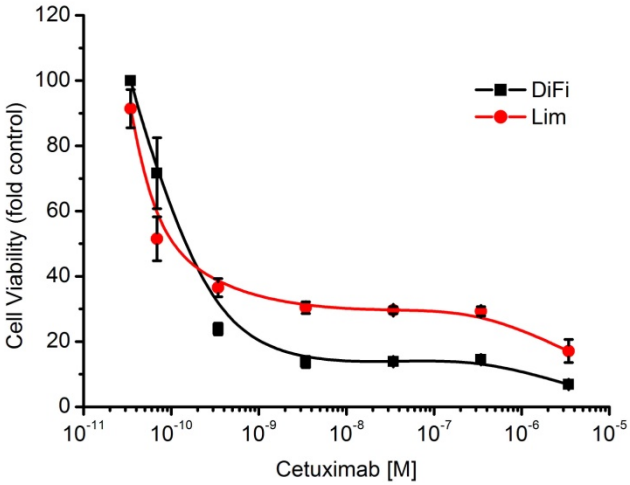
Supplementary Figure 1

a



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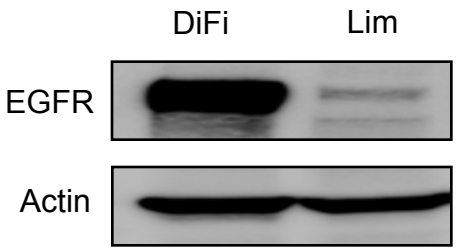

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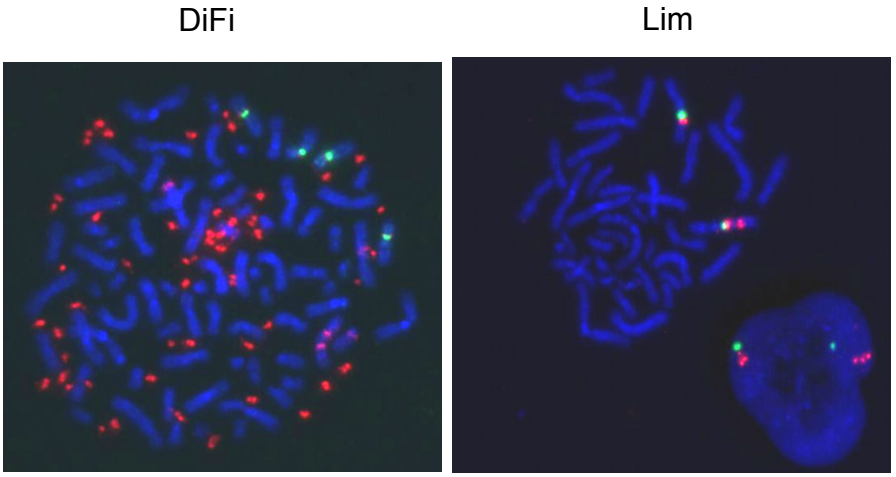
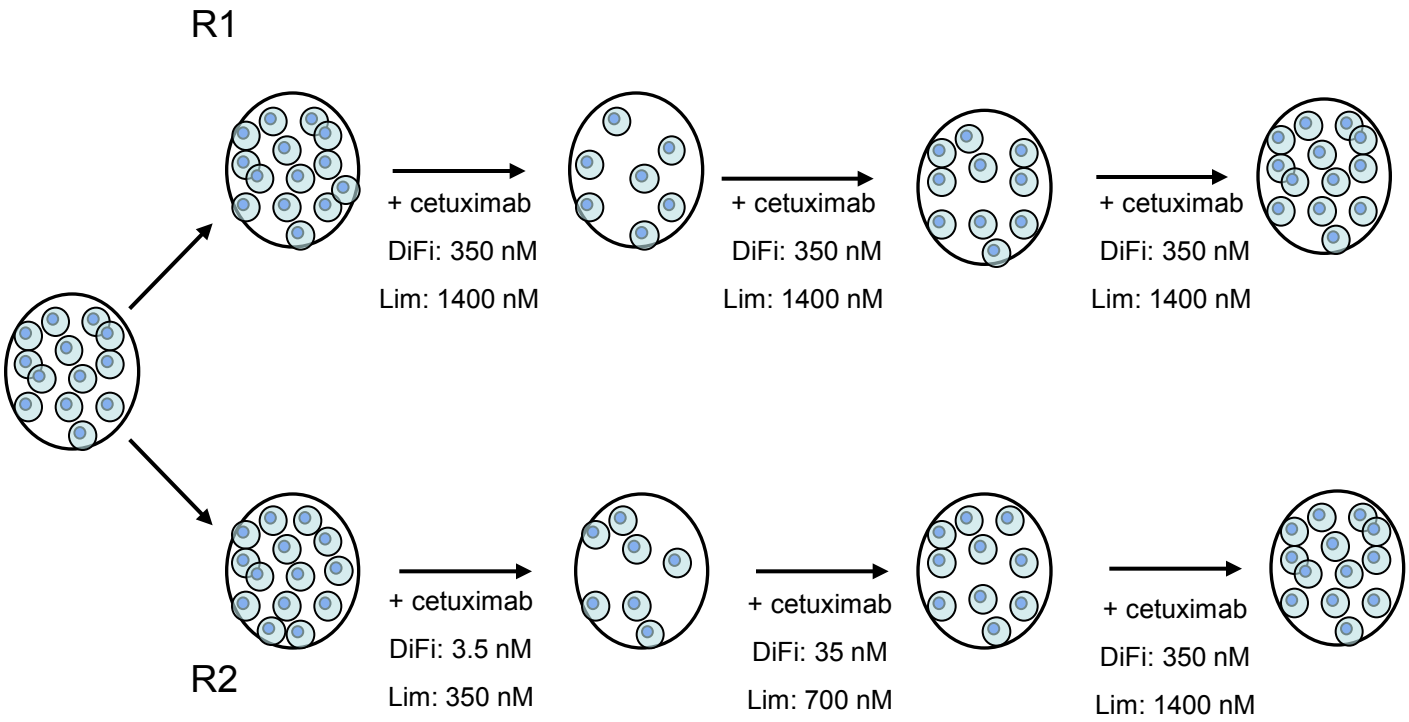


Sensitivity to cetuximab of the DiFi and LIM1215 parental cell lines (a) The indicated cell lines were treated for one week with increasing doses of cetuximab. Cell viability was assayed by the ATP assay. Data points represent means ± SD of three independent experiments. (b) Western blot analysis of EGFR expression levels in DiFi and Lim1215 cells. (c) FISH analysis of the EGFR gene in DiFi and Lim1215 cell lines. Red EGFR gene probe; Green Chr 7 centromeric probe.

Supplementary Figure 2

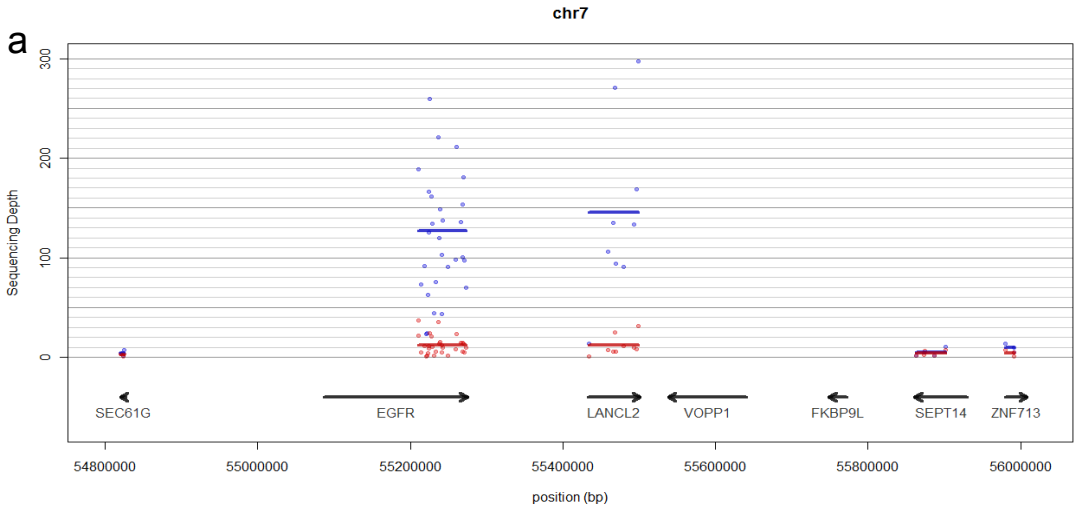


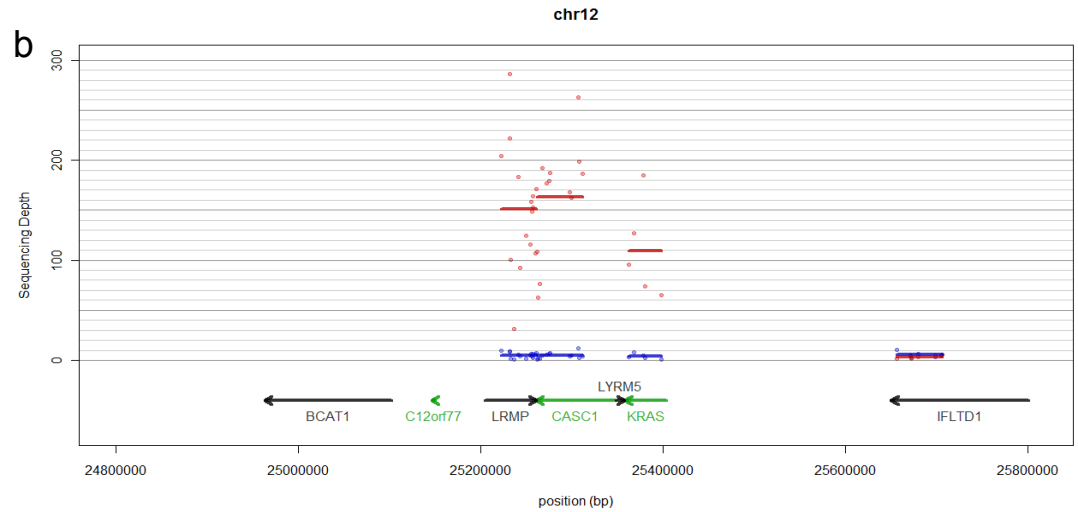
R1: Constant dosage

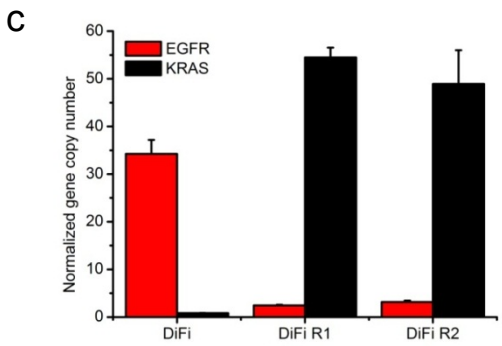
R2: Incremental dosage

Schematic representation of the strategy used to derive cetuximab resistant cell lines. The concentrations of drug and the protocols (constant and incremental) are illustrated.

Supplementary Figure 3

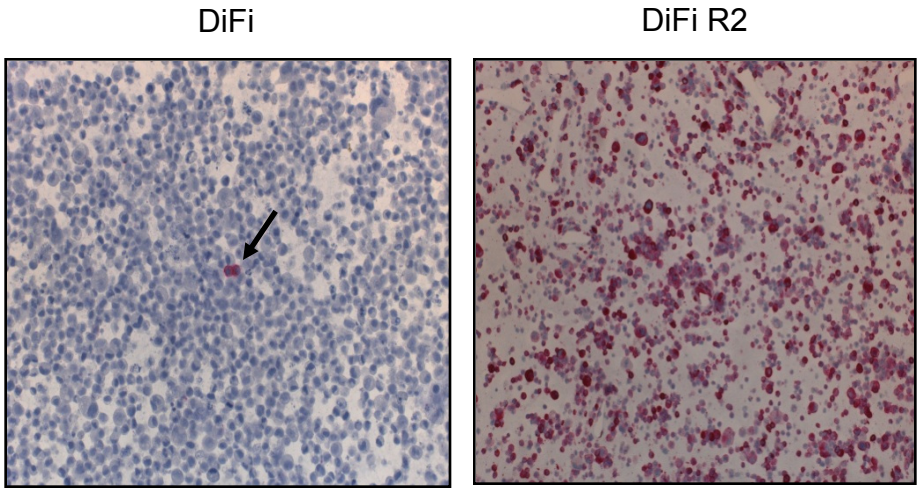






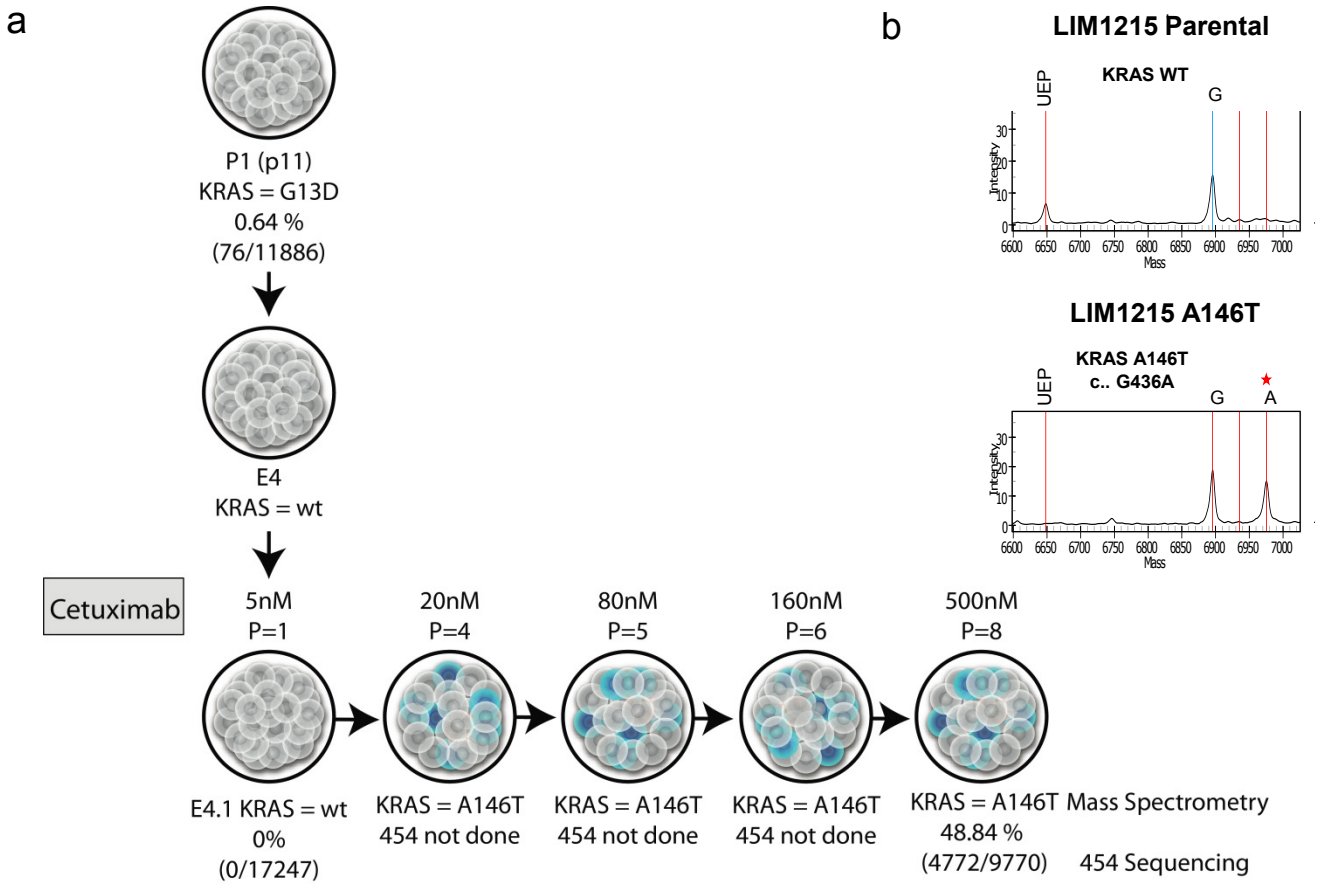
Acquired resistance to cetuximab is associated with focal amplification of the KRAS locus in DiFi cells (a-b) High resolution analysis of EGFR and KRAS amplicons in parental and cetuximab resistant DiFi cells. Dots represent exon-averages while segments are gene-averages of the sequencing depth (blue: parental DiFi; red: resistant DiFi). (c) The number of copies corresponding to the EGFR and KRAS loci was determined by real-time quantitative PCR using gDNA extracted from DiFi parental, R1 and R2 cells. Primers designed to span centromeric regions of chromosomes 7 or 12 were employed to normalize data for aneuploidy. Genomic DNA from a diploid cell line (HCEC) was used as a reference control

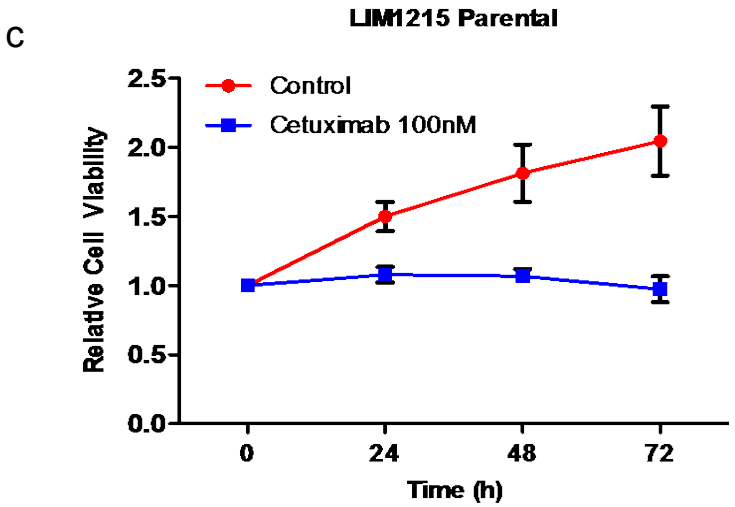
Supplementary Figure 4

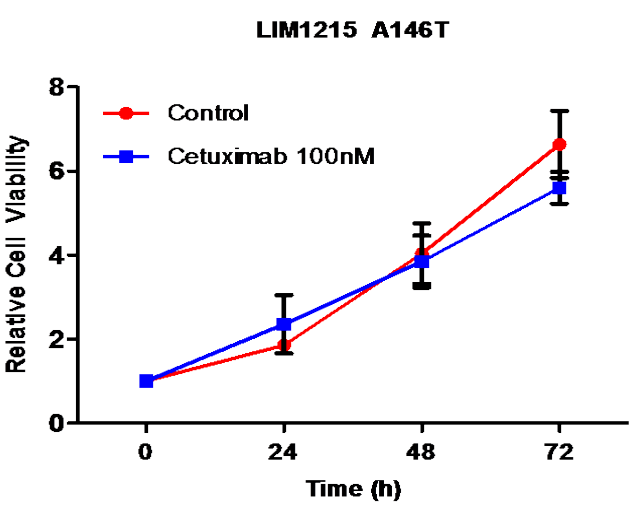


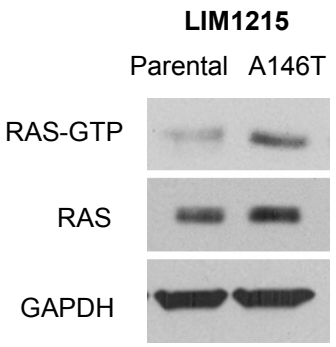
KRAS amplified cells are present in DiFi cells before cetuximab treatment. Immunostaining of KRAS protein in DiFi parental and resistant cells shows the presence of KRAS over expressing cells in the parental population.

Supplementary Figure 5



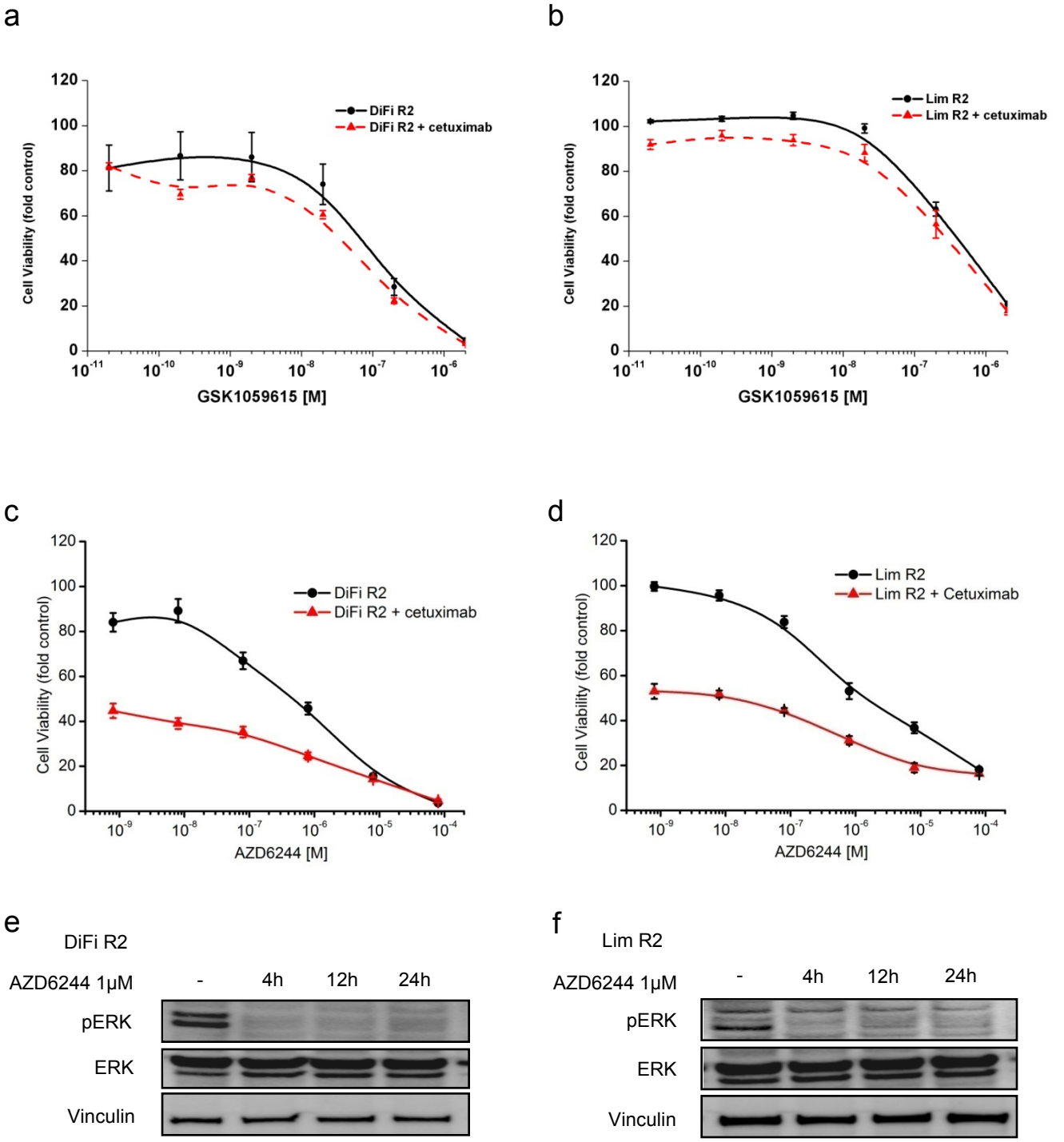






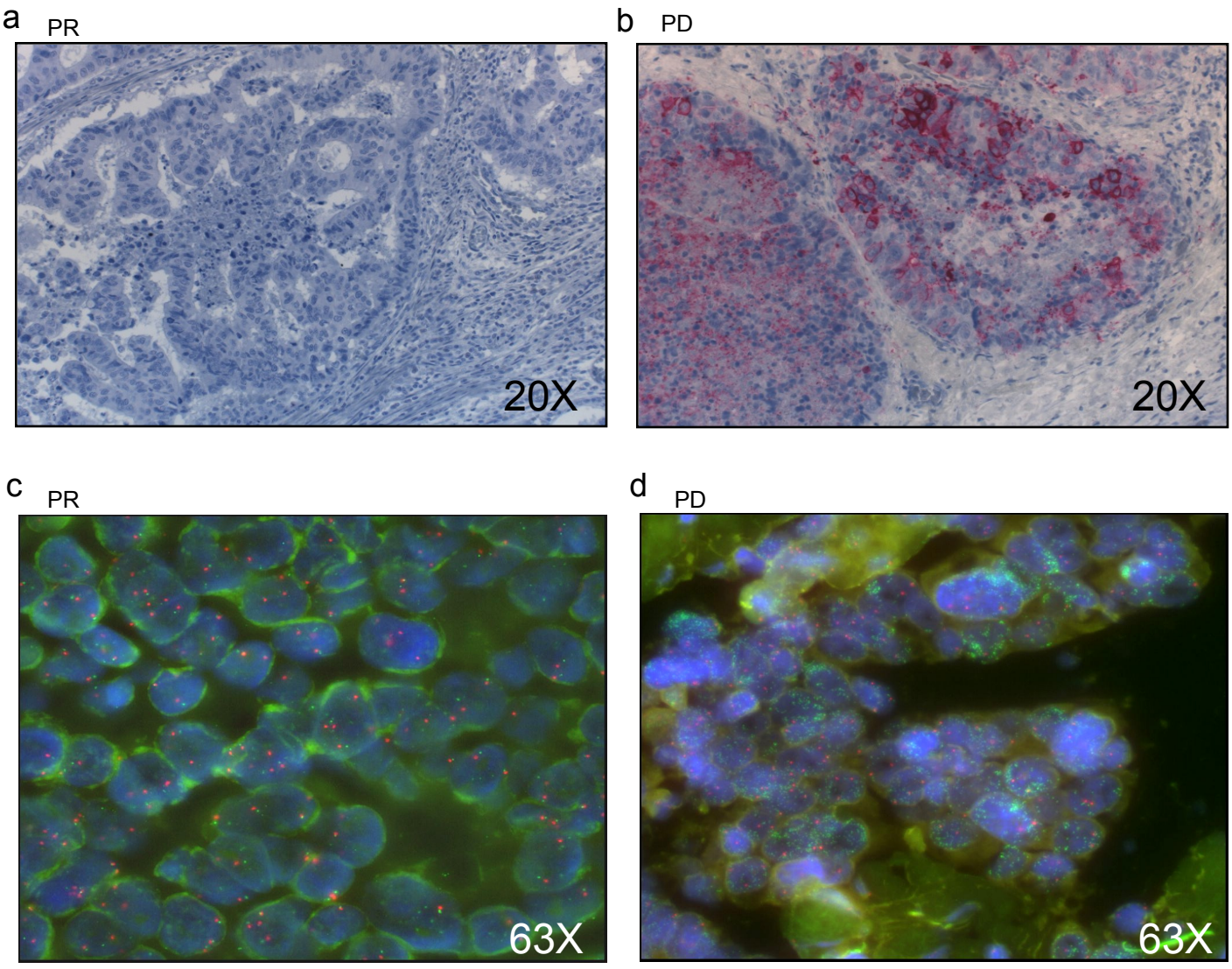
KRAS mutations can arise 'de novo' during cetuximab treatment. (a) Schematic representation of the protocol used to obtain a KRAS wild type Lim1215 clone and to derive its cetuximab resistant variants. (b) Mass Spectrometry analysis of Lim1215 E4.1 cetuximab resistant cells showing the KRAS nucleotide change at codon 146 (G436A). (c) Proliferation of the Lim1215 KRAS WT subclone E4.1 is impaired by cetuximab treatment. (d) KRAS A146T resistant cells derived from the E4.1 subclone are fully insensitive to cetuximab. (e) KRAS A146T cetuximab resistant cells display active GTP-RAS, as assessed by GST-Raf1 pull-down. Whole-cell extracts were blotted total RAS antibody, while GAPDH was included as a loading control.

Supplementary Figure 6



Combinatorial inhibition of EGFR and MEK is effective in cells with acquired resistance to cetuximab (a-d) Cetuximab-resistant DiFi or Lim1215 cells were treated with a constant dose of cetuximab (70 nM) and/or with increasing doses of the PI3K inhibitor GSK1059615 (a-b) or MEK inhibitor AZD6244 (c-d) for one week. Cell viability was assayed by the ATP assay. Data points represent means ± SD of three independent experiments.(e-f) Western blot analysis of phosphorylated ERK expression and activation in the indicated cell lines treated with cetuximab (350 nM for DiFi R2 and 1400 nM for Lim1215 R2) and AZD6244 1 μM.

Supplementary Figure 7



Acquired resistance to cetuximab is associated with focal amplification of the KRAS locus in colorectal tumors (a-b) Immunohistochemical analysis of KRAS protein expression in tumor tissues before (PR) and after (PD) development of resistance to cetuximab. (c-d) FISH analysis of the KRAS gene in the same patient. Red Chr12 centromeric probe; Green KRAS gene probe.

Supplementary Table 1

KRAS Mutations	Lim	Lim R1	Lim R2
G12R	0%	20%	0%
G13D	0.22%	0%	47%

KRAS mutant cells are present in Lim1215 cells before cetuximab treatment. Percentage of KRAS mutant alleles in parental and cetuximab resistant Lim1215 cells as measured by BEAMing.

Supplementary Table 2

Patient ID	Gender	Site of primary tumor	Anti-EGFR treatment monoclonal antibody/CT	Irinotecan refractory	Best Response	Duration of treatment	Time of biopsy after progression	Site of mutational analysis anti-EGFR sensitive	Site of mutational analysis anti-EGFR resistant	FFPE/Frozen	BRAF mutational status	PIK3CA mutational status
Patient #1	M	rectum	panitumumab + irinotecan	yes	SD	20 months	6 months	rectum	liver	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #2	M	ascending colon	panitumumab + irinotecan	yes	PR	6 months	12 months	liver	R chest wall subcut nodule	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #4	F	sigmoid colon	cetuximab + irinotecan	no	SD	5 months	7 months	colon	lung	FFPE	wt (exon 15)	not done
Patient #5	F	sigmoid colon	cetuximab + irinotecan	yes	PR	7 months	1 month	colon	liver	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #6	M	cecum	cetuximab + FOLFIRI	no	PR	21 months	14 months	liver	liver	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #7	M	sigmoid colon	cetuximab + irinotecan; panitumumab + FOLFIRI	no	SD	25 months	1 month	liver	cerebellum	FFPE	wt (exon 15)	not done
Patient #8	M	sigmoid colon	cetuximab + irinotecan	yes	PR	18 months	1 month	liver	liver	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #9	M	rectum	cetuximab + irinotecan	yes	PR	20 months	1 month	liver	liver	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #10	F	sigmoid colon	panitumumab	yes	PR	13 months	4 months	liver	liver	FFPE	wt (exon 15)	wt (exons 9-20)
Patient #11	F	sigmoid-rectum junction	panitumumab	yes	PR	12 months	1 month	paraaortic lymph nodes	liver/paraaortic lymph nodes	FFPE	wt (exon 15)	wt (exons 9-20)

Patient ID	Gender	Site of primary tumor	Previous chemotherapy		Best Response	Duration of treatment	Time of biopsy after progression	Site of analysis		FFPE/Frozen	BRAF mutational status	PIK3CA mutational status
Patient #13	M	rectum	FOLFOX		PR	4 months	4 months	liver		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #14	M	ascending colon	FOLFOX		PR	5 months	1 month	ascending colon		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #15	M	sigmoid colon	FOLFOX + bevacizumab		SD	3 months	2 months	liver		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #16	F	sigmoid colon	FOLFOX + bevacizumab; bevacizumab alone		PR	9 months; 14 months	1.5 months	liver		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #17	M	sigmoid colon	FOLFOX		PR	5 months	2 months	liver		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #18	M	descending colon	FOLFOX; HAI FUDR + 5FU/LV		CR	5 months; 2 months	18 months	liver		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #19	F	ascending colon	capecitabine + bevacizumab		adjuvant	6 months	6 months	liver		FFPE	wt (exon 15)	wt (exons 9-20)
Patient #21	F	sigmoid colon	FOLFOX		PR	3 months	1 month	sigmoid colon		FFPE	wt (exon 15)	wt (exons 9-20)

FFPE:Formalin Fixed Paraffin Embedded

Patients' clinical characteristics

Supplementary Table 3

a

Patient ID	Anti EGFR sensitive tumor KRAS Mutational status (Sanger sequencing on tumor)	Anti EGFR resistant tumor KRAS Mutational status (Sanger sequencing on tumor)
Patient #1	wt	wt
Patient #2	wt	wt
Patient #4	wt	wt
Patient #5	wt	wt
Patient #6	wt	wt
Patient #7	wt	wt
Patient #8	wt	Q61H
Patient #9	wt	wt
Patient #10	wt	wt
Patient #11	wt	wt – KRAS amplified

b

Patient ID	Anti EGFR sensitive tumor KRAS Mutational status (454[a] or BEAMing[b] on tumor)	Anti EGFR resistant tumor KRAS Mutational status (454[a] or BEAMing[b] on tumor)
Patient #2	wt[a]	G13D[a]
Patient #7	wt[a]	wt[a]
Patient #8	wt[b]	Q61H[b]
Patient #9	wt[b]	G12D[b] G13D[b]
Patient #10	wt[b]	wt[b]
Patient #11	wt[b]	wt[b]

Sanger sequencing analysis of KRAS gene (a) Sanger sequencing analysis of KRAS gene in colorectal cancer patients tumors before and after resistance to anti-EGFR therapies. (b) Deep sequencing analysis of KRAS gene in colorectal cancer patients tumors before and after resistance to anti-EGFR therapies.

Supplementary Table 4

a

Patient ID	Anti EGFR sensitive KRAS mutational status **in plasma**			Anti EGFR resistant KRAS mutational status **in plasma**		
	Mutation	Percentage	Events	Mutation	Percentage	Events
Patient #8	wt	0.1%	4/46300	Q61H	1.12%	731/65400
Patient #9	wt	0.03% 0%	3/11600 0/16800	G12D G13D	0.48% 3.3%	89/18400 427/12500
Patient #10	wt	0%	0/85500	wt	0%	0/14200

b

Patient ID	KRAS mutational status **in plasma samples**			KRAS mutational status **in tumor biopsy**
Patient #8	*December 2009*	*April 2010*	*January 2011*	*February 2011*
	0.01% (Q61H)	0.32% (Q61H)	1.12% (Q61H)	17.3% (Q61H)
Patient #9	*January 2011*	*March 2011*	*April 2011*	*September 2011*
	0.03% (G12D) 0% (G13D)	0.71% (G12D) 1.27% (G13D)	0.48% (G12D) 3.3% (G13D)	0.04% (G12D) 0.44% (G13D)
Patient #10	*July 2010*	*April 2011*	*August 2011*	*November 2011*
	0%	0%	0%	0%

BEAMing analysis of KRAS mutational status in plasma samples (a) Detection of mutated KRAS alleles in plasma of colorectal cancer patients before and after resistance to anti EGFR therapies. (b) Measurements of mutant KRAS in serial plasma samples and in biopsies. The time course of plasma and bioptic sampling is indicated.