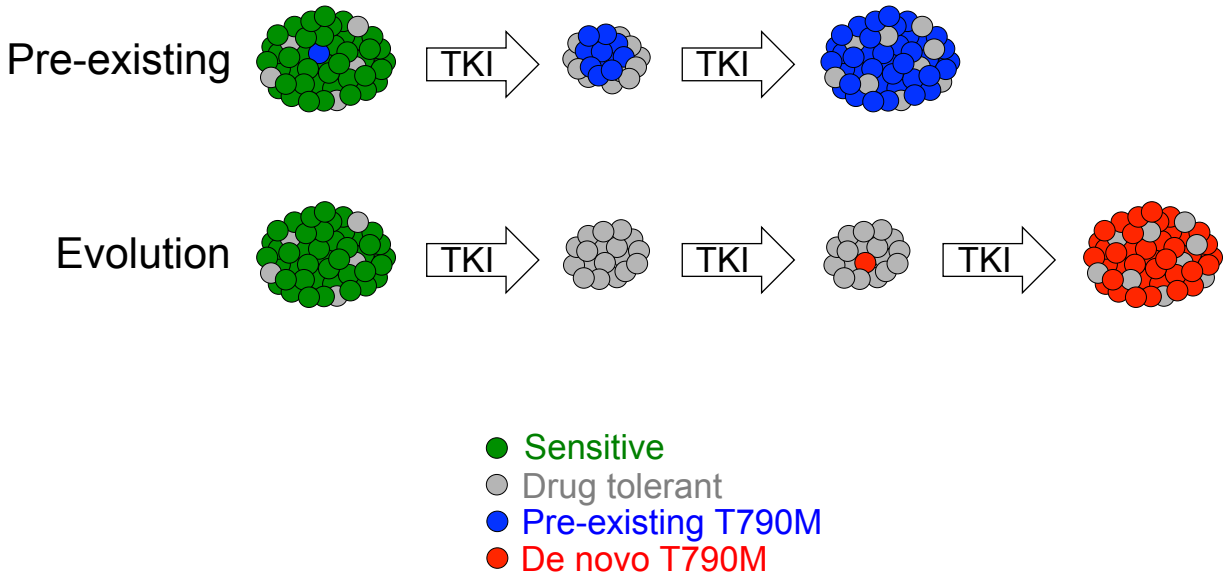
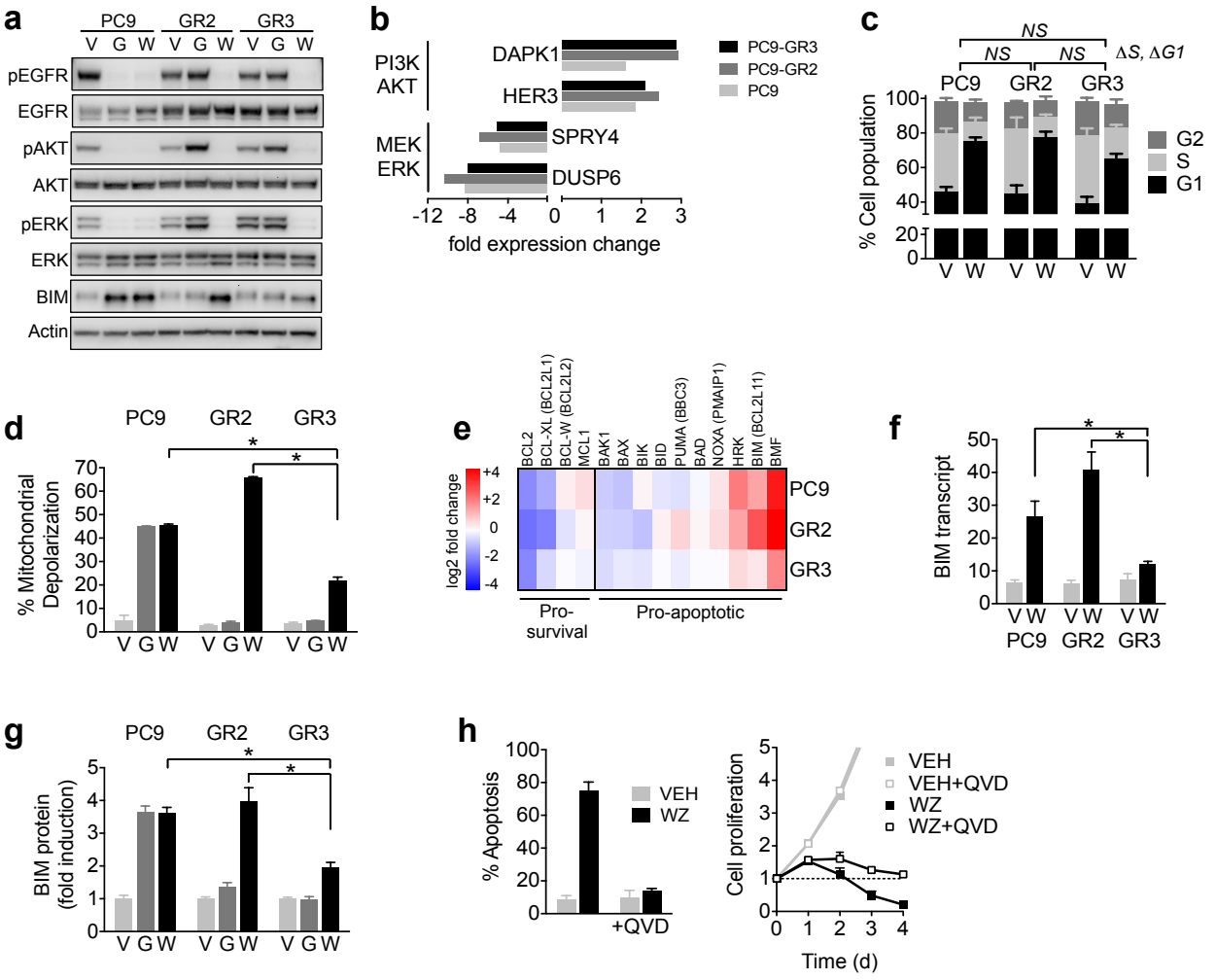
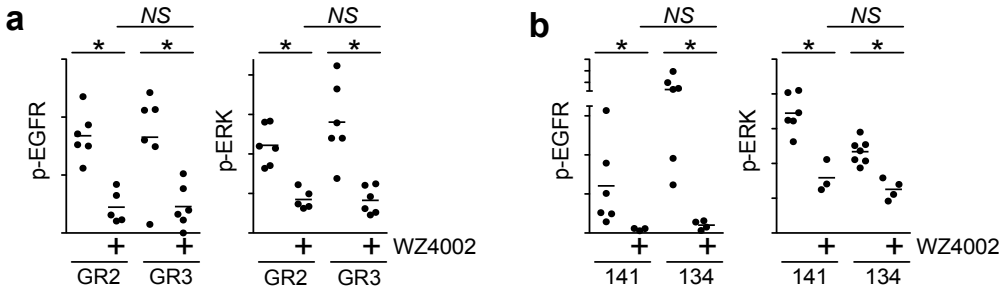


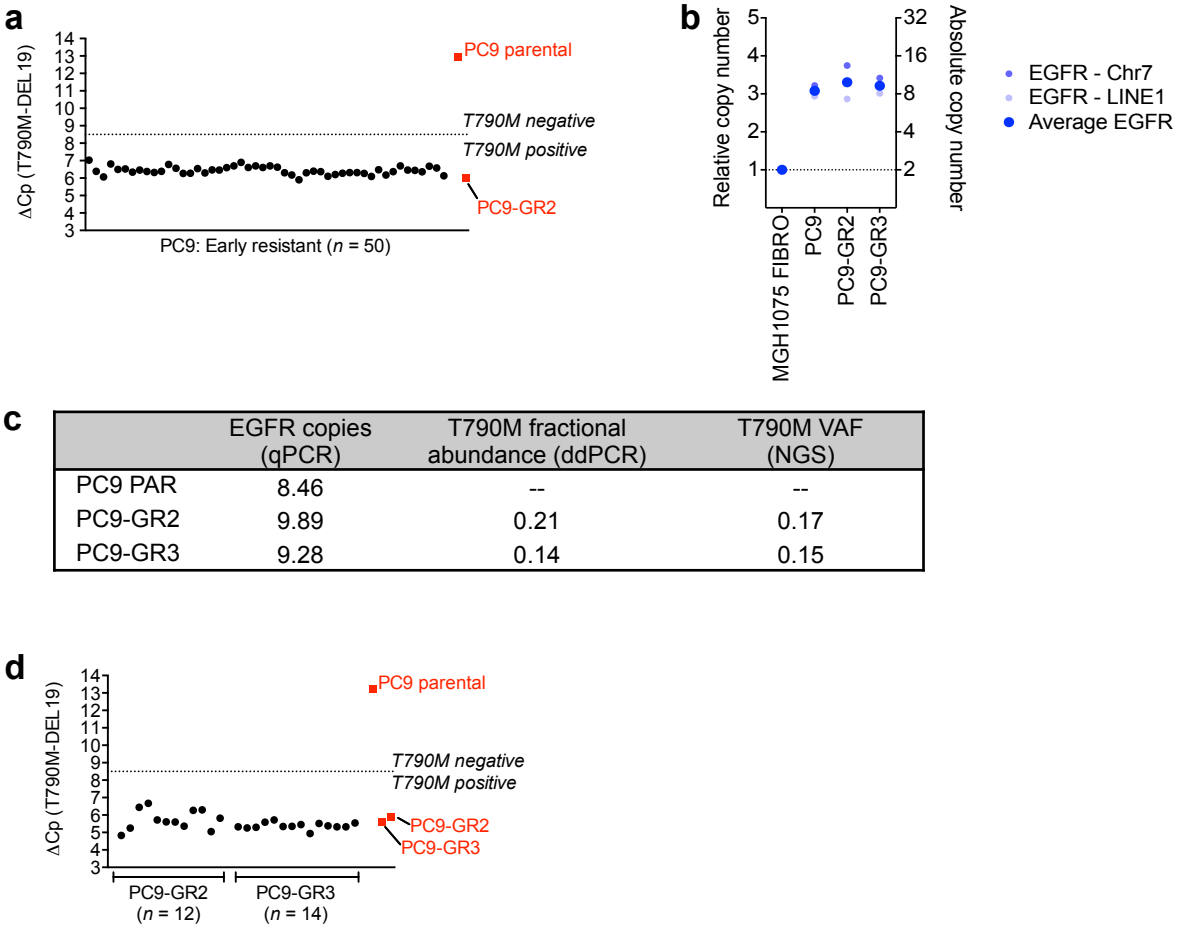
Supplementary Figure 1. Schematic depicting development of acquired resistance by either the emergence of a pre-existing resistant clone (e.g. T790M) versus evolution of a drug tolerant intermediate that acquires the genetic resistance mechanism during the course of drug treatment.



Supplementary Figure 2. WZ4002 inhibits EGFR phosphorylation and downstream MEK and PI3K signaling in PC9, GR2 and GR3 cells, but fails to up-regulate BIM and induce mitochondrial depolarization in PC9-GR3 cells. (**a)** Cells were treated with 1 μM gefitinib (G), WZ4002 (W) or vehicle (V) for 24 hours and lysates harvested for western blotting. (**b)** Transcriptional outputs of MEK/ERK (DUSP6, SPRY4) and PI3K/AKT signaling (HER3, DAPK1) are modulated equally by WZ4002 in PC9, GR2 and GR3 cells. Cell were treated with 1 μM WZ4002 or vehicle for 24 hours. Gene transcript levels were determined by quantitative RT-PCR and normalized to ACTIN. Values shown are fold difference in transcript level after WZ4002 treatment relative to vehicle control. (**c)** Cells were treated with 1 μM WZ4002 for 18 hours and cell cycle populations were determined by propidium iodide staining. There was no statistical difference between the % increase in cells in G1 or % decrease in cells in S phase after WZ4002 treatment between any of the three cell lines. **(d)** Cells were treated with 1 μM gefitinib (G), WZ4002 (W) or vehicle (V) in the presence of the 10 μM QVD-Oph (pan-caspase inhibitor) in triplicate. After 48 hours, cells were harvested and stained with JC-1 dye and analyzed by flow cytometry. **P* < 0.05, two-tailed t-test. (**e)** Cells were treated with 1 μM WZ4002 for 24 hours in duplicate and RNA harvested for RNA-Seq analysis. Values shown are $\log_2$ fold change after WZ treatment. (**f)** BIM mRNA transcript levels were quantified by quantitative RT-PCR following treatment with 1 μM WZ4002 for 24 hours and normalized to GAPDH (mean and standard error of 3 independent experiments, **P* < 0.05, two-tailed t-test). **g,** Cells were treated with 1 mM gefitinib (G), WZ4002 (W) or vehicle (V) and harvested for western blotting. Band intensities were quantitated by densitometric analysis and normalized to vehicle control (mean and standard error of 4 independent experiments, **P* < 0.05, two-tailed t-test). **h,** Induction of apoptosis is necessary for cytotoxic response of PC9-GR2 cells to WZ4002 in vitro. Cells were treated with vehicle or 1 μM WZ4002 in the presence or absence of QVD-Oph. Apoptosis was determined by annexin staining after 72 hours. Cell proliferation was determined by CellTiter-Glo assay. Blocking the apoptotic response in PC9-GR2 cells resulted in cytostatic response similar to PC9-GR3 cells (see **Fig. 1c**).



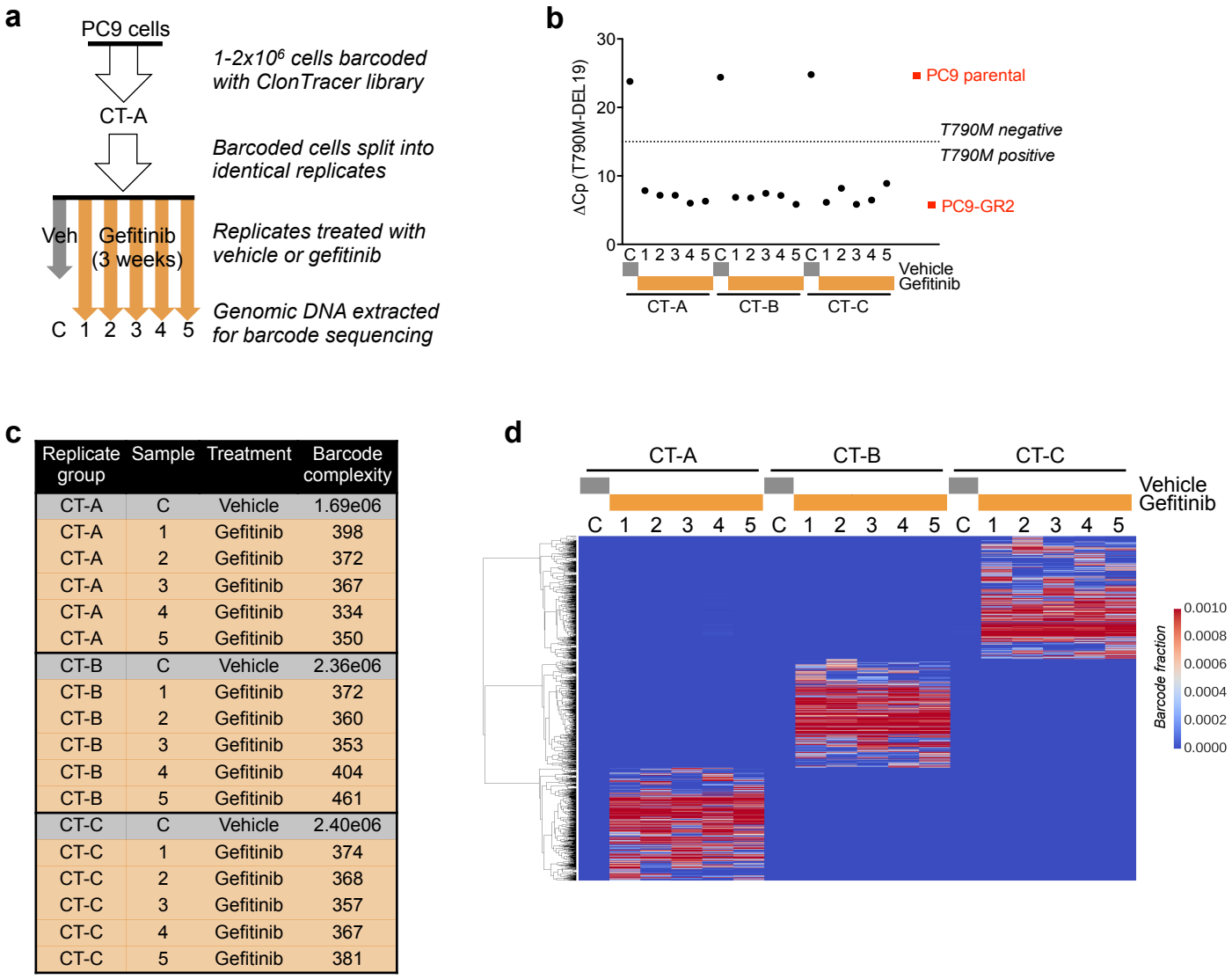
Supplementary Figure 3. Inhibition of EGFR and ERK phosphorylation after WZ4002 treatment in xenograft tumors. Mice were treated with 50 mg/kg/day WZ4002 daily and tumors were harvested 3 hours after the day 3 dose for western blot analysis. Phosphorylated EGFR and phosphorylated ERK1/2 band intensities were quantified by densitometric analysis, and each data point is the relative phospho-protein abundance from an individual mouse. Asterisks indicate that statistically significant decreases in EGFR and ERK phosphorylation were observed after drug treatment for all models. There were no significant differences in phosphorylation levels in drug treated tumors between (a) GR2 versus GR3 and (b) MGH141 versus MGH134 models. *$P < 0.05$, two-tailed t-test.



c

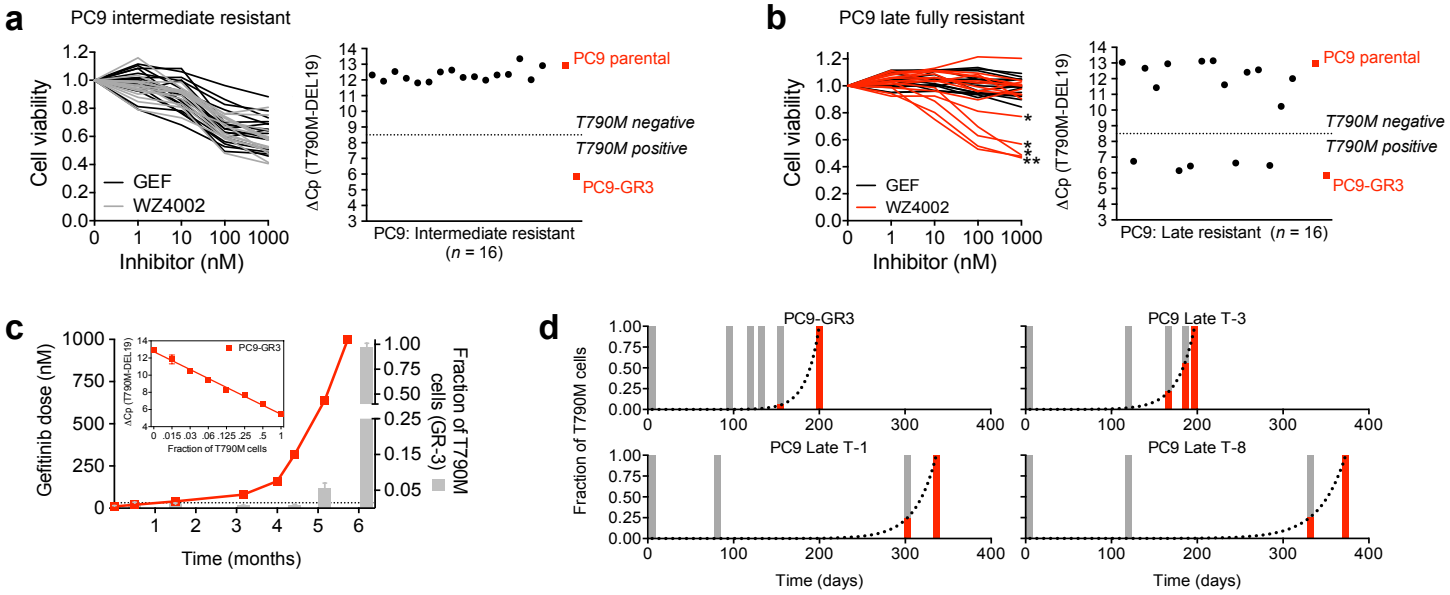
	EGFR copies (qPCR)	T790M fractional abundance (ddPCR)	T790M VAF (NGS)
PC9 PAR	8.46	--	--
PC9-GR2	9.89	0.21	0.17
PC9-GR3	9.28	0.14	0.15

Supplementary Figure 4. Characterization of T790M alleles in gefitinib resistant PC9 cells. (a) T790M allele specific quantitative PCR performed on genomic DNA isolated from 50 early gefitinib resistant clones revealed that all harbor the T790M mutation. Values shown are the difference in Cp values between the T790M specific probe and exon 19 deletion control. **(b)** EGFR is amplified in PC9 cells. EGFR amplification was assessed by quantitative PCR on genomic DNA. Copy number relative to LINE1 and chromosome 7 was calculated after normalization to MGH1075 (stromal fibroblast DNA) diploid control. **(c)** T790M allelic abundance in PC9-GR2 and PC9-GR3 cells as determined by droplet digital PCR and next generation sequencing corresponds to one T790M mutant allele per 8-10 total EGFR alleles. **(d)** Single cell clones of PC9-GR2 and PC9-GR3 lines were established and assayed for the presence of T790M. All clones examined were T790M positive, ruling out the alternative possibility that the observed T790M fractional abundance (ddPCR) and VAF (NGS) is due to a mixed population of T790M-positive and negative cells.



Replicate group	Sample	Treatment	Barcode complexity
CT-A	C	Vehicle	1.69e06
CT-A	1	Gefitinib	398
CT-A	2	Gefitinib	372
CT-A	3	Gefitinib	367
CT-A	4	Gefitinib	334
CT-A	5	Gefitinib	350
CT-B	C	Vehicle	2.36e06
CT-B	1	Gefitinib	372
CT-B	2	Gefitinib	360
CT-B	3	Gefitinib	353
CT-B	4	Gefitinib	404
CT-B	5	Gefitinib	461
CT-C	C	Vehicle	2.40e06
CT-C	1	Gefitinib	374
CT-C	2	Gefitinib	368
CT-C	3	Gefitinib	357
CT-C	4	Gefitinib	367
CT-C	5	Gefitinib	381

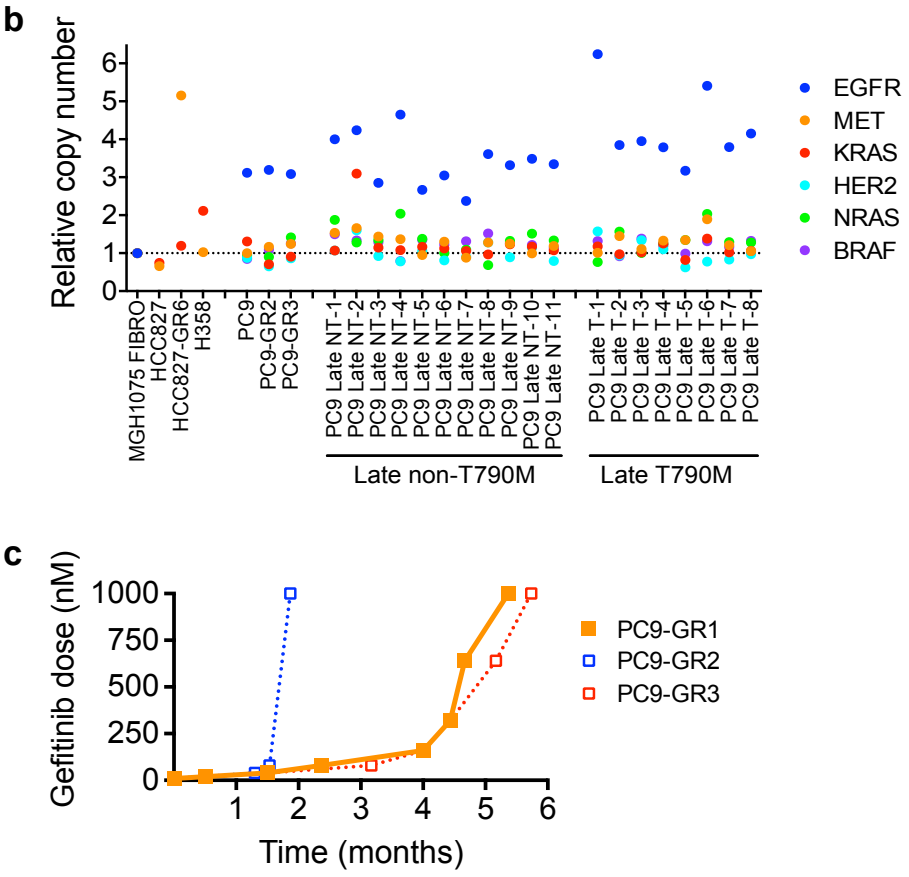
Supplementary Figure 5. ClonTracer barcode analysis of gefitinib treated PC9 cells. (a) Schematic of ClonTracer (CT) experimental setup. Three independent barcoded PC9 populations were generated (CT-A, CT-B, CT-C) and treated in identical fashion, **(b)** T790M allele specific PCR demonstrates that gefitinib treated barcoded PC9 populations are enriched for early resistant T790M clones. **(c)** Barcode complexity of vehicle treated (pre-treatment) and gefitinib selected PC9 populations as determined by next generation sequencing of barcodes. To accomodate the barcode complexity observed in vehicle treated groups, top 95% of the total cell population was used for estimating the number of unique barcodes. **(d)** Heatmap depicting barcode abundance in vehicle (pre-treatment) and gefitinib treated PC9 populations. Note that replicates of the same transduced pool exhibit substantial overlap of enriched barcodes, whereas there is negligible overlap of barcodes between each of the three independent pools.



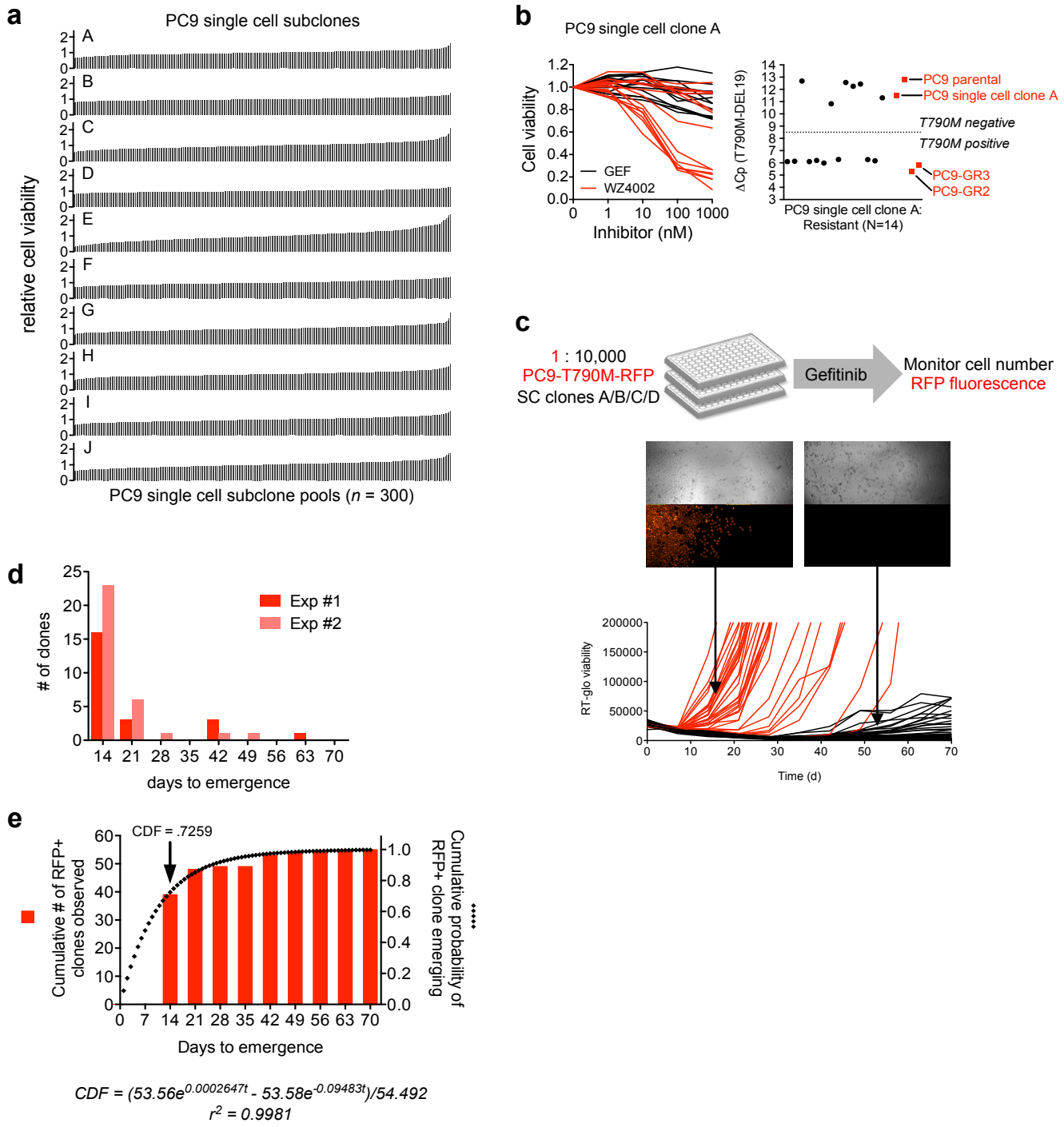
Supplementary Figure 6. Emergence of T790M resistant clones from drug tolerant cells.
(a) Intermediate resistant PC9 cells expanded from drug tolerant cells (after 12-16 weeks gefitinib treatment) are negative for T790M by allele specific qPCR. **(b)** A subset of late (fully) gefitinib resistant PC9 cells derived from drug tolerant cells acquire T790M. Asterisks denote WZ4002 sensitivity of T790M positive late resistant cells. **(c)** Fractional abundance of T790M cells can be determined by T790M allele specific qPCR. Intermediate freeze-downs of PC9-GR3 cells during the development of gefitinib resistance were assayed for the presence of T790M. Red line shows gefitinib dose tolerated as a function of time and is replotted from **Fig. 1a**. Gray bars show fraction of T790M cells in the total population. Inset shows calibration of standard curve. No T790M was detected prior to 5 months (threshold for detection 1.5%), whereas 6% of population was T790M positive at 5.5 months. **(d)** Fraction of T790M cells (red bar) at intermediate time points for selected late resistant T790M clones. PC9-GR3 is shown for comparison. The dotted line depicting an exponential growth curve fit to the data is shown for illustrative purposes.

a

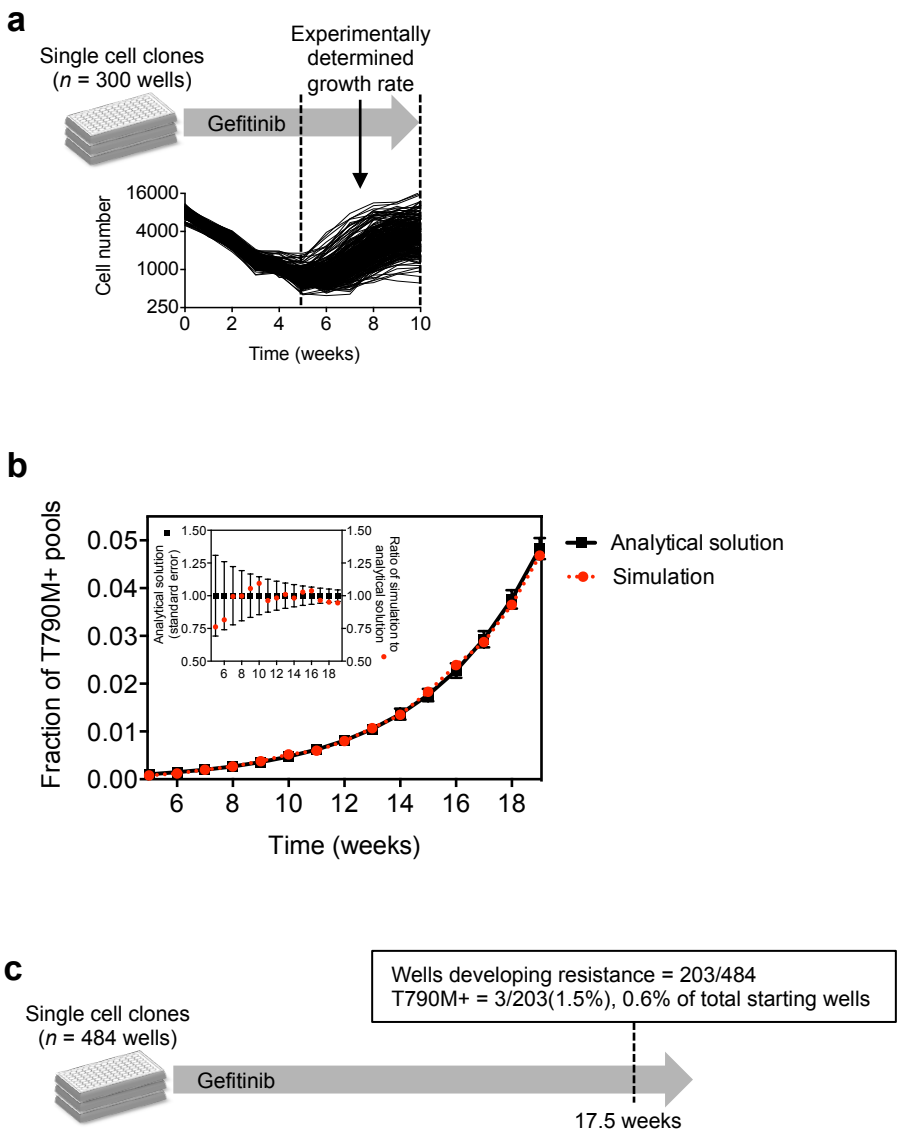
	Parental Variant 1	Parental Var. 2	Parental Var. 3	Parental Var. 4	Novel Var. 1	AF	Novel Var. 2	AF	Novel Var. 3	AF
PC9 Late NT-1	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	NRAS p.G12V	13%				
PC9 Late NT-2	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	KRAS p.K147E	77%				
PC9 Late NT-3	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	NRAS p.A146T	37%				
PC9 Late NT-4	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	NRAS p.G12A	14%				
PC9 Late NT-5	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	None					
PC9 Late NT-6	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	None					
PC9 Late NT-7	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	None					
PC9 Late NT-8	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	RET p.Leu923R	21%				
PC9 Late NT-9	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	BRAF p.G469A	15%				
PC9 Late NT-10	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	CDH1 p.T40K	NRASp.G12R	10%	NRAS p.E63K	10%	KRAS p.G12R	10%
PC9 Late NT-11	EGFR p.E746_A750del	TP53 p.R248Q	CDKN2A p.G67V	LOST	NRAS p.Q61P	25%				



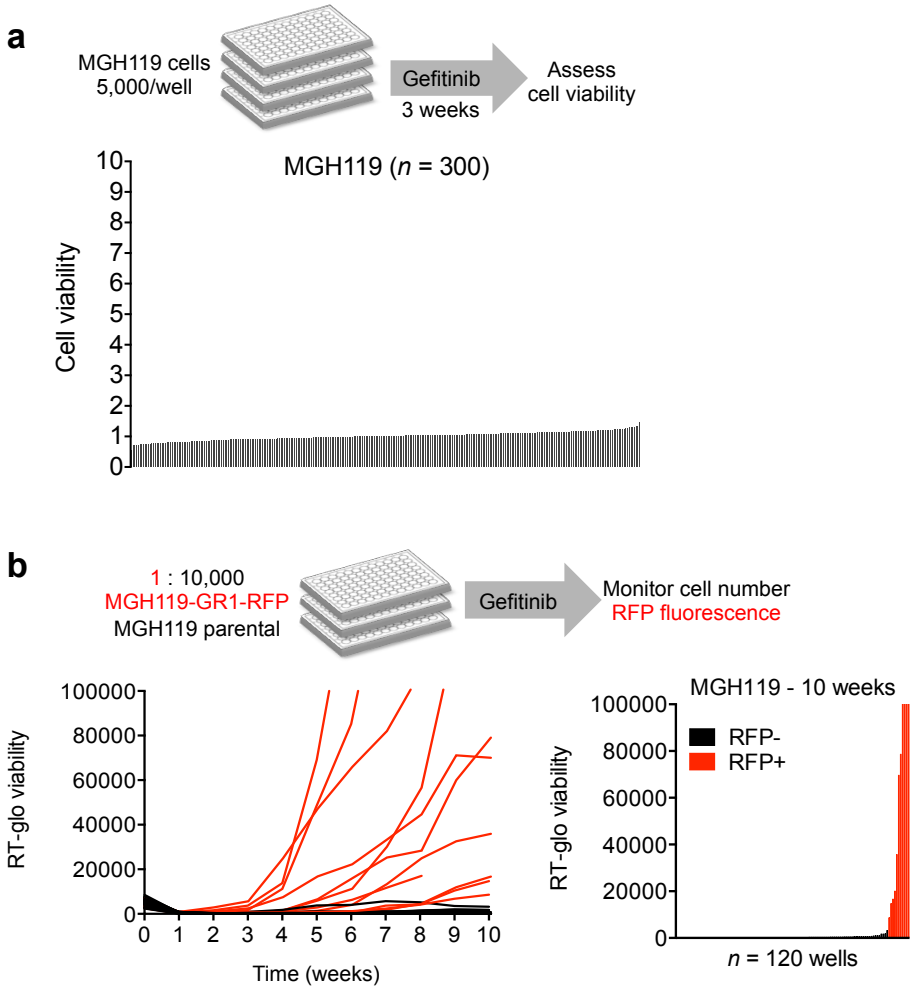
Supplementary Figure 7. Assessment of mechanisms of resistance in late non-T790M resistant clones. **(a)** MGH Snapshot NGS genotyping of late non-T790M gefitinib resistant PC9 clones. **(b)** Assessment of MET, KRAS, EGFR, HER2, NRAS and BRAF amplification by quantitative PCR for gefitinib resistant PC9 clones. Copy number relative to LINE1 (MET, KRAS, EGFR, HER2, NRAS and BRAF), chromosome 12 (KRAS) or chromosome 7 (MET, EGFR, BRAF) was calculated after normalization to MGH1075 (stromal fibroblast DNA) diploid control. HCC827-GR6 is MET amplified positive control, H358 is KRAS amplified positive control. **(c)** PC9 parental cells were cultured in escalating concentrations of gefitinib as tolerated until fully resistant, which was defined as lack of inhibitory effect of drug on cell proliferation. PC9-GR1 developed at a nearly identical rate as PC9-GR3 cells but did not harbor T790M and were sensitive to combination EGFR+MEK inhibition (Ref. 14). PC9-GR2 and PC9-GR3 cells are replotted from Figure 1a for comparison.



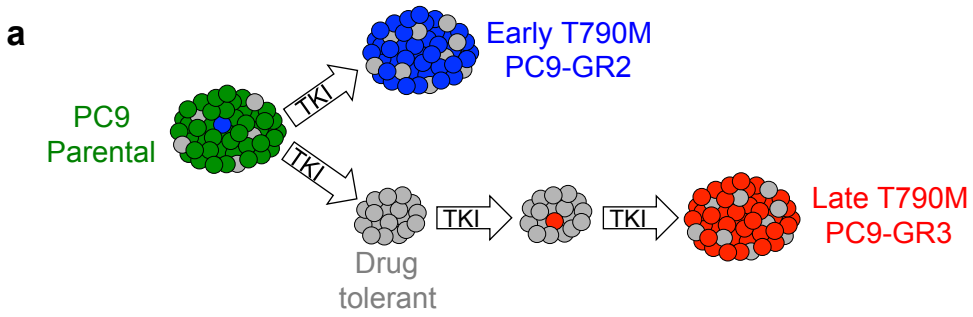
Supplementary Figure 8. T790M can emerge from drug tolerant clones. (**a)** No early T790M resistant clones were observed after treating ten independent PC9 single cell sub-clones (5,000 cells/well) with gefitinib for two weeks. **(b)** A subset of gefitinib resistant clones emerging from PC9 single cell sub-clone A develop T790M. **(c)** Introduction of pre-existing T790M cells into single cell subclone populations recapitulates emergence of early resistant T790M clones. An early T790M resistant clone (see Fig. 2b) was labeled with RFP to generate PC9-T790M-RFP cells, which were spiked into a combined background of unlabeled single cell-derived subclones A-D at a frequency of 1:10,000. Cells were treated with gefitinib and cell viability was measured weekly with RealTime-Glo assay. **(d)** The number of resistant wells that were newly detected at each time interval for two independent experiments. **(e)** A cumulative density function modeling the emergence of PC9-T790M-RFP clones indicates that 72.6% of emerging pre-existing T790M clones are detectable 2 weeks after starting gefitinib treatment. We observed that 4 out of 4 single cell subclones (A-D) yielded late T790M resistant clones, but no resistant clones at 2 weeks. The probability that these late T790M clones emerged from undetected pre-existing T790M cells is $0.274^4 = 0.006$.



Supplementary Figure 9. Mathematical modeling and experimental determination of acquisition of T790M during drug treatment. (a) PC9 pools (*n* = 300) were treated with 300 nM gefitinib and cell number tracked over time using RealTime-Glo assay. The population sizes between weeks 5-10 were used to fit growth parameters for mathematical modeling of evolution of T790M from drug tolerant cells. **(b)** To mathematically model the acquisition of T790M during drug treatment, we applied a simple branching model, similar to Chmielecki et al. (Ref. 43), estimating the range of the birth rate of drug tolerant cells calculated from previously reported single cell imaging experiments of PC9 cells treated with erlotinib (Ref. 44). The growth rate and the initial population size of the drug tolerant PC9 cells were determined by experimentally measuring the proliferation of drug tolerant PC9 cell pools over time (see **Supplementary Fig. 9a**). We anlaytically solved and simulated this model. Comparison between the analytical solution (black squares) and the Monte Carlo simulation (red circles) shows very close correlation between predicted frequency of acquiring the T790M mutation in drug tolerant cells (error bars indicate the standard error of the mean). The inset compares the ratio of the simulation (10,000 runs for every week) and the analytical solution (red circles) to the calculated theoretical prediction (error bars indicate the standard error divided by the analytical values). Parameter values are the same as in **Fig. 3d**. **(c)** 484 pools of 5,000 PC9 cells representing 8 different single cell clonal lines were treated with 300 nM gefitinib for 17.5 weeks (we estimated that a cell acquiring T790M would take ~1.5 weeks to reach sufficient population size to be detected by our genotyping assay). 203 pools with developing resistance had expanded to sufficient numbers to perform T790M genotyping. 3/203 were found to be T790M positive.



Supplementary Figure 10. The MGH119 cell line does not harbor pre-existing T790M cells. (a) No early resistant colonies emerge from MGH119 cells upon gefitinib treatment. MGH119 cell pools (5,000/well) were treated with 300 nM gefitinib for three weeks and cell viability determined with CellTiter-Glo assay. **(b)** Modeling emergence of pre-existing MGH119 T790M clones. MGH119-GR1 cells were labeled with RFP and introduced at low frequency into a background of unlabeled MGH119 parental cells. Cells (120 wells, 5,000 cells/well) were treated with 300 nM gefitinib and emerging resistant colonies were monitored by RealTime-Glo assay and RFP fluorescence. Note that all emerging resistant colonies derive from the introduced RFP-labeled pre-existing T790M cells. Right panel shows cell viability for all wells (each bar represents one well) at 10 weeks.



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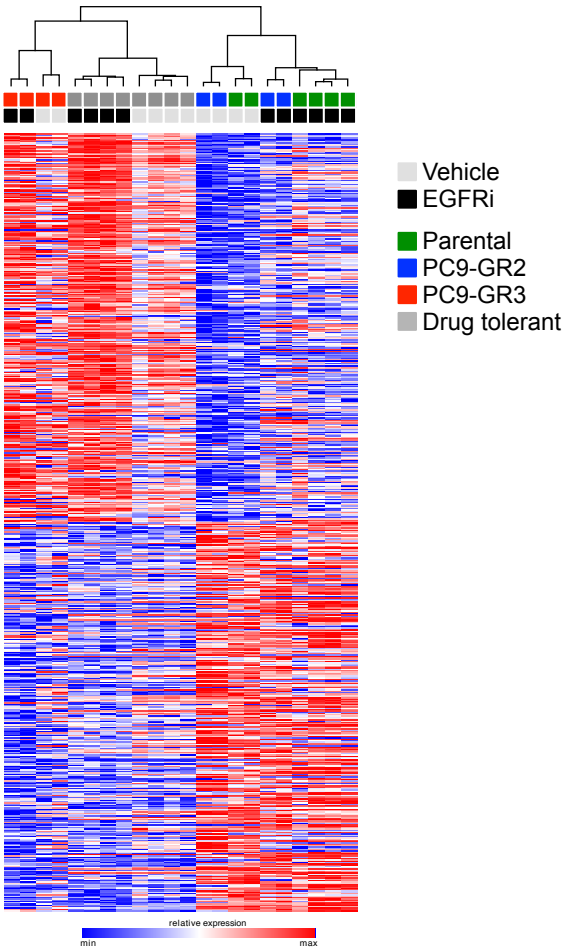
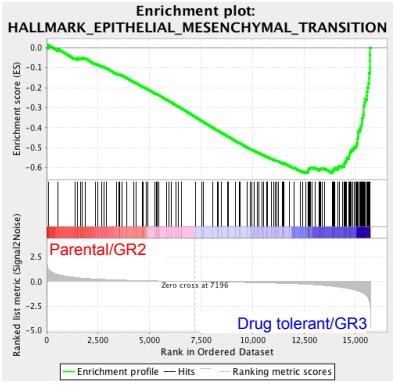


c

NAME	GENE SET	SIZE	ES	NES	NOM p-val	FDR q-val	FWER p-val
HALLMARK	HALLMARK_EPITHELIAL_MESENCHYMAL_TRANSITION	141	-0.6284	-2.0851	0	0	0
ONCOGENIC SIG	E2F3_UP.V1_UP	145	-0.5963	-1.9976	0	0	0
HALLMARK	HALLMARK_INTERFERON_GAMMA_RESPONSE	150	-0.5779	-1.9388	0	0	0
HALLMARK	HALLMARK_INTERFERON_ALPHA_RESPONSE	80	-0.6005	-1.8732	0	0	0
ONCOGENIC SIG	CORDENONSI_YAP_CONSERVED_SIGNATURE	53	-0.6403	-1.8571	0	0.003674	0.007
HALLMARK	HALLMARK_ALLOGRAFT_REJECTION	115	-0.5231	-1.6907	0	0.008139	0.031

d



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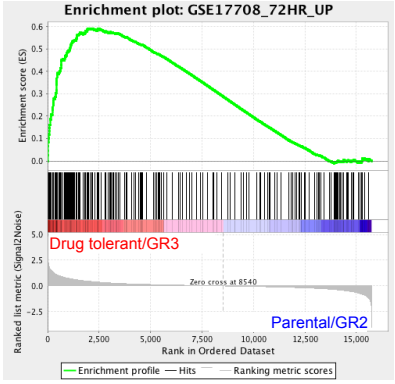
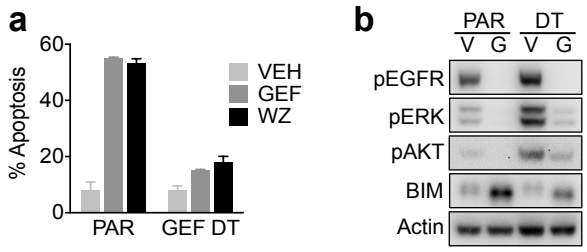
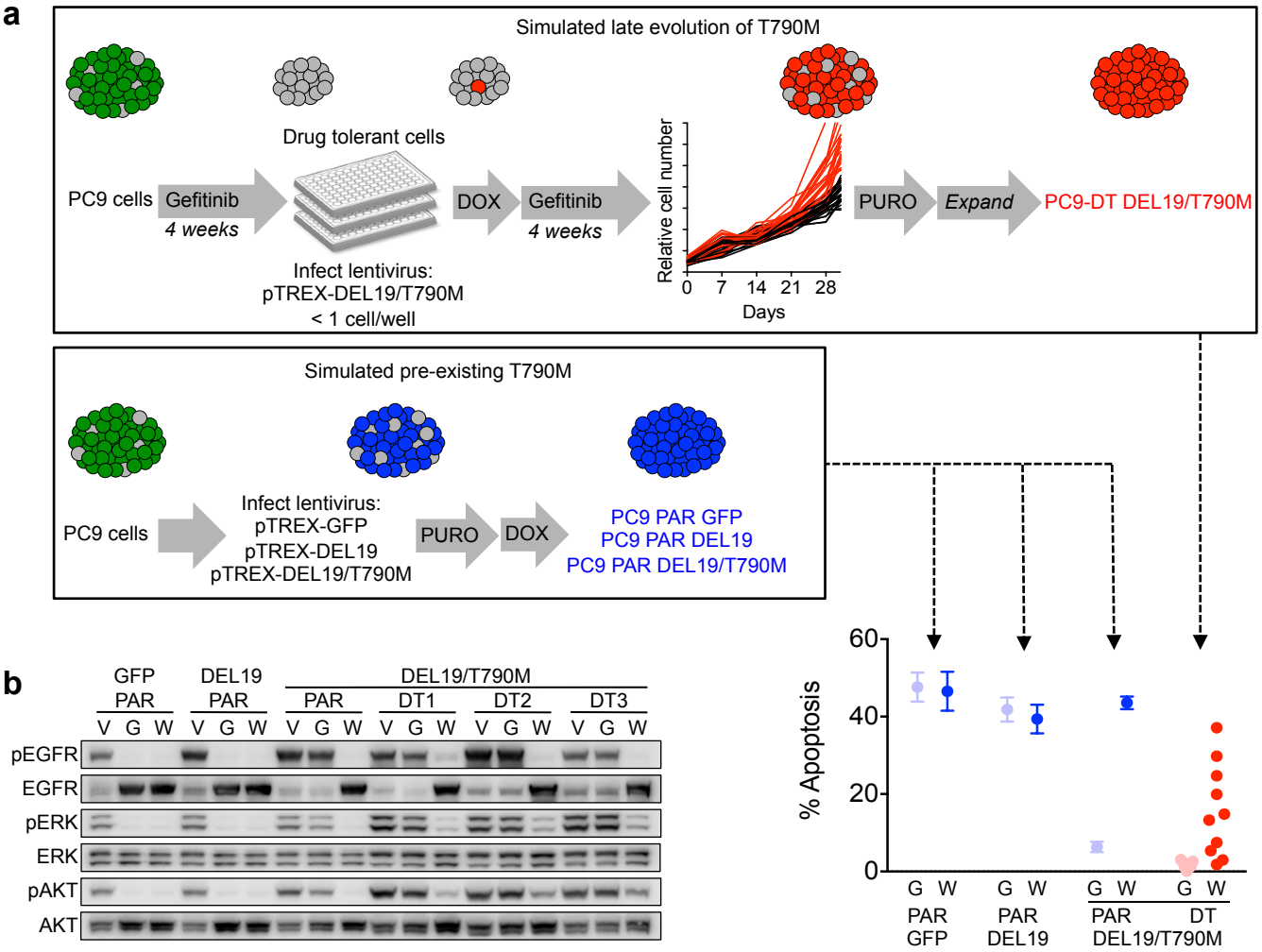


Enrichment Score (ES)	0.5921152
Normalized Enrichment Score (NES)	2.0599198
Nominal p-value	0.0
FDR q-value	0.0
FWER p-Value	0.0

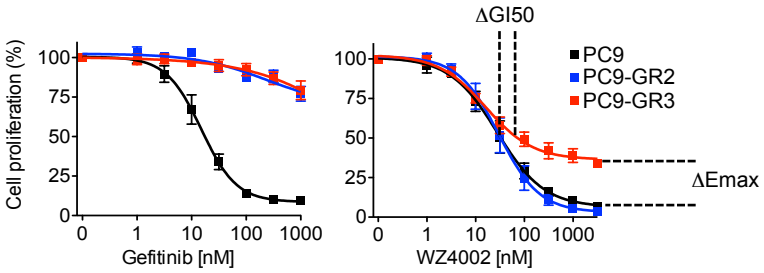
Supplementary Figure 11. RNA-Seq analysis of PC9 cell populations. (a) Proposed evolutionary relationship of samples analyzed by RNA-Seq. **(b)** Heatmap of top and bottom 600 genes contributing most strongly to variance along principal component 2 (PC2) demonstrates similarity of expression patterns in PC9-GR3/drug tolerant versus PC9-GR2/parental cells. **(c)** Gene set enrichment analysis was performed by assessing the enrichment of various Hallmark and Oncogenic Signature sets from the Molecular Signatures Database (MSigDB) among genes ranked by differential expression between PC9-GR3/drug tolerant and PC9-GR2/parental cell groups. Six gene sets were significantly enriched with a FDR q-value less than 0.01. Epithelial-to-mesenchymal transition signature was the top significantly enriched set among genes upregulated in PC9-GR3/drug tolerant cells (EMT-related gene sets are highlighted in yellow.) **(d)** Significant enrichment of genes associated with TGF-β induced EMT in lung cancer cells (GSE17708) was also observed in PC9-GR3/ drug tolerant cells.



Supplementary Figure 12. Drug tolerant cells are characterized by loss of apoptotic response despite inhibition of EGFR signaling. (a) Gefitinib tolerant PC9 cells have decreased apoptotic response to EGFR inhibition. PC9 drug tolerant cells were selected in gefitinib for two weeks and apoptotic response to 1 μM gefitinib or WZ4002 was determined (PAR = parental cells, GEF DT = gefitinib tolerant cells). **(b)** Drug tolerant cells maintain residual pAKT and pERK signaling in the presence of gefitinib and have impaired upregulation of BIM.



Supplementary Figure 13. Acquisition of T790M by drug tolerant cells leads to emergence of T790M clones with reduced apoptotic sensitivity to WZ4002. (a) To model evolution of T790M from drug tolerant cells, PC9 cells were treated with 300 nM gefitinib for 4 weeks to generate T790M-negative drug tolerant cells. Pools of 1,000 drug tolerant cells were infected with lenti pTREX-DEL19/T790M at a low titer to infect 1 cell per every other well. Cells were treated with doxycycline (DOX) to induce expression of T790M and then cultured in the presence of gefitinib. Cell number was assayed weekly using RealTime-Glo assay. After 2-3 weeks, pTREX-DEL19/T790M resistant colonies were detected above background drug tolerant cells. Red lines indicate wells with emerging pTREX-DEL19/T790M clones, black lines indicate wells with only drug tolerant cells. After 30 days, all cells were treated with puromycin to distinguish which drug tolerant clones contained the pTREX-DEL19/T790M transgene (designated PC9 DT-DEL19/T790M) and to eliminate uninfected drug tolerant cells. Nine out of ten clones tested had reduced apoptotic response to 1 μM WZ4002 compared to PC9 parental cells infected with lenti pTREX-DEL19/T790M (and pTREX-DEL19 and pTREX-GFP controls), which simulate pre-existing T790M cells (G = Gefitinib, W= WZ4002; data are expressed as % apoptosis above vehicle control). **(b)** Western blot confirmation of expression of T790M EGFR in three pTREX-DEL19/T790M drug tolerant clones. Cells were treated with 1 μM geftinib or WZ4002 for 24 hours. **(c)** Western blot confirmation of expression of T790M EGFR in MGH119-pTREX- T790M cells (see **Fig. 4b**).



Supplementary Figure 14. Rationale for re-analyzing patient-derived cell line combination drug screen data. Loss of apoptotic response in PC9-GR3 cells manifests more prominently as a change in Emax rather than a shift in GI50.

Supplementary Table 1. Characteristics of patient tumors and corresponding cell lines.

	Patient characteristics					Cell line	
	1° EGFR mutation	EGFR TKI	TTP (Months)	Post-resistant sample	Clinical resistance mutation	1° EGFR mutation	Resistance mutation
MGH121	Ex 19 del	Erlotinib	6.4	Pleural Effusion	T790M	Ex 19 del	T790M
MGH125	L858R	Erlotinib	13.0	Pleural Effusion	T790M	L858R	T790M
MGH134	L858R	Erlotinib	7.0	Pleural Effusion	T790M	L858R	T790M
MGH138	Ex 19 del	Erlotinib/Gefitinib	36.5	Pleural Effusion	T790M	Ex 19 del	T790M
MGH141	Ex 19 del	Erlotinib	5.1	Pleural Effusion	T790M	Ex 19 del	T790M
MGH157	Ex 19 del	Erlotinib	10.9	Core Biopsy (bone)	T790M	Ex 19 del	T790M
MGH164	Ex 19 del	Erlotinib	6.0	Pleural Effusion	T790M	Ex 19 del	T790M