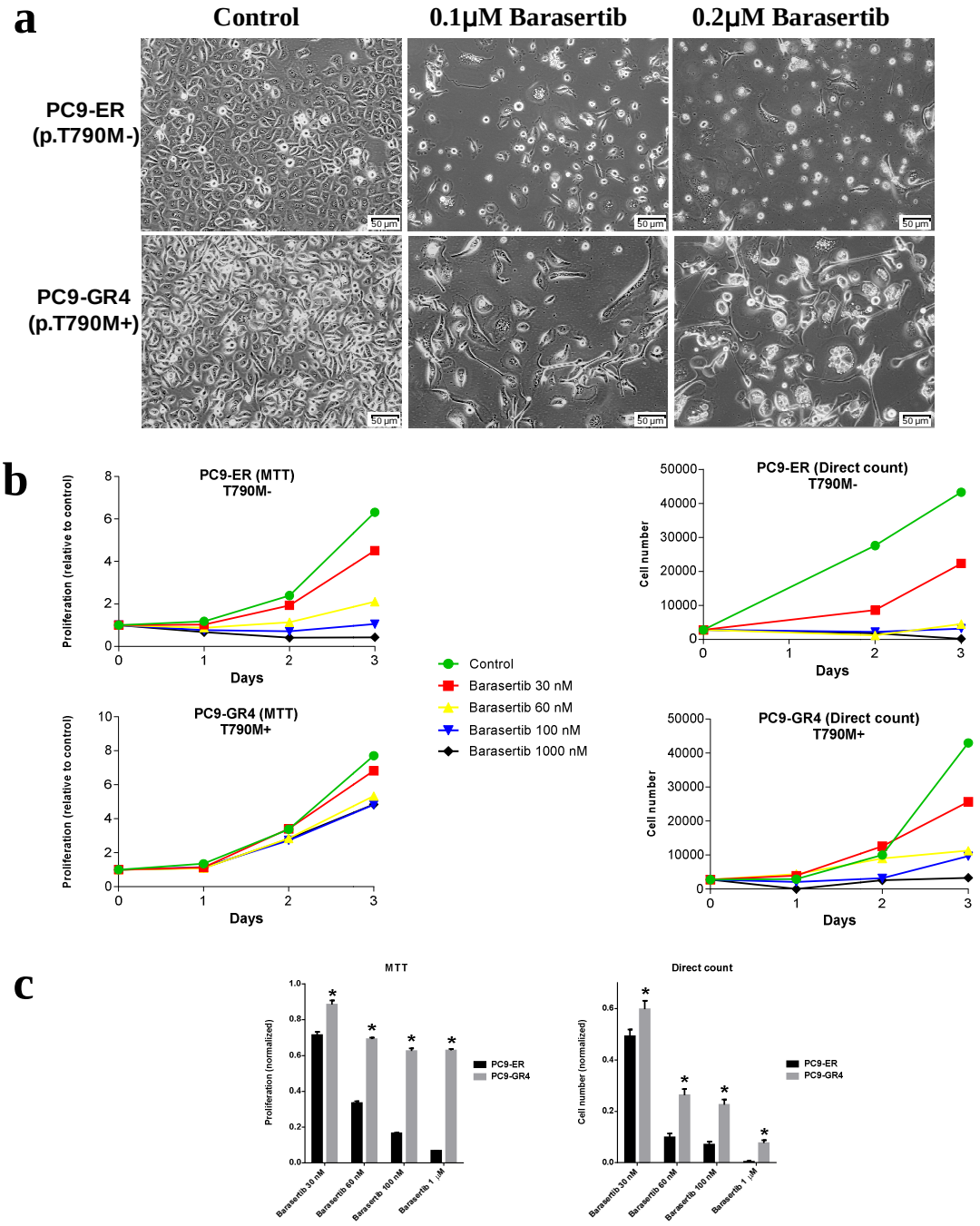
Supplementary Fig. 2: Dose-response plots of PC9-ER cells treated with BGB324, BGB324 in combination with capmatinib and S49076. Experiments were conducted in triplicates, values shown are means ± S.D.



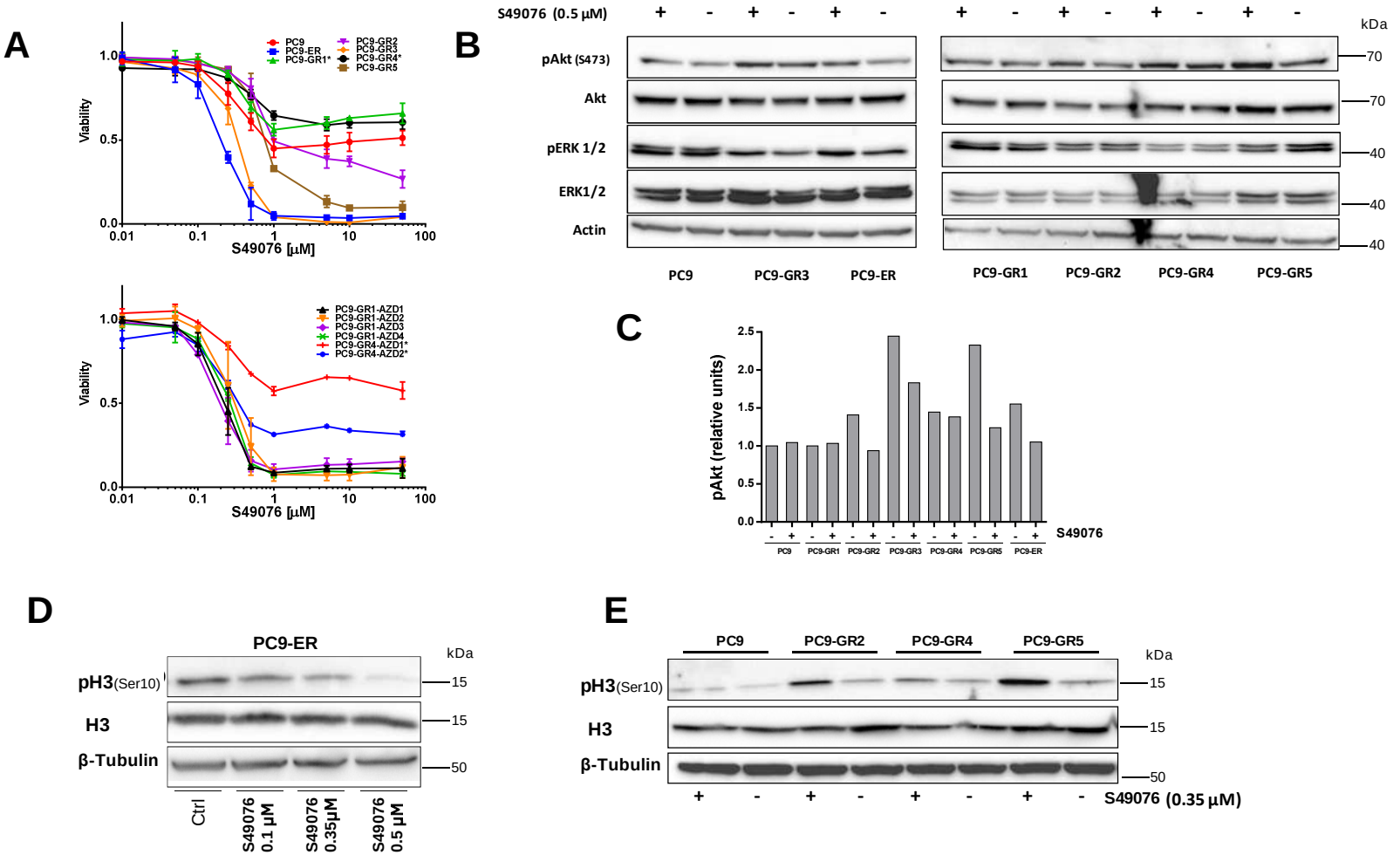
Supplementary Fig. 3: a Effects of S49076, crizotinib and nintedanib and BGB324 on the phosphorylation of MET, FRS and AXL. **b** Expression of AXL by Western blotting in the control and the silenced clones. **c, d** Dose-response plots to gefitinib and S49076 of the PC9-ER control clones and the clones with stable *AXL* silencing. Experiments were conducted in triplicates, values shown are means



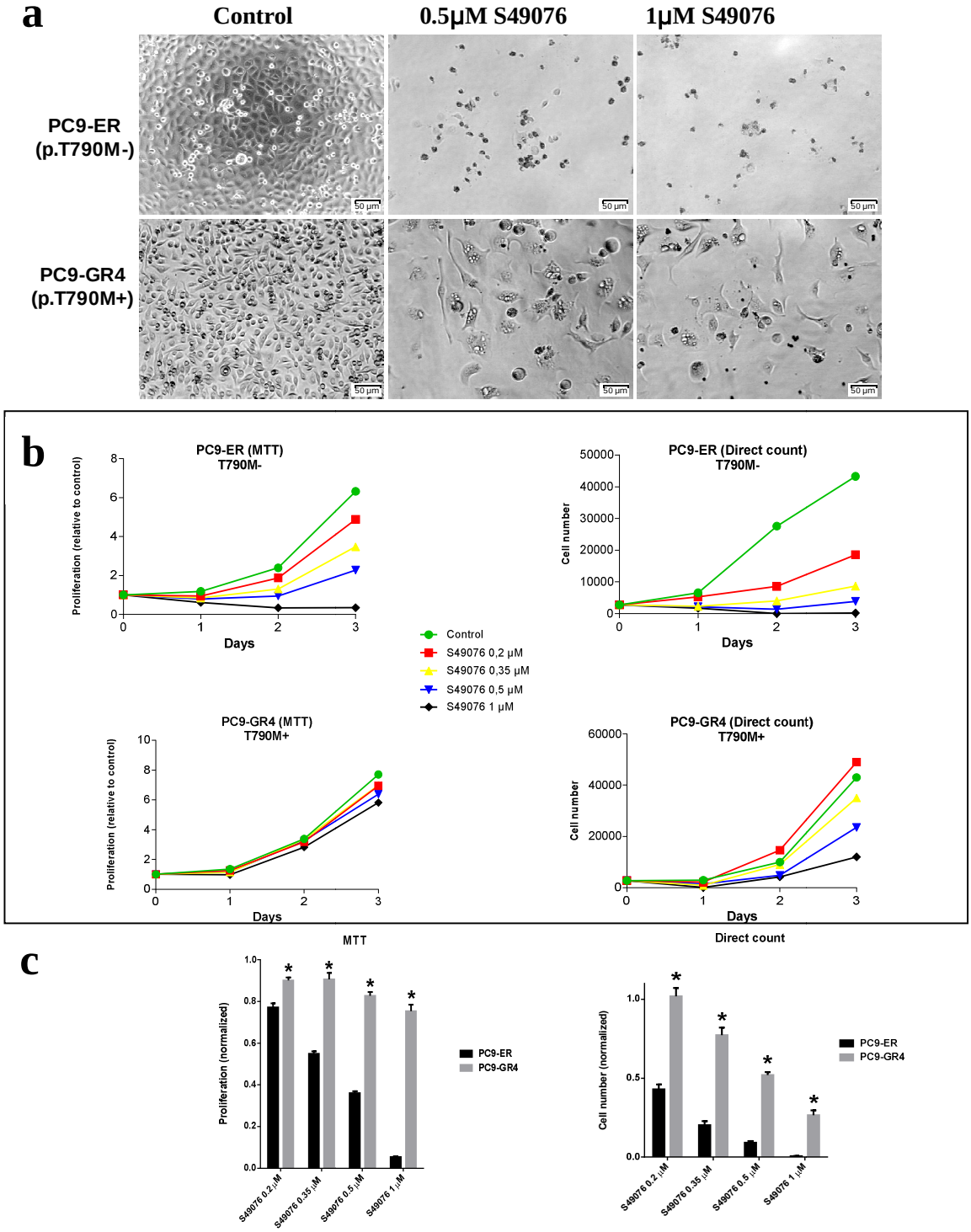
Supplementary Fig. 4: Dose-response curves of selected parental and EGFR TKI resistant cells to the AURKB inhibitor barasertib at 24 (**a**) and 48 h (**b**). Asterisks indicated cell lines harboring the T790M resistant mutation. Experiments were conducted in triplicates, values shown are means ± S.D.



Supplementary Fig. 5: Effects of Barasertib on PC9-ER (p.T790M-negative) and PC9-GR4 (p.T790M-positive) cell lines. **a** Microscopic photographs of the cells at different concentrations of the inhibitor. Scale bars indicate 50 µm. **b** Growth curves of the cell lines in presence of the inhibitors, determined by MTT (left) or direct counting (right). **c** Comparison of the final numbers of cells of the growth curves presented in (**b**), as estimated by MTT (left) or direct count (right). Experiments were conducted in triplicates, values shown are means ± S.D. and asterisks indicate statistical significance (P<0.05 in a Student's t test)

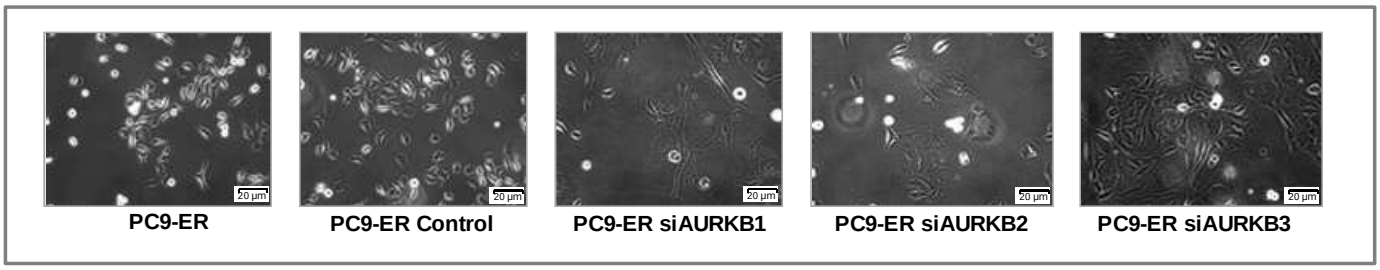


Supplementary Fig. 6: Effects of the MET/AXL/FGFR/AURKB inhibitor S49076 on EGFR TKI resistant cell lines. **a** Dose-response curves of the parental and the EGFR TKI resistant cells to S49076. Experiments were conducted in tri or quadruplicates and values shown are means ± S.D. **b** Effects of the S49076 on the phosphorylation of Erk1/2 and Akt. Incubation time was 2 h. **c** Quantification of the Western blot for pAkt. **d** Western blot showing the dose-dependent decrease of pH3 levels induced by S49076 at 24 h. **e** Western blot demonstrating the inhibition of pH3 by S49076 after 24 h in EGFR-mut cell lines

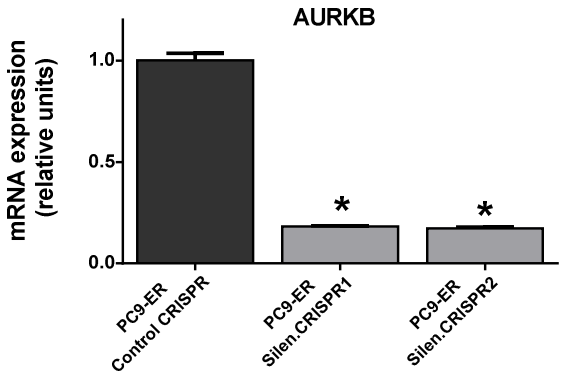


Supplementary Fig. 7: Effects of S49076 on PC9-ER (p.T790M-negative) and PC9-GR4 (p.T790M-positive) cell lines. **a** Microscopic photographs of the cells at different concentrations of the inhibitor. Scale bars indicate 50µm **b** Growth curves of the cell lines in presence of the inhibitors, determined by MTT (left) or direct counting (right). **c** Comparison of the final numbers of cells of the growth curves presented in (**b**), as estimated by MTT (left) or direct count (right). Values shown are means ± S.D. of six replicates and asterisks indicate statistical significance (P<0.05 in a Student's t test)

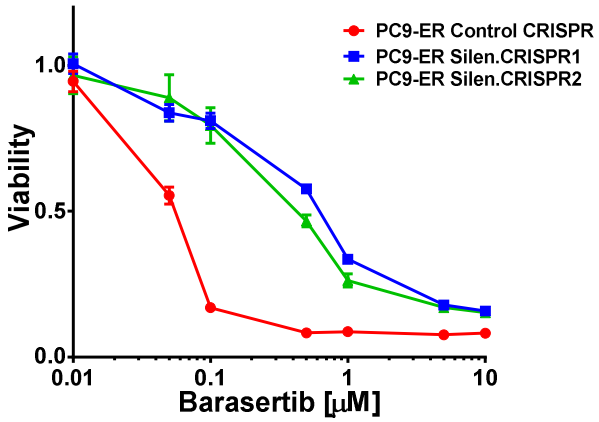
a



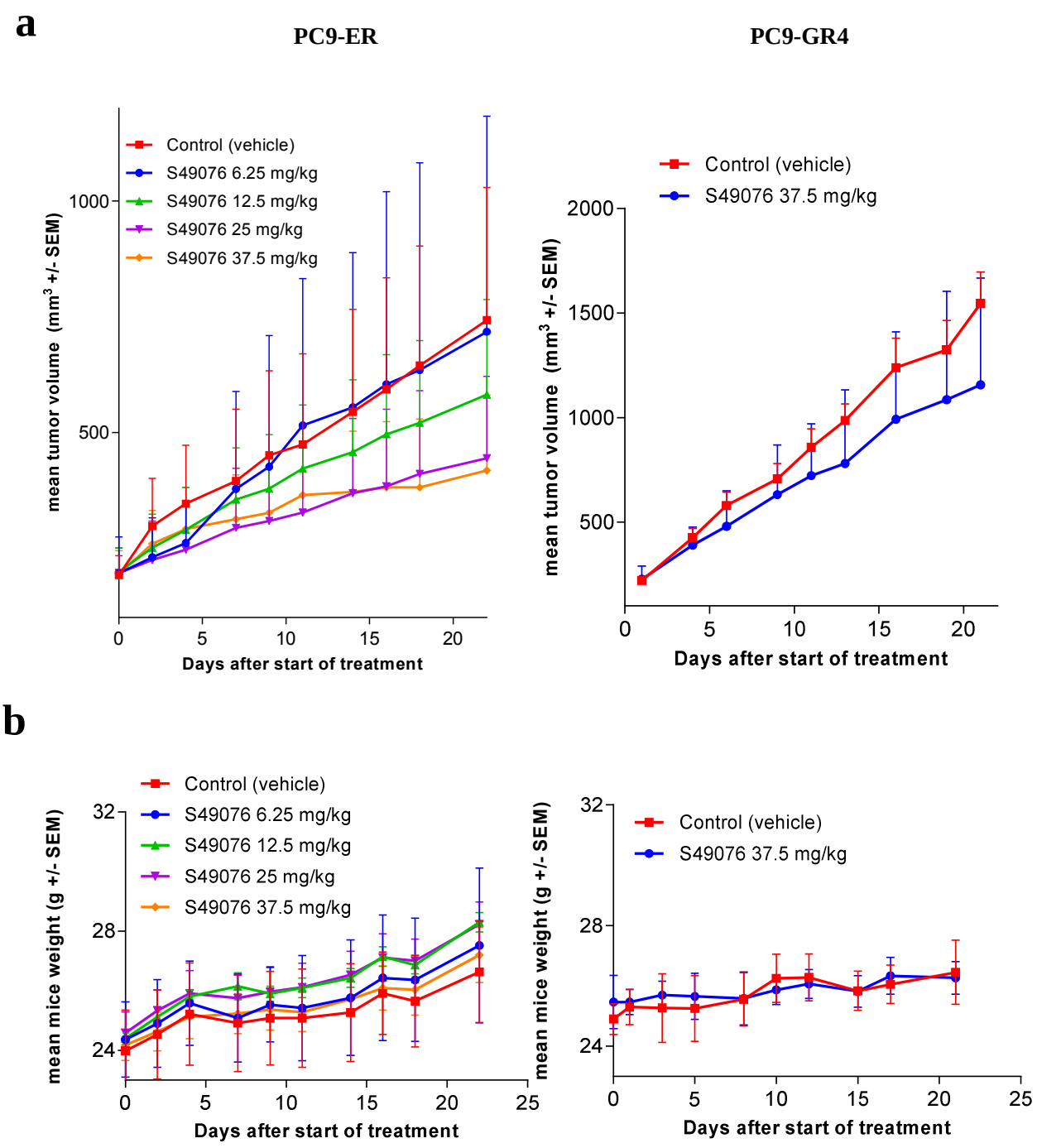
b



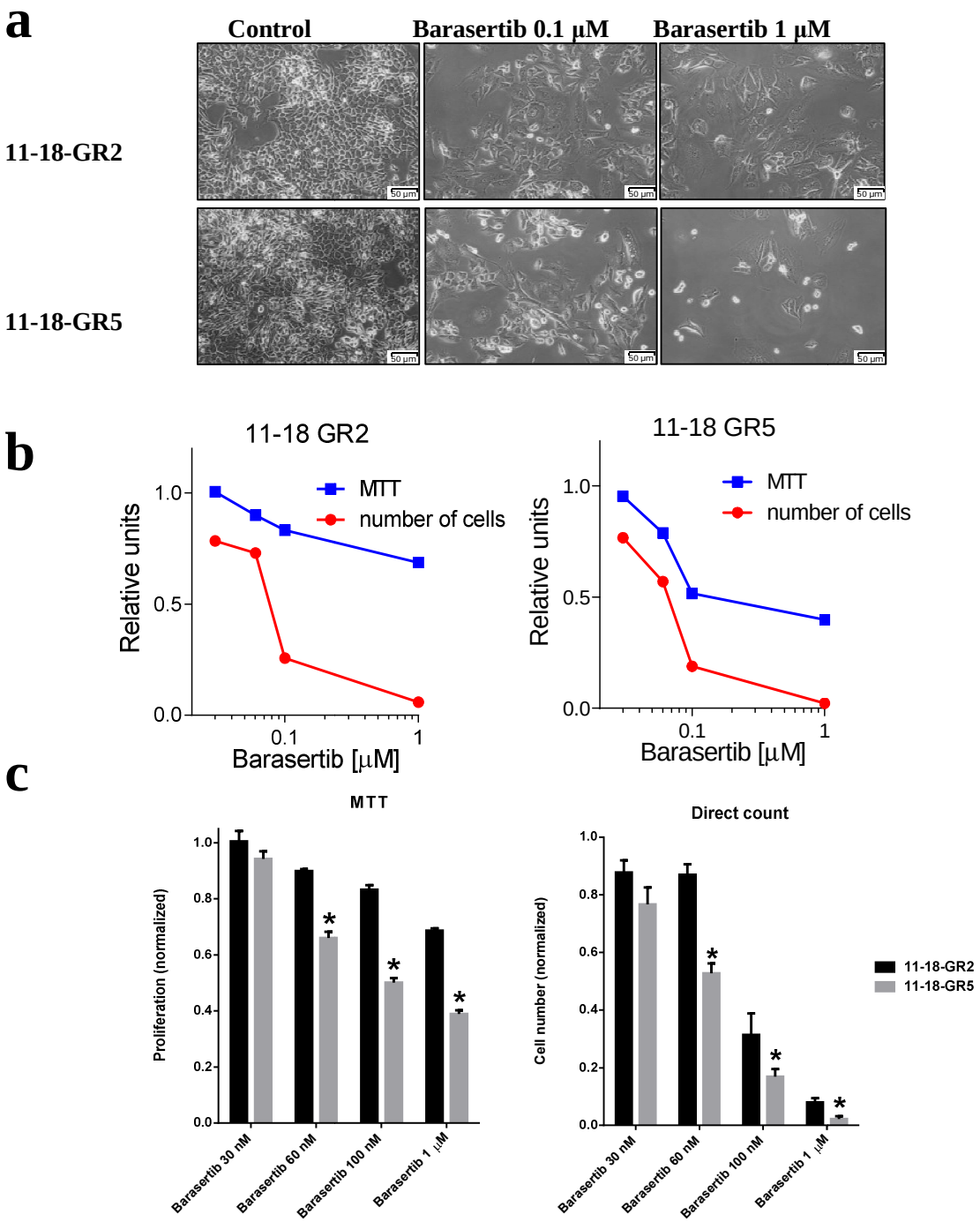
c



Supplementary Fig. 8: a Microscopic photographs of the parental PC9-ER cells, the clones partly silenced for *AURKB* (siAURKB1-3) and the clone transfected with a control plasmid. Scale bars indicate 20µm **b** Relative expression levels of the *AURKB* mRNAs in the PC9-ER control clone and the clones with AURKB CRISPR silencing**.** Values shown are means ± S.D. of three replicates and asterisks indicate statistical significance ($P<0.05$ in a Student's t test). **c** Dose-response plots to barasertib of the PC9-ER control clone and the clones with AURKB CRISPR silencing. Values shown are means ± S.D. of six replicates

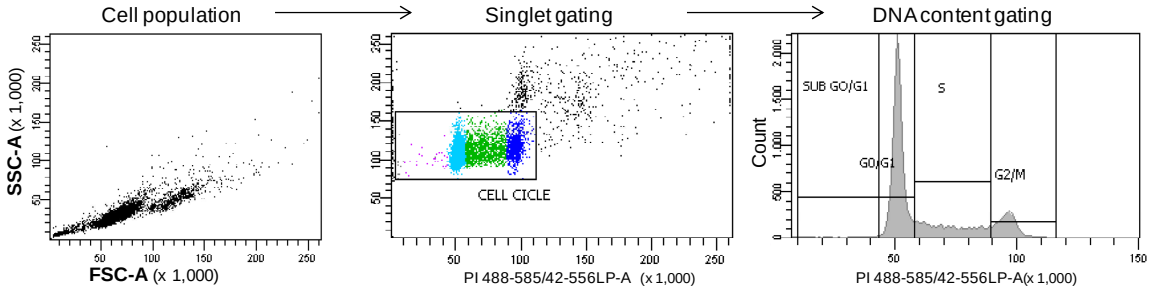


Supplementary Fig. 9: Effects of S49076 on the growth of subcutaneous PC9-ER (left panels) and PC9-GR4 xenografts (right panels). **a** Time course assessment of total tumor volume. **b** Weight of the animals used in the study. Values shown are means ± S.E.M of six replicates



Supplementary Fig. 10: Effects of Barasertib on 11-18-GR5 (*NRAS* and T790M-negative) and 11-18-GR2 (*NRAS* Q61L) cell lines. **a** Microscopic photographs of the cells at different concentrations of the inhibitor. Scale bars indicate 50 µm**. b** Comparison of the dose-response to barasertib, as determined by MTT or direct counting in 11-18 GR2 (left) or 11-18 GR5 (right). **c** Comparison of the dose-response to barasertib of 11-18 GR2 and GR5, as determined by MTT (left) or direct counting (right). Values shown are means ± S.D. of six replicates and asterisks indicate statistical significance ($P<0.05$ in a Student's t test).

a



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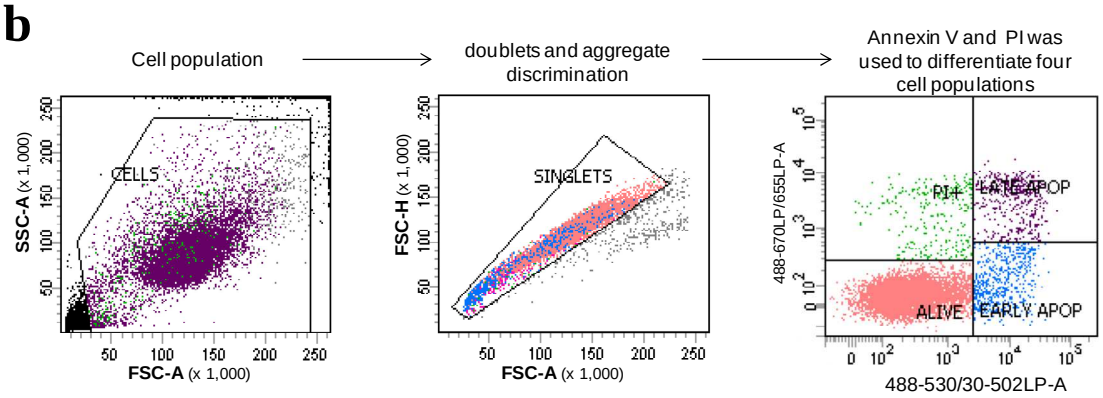

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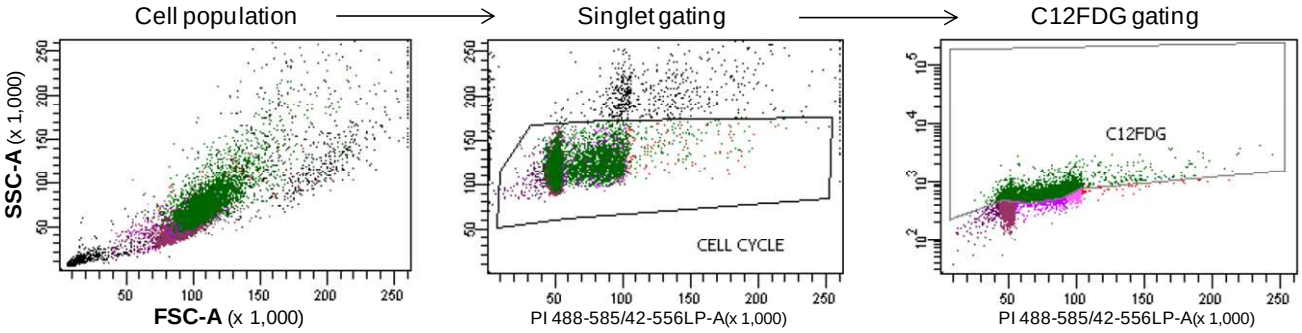

Supplementary Fig. 11: Representative FACS plots showing the gating strategy for flow cytometry analysis of: **a** cell-cycle profile (Propidium Iodide staining), **b** cell death (Annexin-V and Propidium Iodide staining), **c** senescent cells (C12FDG).

SUPPLEMENTARY TABLES

Supplementary Table 1: Doubling times and IC50 of selected inhibitors in the *AURKB* and *AXL* silenced clones derived from PC9-ER and used in this study. Values shown are the means of at least three different experiments. ND, not determined.

Cell line/Clone	Doubling time (h)	S49076	Barasertib	Tozasertib	BGB324	Foretinib	Crizotinib	Gefitinib
PC9-ER	27±2	0.3	0.06	0.2	2.3	0.7	1.4	12.2
PC9-ER siCont 1	27±4	0.3	0.04	0.2	2.1	0.6	1.6	10.4
PC9-ER siCont 2	31±3	0.4	0.05	0.2	2.3	0.6	1.8	8.8
PC9-ER siAXL1	31±7	0.5	0.05	ND	1.5	ND	1.7	10.6
PC9-ER siAXL2	31±2	0.4	0.05	ND	1.2	ND	1.6	10.3
PC9-ER siAXL3	ND	0.5	0.06	ND	1.0	ND	1.4	8.9
PC9-ER siAURKB1	28±3	>50	16.0	3.2	1.7	1.6	4.8	14.3
PC9-ER siAURKB2	30±4	>50	22.6	10.1	2.7	1.1	3.5	16.5
PC9-ER siAURKB3	28±4	>50	17.2	3.7	1.7	1.2	4.5	14.2

Supplementary Table 2: Characteristics of the 59 patients with *EGFR* mutant NSCLC treated with first line EGFR TKIs (pre-treatment samples) included in the study.

Gender	**no. (%)**
Male	15 (25.4)
Female	44 (74.6)
Age - years	
Median	66
Range	36−90
ECOG performance status	
0	15 (25.4)
1	26 (44.1)
2	15 (25.4)
3	3 (5.1)
Smoking status	
Never	41 (69.5)
Former	14 (23.7)
Current	4 (6.8)
Disease stage	
IIIB	14 (23.7)
IV	45 (76.3)
Brain metastasis	
No	40 (67.8)
Yes	19 (32.2)
Bone metastasis	
No	33 (55.9)
Yes	26 (44.1)
Other metastatic sites	
No	23 (39.0)
Yes	36 (61.0)
Type of EGFR mutation	
Exon 19 deletion	36 (61.0)
L858R	19 (32.2)
Other	4 (6.8)
Type of EGFR TKI	
Erlotinib	44 (58.7)
Gefitinib	29 (38.7)
Afatinib	2 (2.6)
Response*	
PD	6 (10.1)
SD	19 (32.2)
PR	32 (54.2)
CR	2 (3.4)

*PD, progressive disease; SD, stable disease; PR, partial response; CR, complete response

Supplementary Table 3: Overall survival of *EGFR*-mut patients treated with EGFR TKIs (n=59) classified according to IHC H-scores.

H-score	**OS / Q4**	**OS / Q1-Q3**	**p (Long-rank)**
Non-mitotic pH3	18.8 months	34.9 months	0.413
Mitotic pH3	20.8 months	35.4 months	0.126
Ki67	21.9 months	36.7 months	0.071

Supplementary Table 4: Antibodies used in this study.

Antibody	Vendor (IHC)	Vendor (Western blotting)	Dilution
EGFR	--	Cell Signaling Technology (CST)-Cat#4267	1:1000
pEGFR	--	CST-Cat#3777	1:500
AXL	CST-Cat#8661	CST-Cat#8661	1:100 (IHC); 1:1000 (WB)
GAS6	R&D Systems-Cat# AF885	--	1:50
MET	Roche Diagnostics, Clone SP44-Cat#790-4430	CST-Cat#8198	Ready to use (IHC); 1:1000 (WB)
pMET	--	CST-Cat#3077	1:500
FGFR1	--	CST-Cat#9740	1:1000
FRS	--	R&D Systems-Cat#AF4069	1:1000
pFRS	--	CST-Cat#3861	1:500
ERK1/2	--	CST-Cat#9102	1:1000
pERK1/2	--	CST-Cat#9101	1:500
Akt	--	CST-Cat#9272	1:1000
pAkt	--	CST-Cat#9271	1:500
AURKB	Abcam-Cat# ab2254*	CST-Cat#3094	1:50 (ICC); 1:1000 (WB)
Ki67	Roche Diagnostics, Clone 30-9-Cat#790-4286	--	Ready to use
H3	--	Abcam-Cat# ab1791	1:1000
pH3	Abcam-Cat# ab5176	Millipore-Cat#06-570*	1:200 (IHC); 1:2000 (ICC); 1:250 (WB)
PARP		CST-Cat#9542	1:1000
Actin	--	Sigma Aldrich-Cat#A5441	1:2000
Tubulin	--	Sigma-Aldrich-Cat#T9026	1:2000

*Indicates the antibodies employed for ICC

Supplementary Table 5: Primers and probes used in this study

Gene		Sequence
AURKB	F	5´ CATGAGCCGCTCCAATGTC 3´
	R	5´ TGCTATTCTCCATCACCTTCTGG 3´
	Probe	6FAM 5´ AGCCCACAGCTGCC 3´ MGB
ATM	F	5´ TGGTGTGAAATATCTCAGCAACAGT 3´
	R	5´ CTTGTGAAGGTTTCAGATAGAGCCT 3´
	Probe	6FAM 5´ AGAATTGTTCTCTGTGTACTT 3´ MGB
NFKBIA	F	5´ TCCTCAACTTCCAGAACAACCTG 3´
	R	5´ TTCTGGCTGGTTGGTGATCA 3´
	Probe	6FAM 5´ CAGCAGACTCCACTCC 3´ MGB
53BP1	F	5´ GTCAGGTCATTGAGCAGTTACCTC 3´
	R	5´ TCCTCCACAGCAGGAGCAG 3´
	Probe	6FAM 5´ GGACAAGCAGTGTTCT 3´ MGB
CHEK2	F	5´TCCAAAGGCACGTTTTACGAC 3´
	R	5´TGTCTTCATCCTGAAGCCACG 3’
	Probe	6FAM 5´AGAAGAAGCCTTAAGACACC 3´ MGB
AXL silencing	shRNA	5´CCGGCGTGGAGAACAGCGAGATTTACTCGAGTAAA TCTCGCTGTTCTCCACGTTTTTTG 3´
AURBK silencing	shRNA	5´CCGGCCTGCGTCTCTACAACTATTTCTCGAGAAATA GTTGTAGAGACGCAGGTTTTT 3´
AURBK silencing (CRISPR)	sgRNA	5’ ATTCTAGAGTATGCCCCCCGCGG 3’