Supplemental Figures: Integrated time-course omics analysis distinguishes immediate therapeutic response from acquired resistance

Supplemental Figure 1 – Time course approach to induce resistance to cetuximab and measure gene expression and DNA methylation changes. Intrinsic cetuximab sensitive HNSCC cell line SCC25 were treated with cetuximab (red) or PBS (black) for 7 days. In the eighth day, cells were collected and pooled from multiple replicate cultures to provide adequate amounts for total RNA isolation for RNA-seq, genomic DNA isolation for DNA methylation array, proliferation assay (flow), for storage (frozen) and to be plated again to continue treatment until resistance to cetuximab developed. Each collection point was called a generation (from CTX-G0 to CTX-G11).

Supplemental Figure 2 – Colony formation assay in matrigel for anchorage-independent growth confirmed acquired cetuximab resistance of CTX-G10 (red) relative to the parental cell line (CTX-G0, black) at different concentrations of cetuximab (0nM, 10nM, 100nM and 1000nM).

Supplemental Figure 3 – Heatmap and hierarchical clustering of gene expression values in 11 generations of SCC25 cells treated with PBS as control (black columns) and with 100nM of cetuximab (red columns) to acquire resistance.

Supplemental Figure 4 – **A.** Heatmap of gene expression values in 11 generations of SCC25 cells treated with 100nM of cetuximab (red columns) to acquire resistance and with PBS as control (black columns). Genes selected for visualization were associated with cetuximab resistance from previous gene expression studies comparing sensitive and resistant cells without regard for timing. These studies provided three gene sets, colored along rows of the heatmap. **B.** Average of z-score gene expression values for genes in each of the resistance signatures over generations of PBS control (black lines) or treatment with 100nM of cetuximab (red lines).

Supplemental Figure 5 – Expected gene expression values for genes in each CoGAPS pattern inferred from gene expression data over generations of PBS control (black lines) or treatment with 100nM of cetuximab (red lines). Patterns included a pattern reflecting technical artifacts between untreated controls at time 0 and subsequent generations (pattern 4) and a flat pattern for highly expressed genes (pattern 5), excluded from analysis in main figures. Heatmap of gene expression values for PatternMarker genes identified for all of these patterns. Rows were colored according to which CoGAPS pattern the PatternMarker statistic assigned each gene, and sorted by the PatternMarker statistic.

Supplemental Figure 6 – Heatmap of gene set analysis scores for targets of transcription factors in the EGFR network, targets of the AP-2alpha transcription factors associated with cetuximab response, and cetuximab resistance signatures in CoGAPS patterns. A score of 100 indicated upregulation of the targets with a p-value of 0 and -100 downregulation with p-values of 0. Matrix elements with a star indicated p-values below 0.05 for either up or down-regulation of the gene set. Gene expression heatmap was colored on a red-green scale where as the gene set statistics heatmap was colored on a blue-red scale, with values indicated in the respective color keys.

Supplemental Figure 7 – **A.** Heatmap of Pearson correlation coefficients between CoGAPS gene expression and DNA methylation patterns. Row colors for expression patterns match the colors for patterns in Figure 2,3. The column colors for methylation patterns are selected to match the color of the corresponding expression pattern with maximum anti-correlation. **B.** As in **A** for CoGAPS gene weights (meta-pathway values) corresponding to patterns in DNA methylation (columns) and gene expression (rows).

Supplemental Figure 8 – Heatmap of gene expression values for 11 generations of SCC25 cells treated with PBS as control (black columns labeled PBS) and with 100nM of cetuximab (red columns labeled cetuximab) to acquire resistance and gene expression data from independent, stable cetuximab resistant clones in absence of cetuximab treatment (CTX resistant clones).

Supplemental Figure 9 – Heatmap of DNA methylation values for 11 generations of SCC25 cells treated with PBS as control (black columns labeled PBS) and with 100nM of cetuximab (red columns labeled cetuximab) to acquire resistance and gene expression data from independent, stable cetuximab resistant clones in absence of cetuximab treatment (CTX resistant clones).

Supplemental Figure 10 – Brightfield microscopy images of the representative clones' morphology above with corresponding clone specific CoGAPS patterns of DNA methylation above.

Supplemental Figure 11 – Epigenetically regulated pattern marker genes associated with resistance having significant anti-correlation between gene expression and DNA methylation in the cetuximab single cell resistant clones.

Supplemental Figure 12 – Cell proliferation assay using AlamarBlue (Invitrogen, Carlsbad, CA) to compare proliferation rates under different concentrations of cetuximab in the resistant single cell clones (CTXR4, 7, 10 and 11) and the parental SCC25 cell line to confirm resistance when treated with different concentrations of cetuximab.

Storage
RNA-seq
DNA methylation
Proliferation assay

Generation 0 (G0)
cetuximab sensitive parental cell line (SCC25)

60,000 cells

60,000 cells

cetuximab resistant generations (CTX-G1 to -G11)

Treatment with 100nM cetuximab every 3 days

7 days

Collect and pool cells
Resistant Generation n+1

Storage
RNA-seq
DNA methylation
Proliferation assay

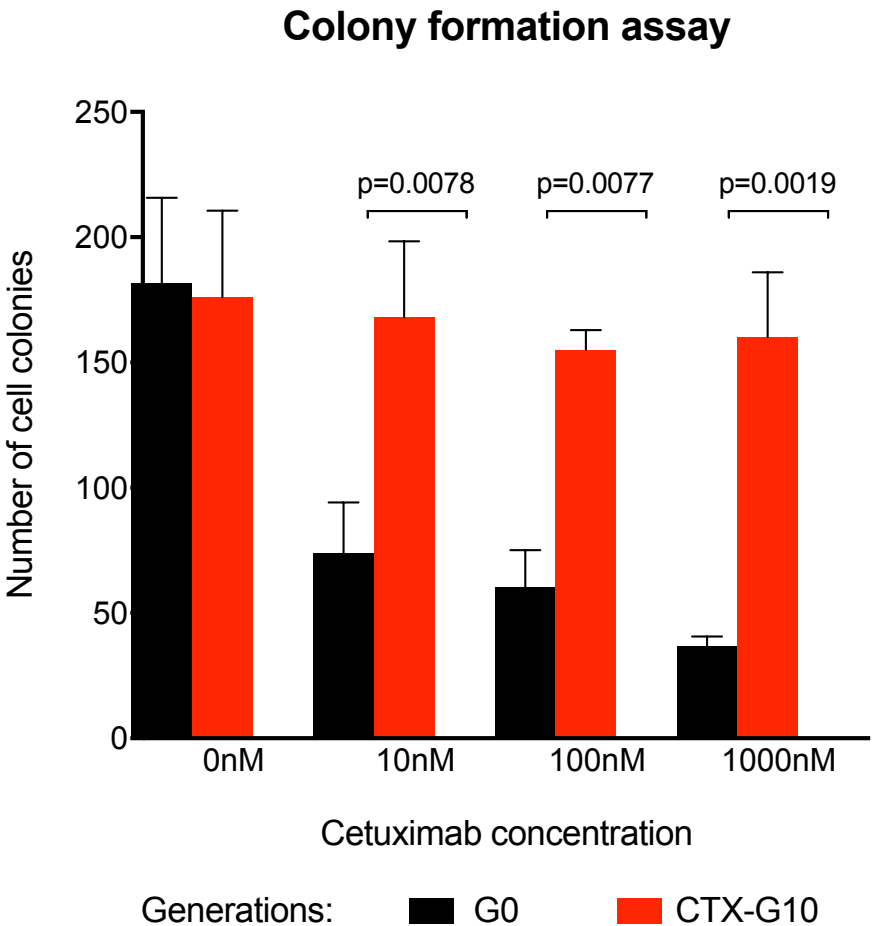
control PBS generations (PBS-G1 to -G11)

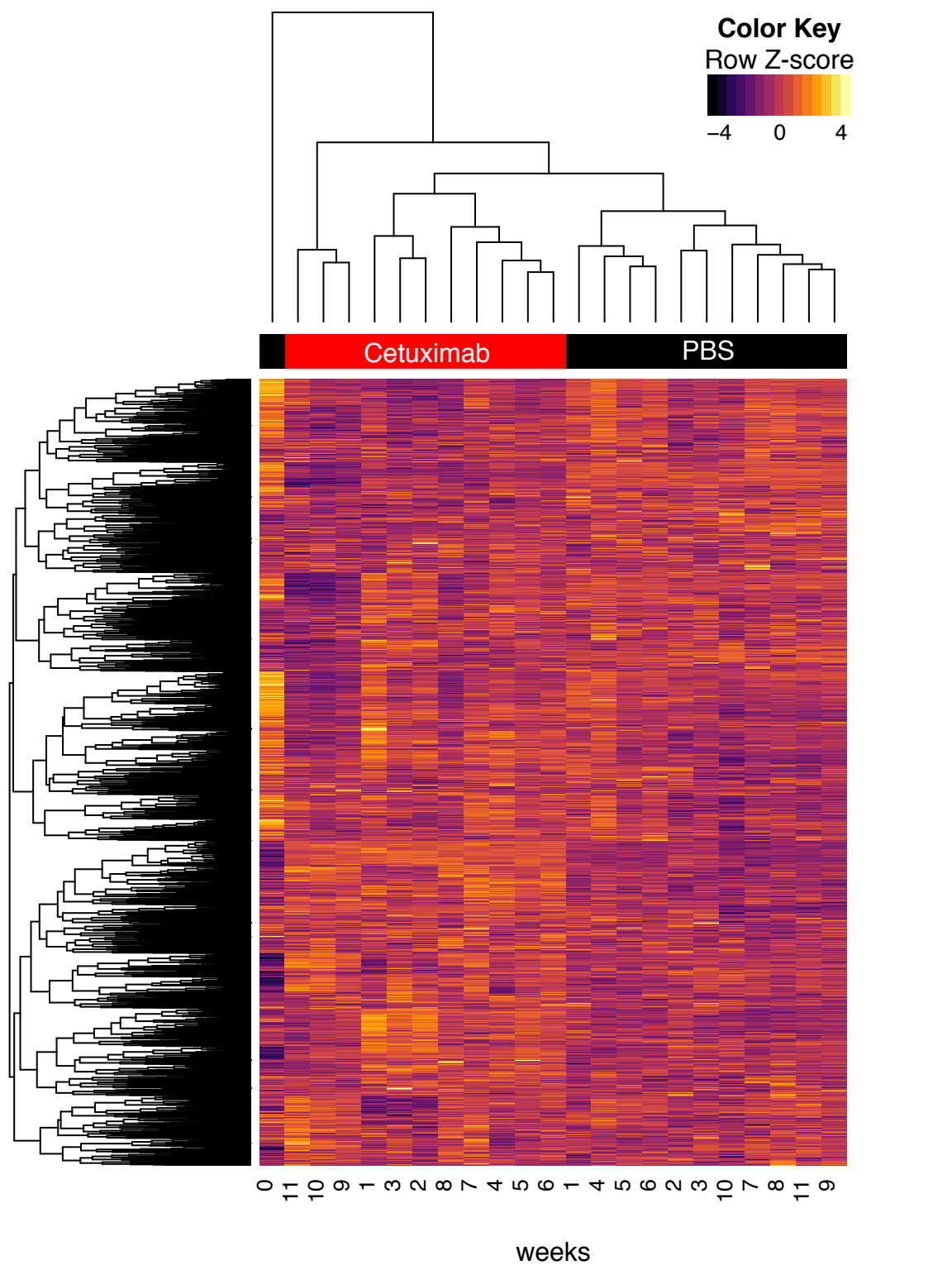
Treatment with PBS every 3 days

7 days

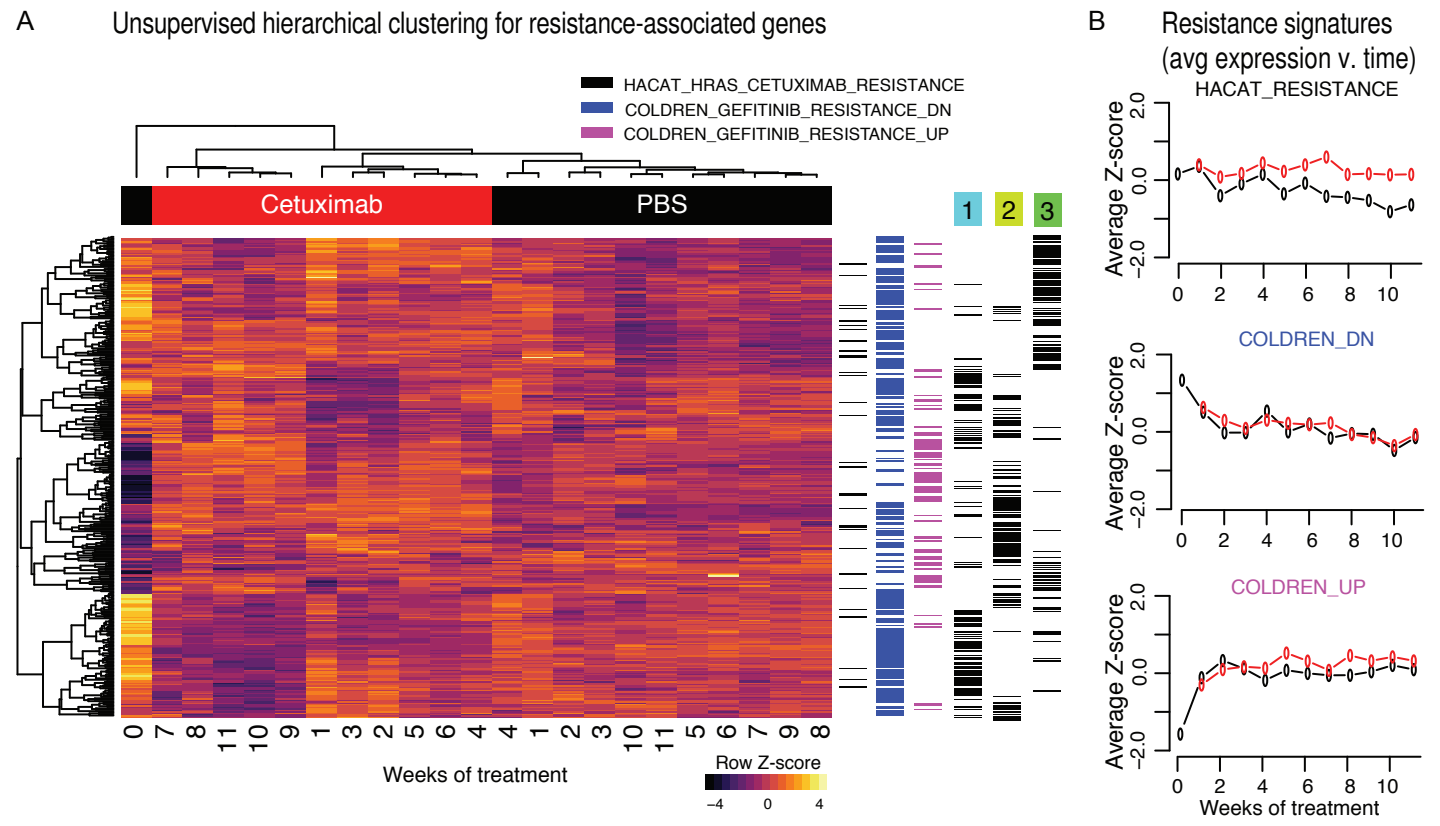
Collect cells
PBS Generation n+1

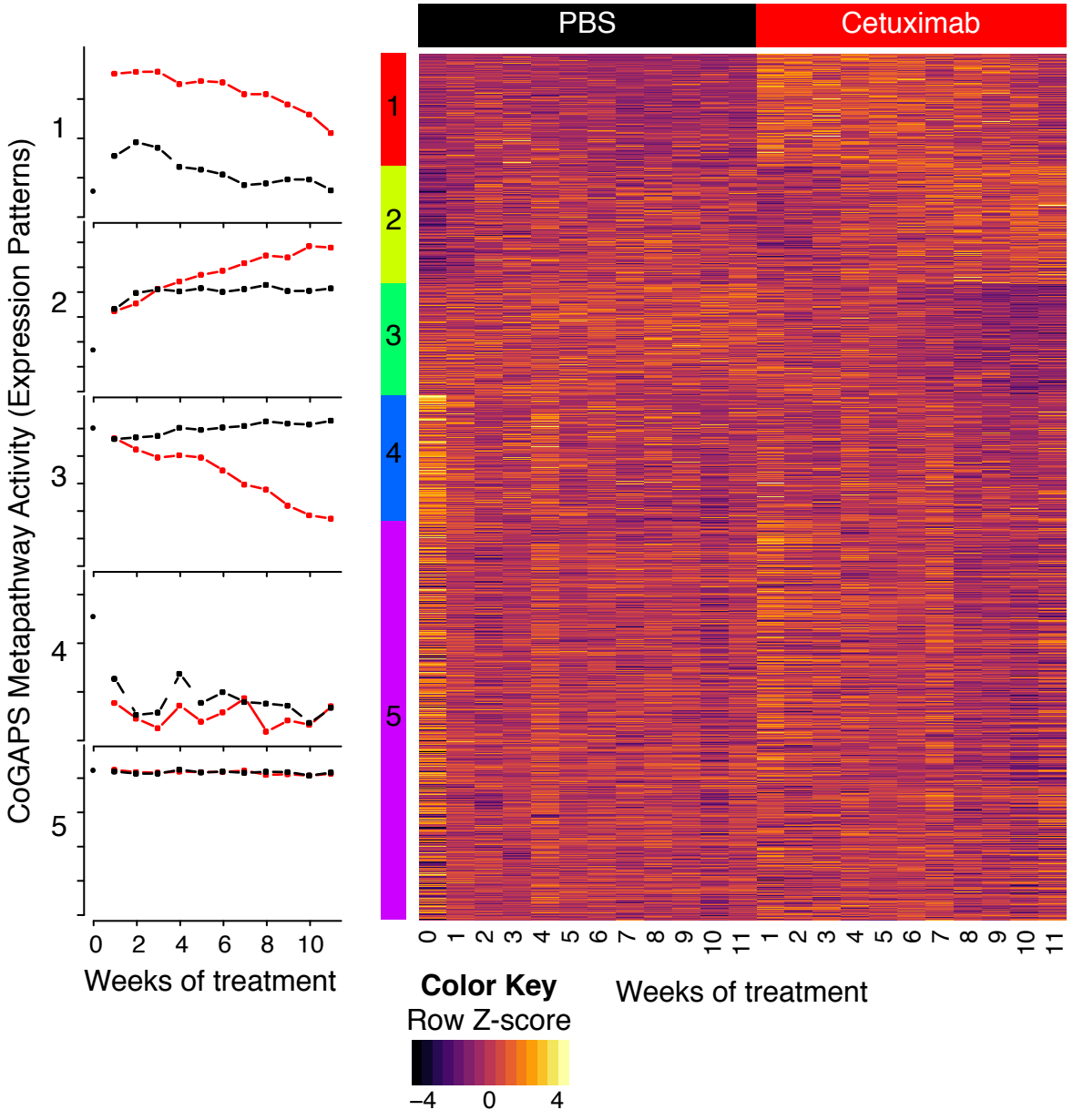
Storage
RNA-seq
DNA methylation
Proliferation assay


Colony formation assay
250
200
150
100
50
0
Number of cell colonies
p=0.0078
p=0.0077
p=0.0019
0nM
10nM
100nM
1000nM
Cetuximab concentration
Generations:
G0
CTX-G10

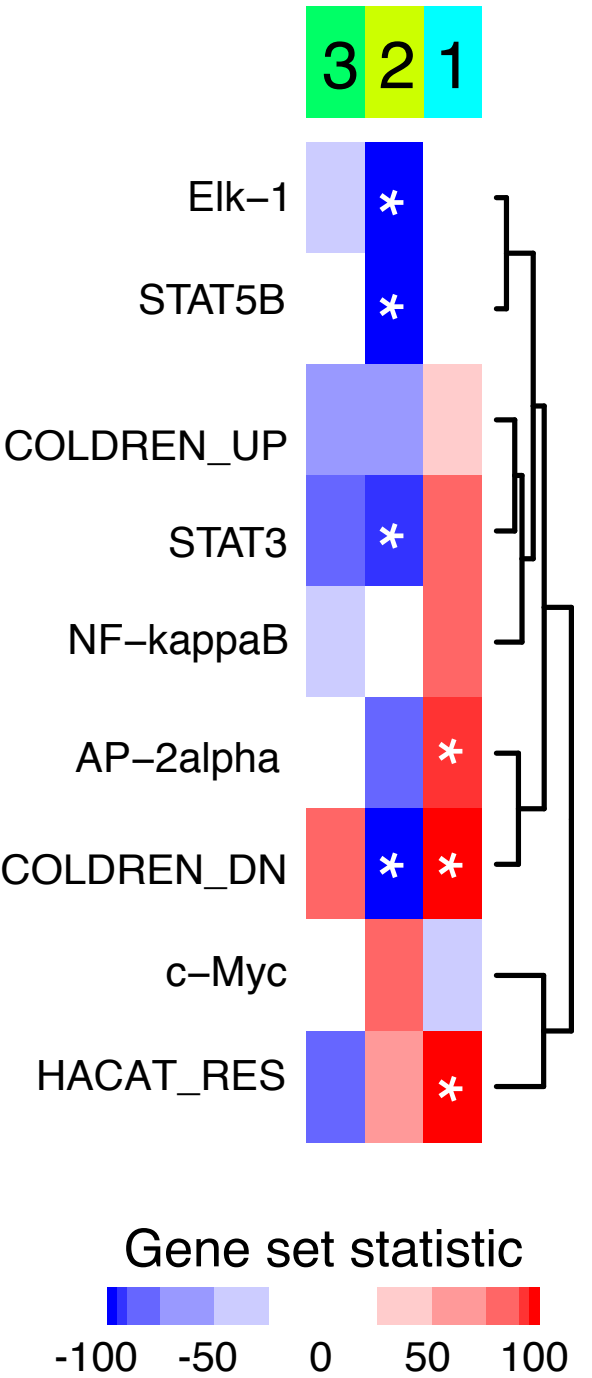

Color Key
Row Z-score
−4
0
4
Cetuximab
PBS
0
11
10
9
1
3
2
8
7
4
5
6
1
4
5
6
2
3
10
7
8
11
9
weeks

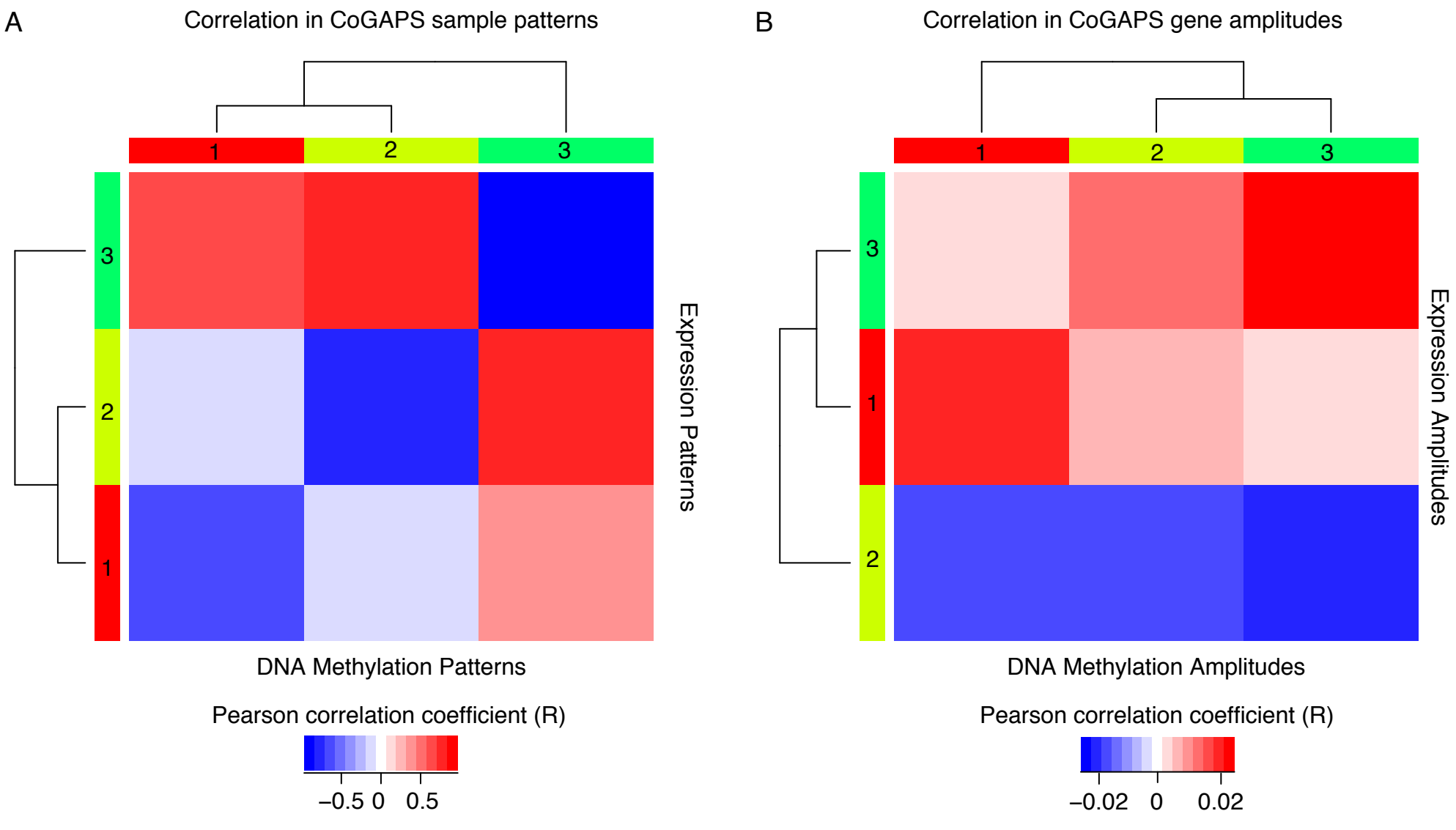
Clustering of resistance signatures

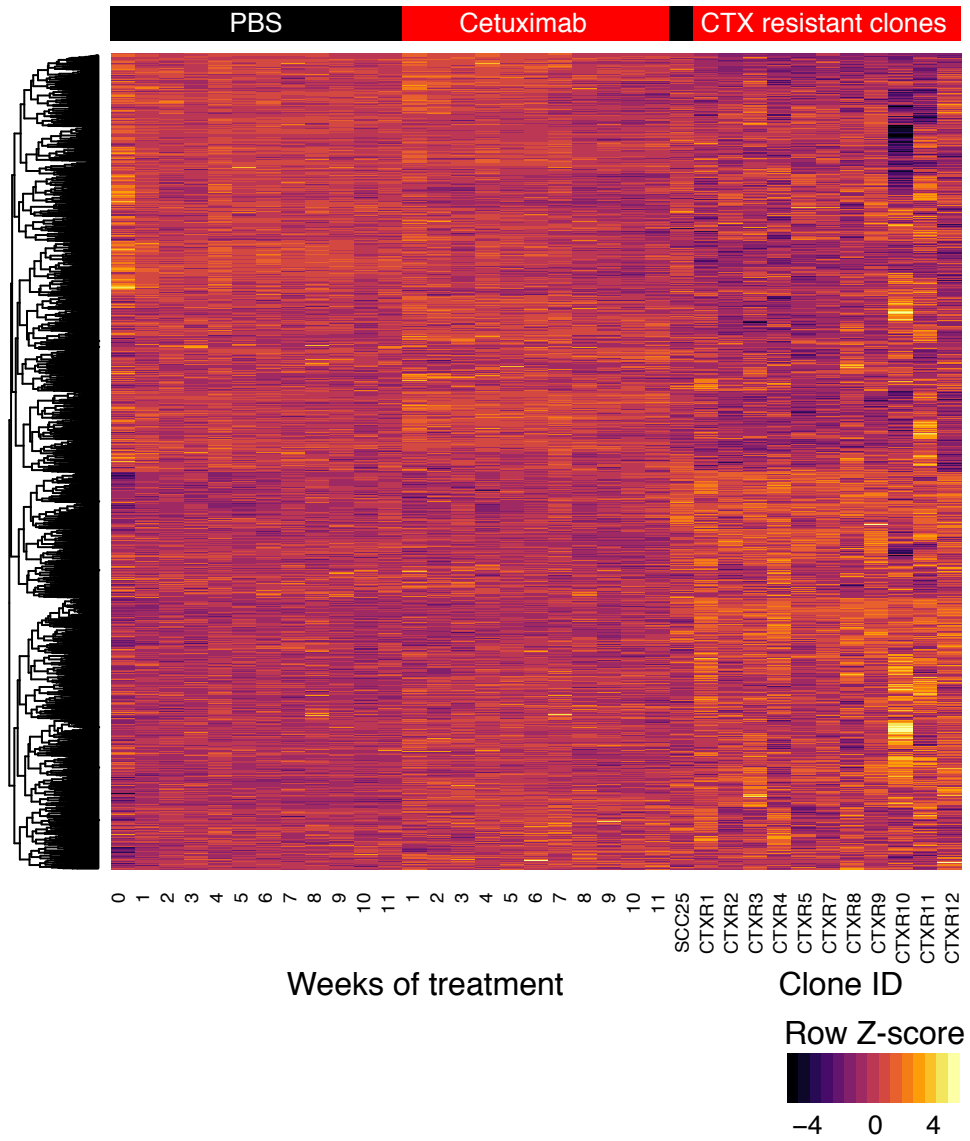


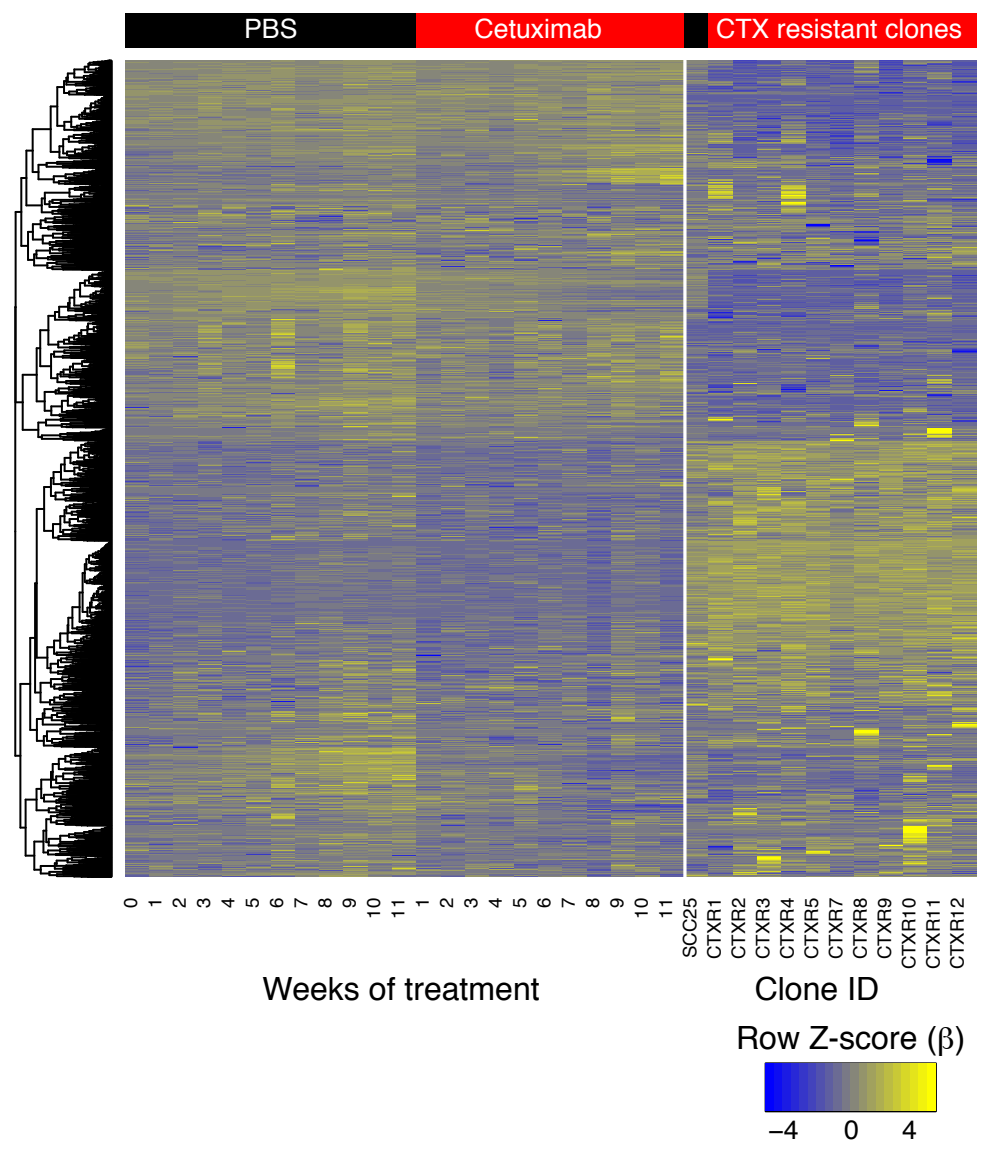

PBS
Cetuximab
CoGAPS Metapathway Activity (Expression Patterns)
1
2
3
4
5
0 2 4 6 8 10
Weeks of treatment
0 1 2 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11
Weeks of treatment
Color Key
Row Z-score
−4 0 4

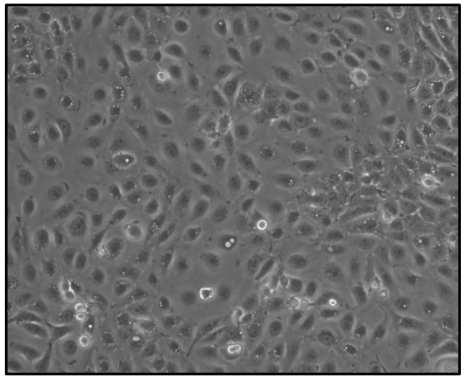
Pattern geneset enrichment



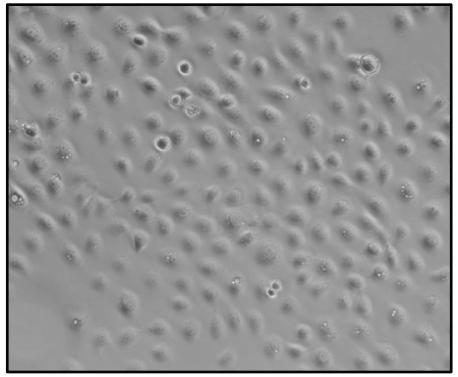

A
Correlation in CoGAPS sample patterns
1
2
3
3
2
1
Expression Patterns
DNA Methylation Patterns
Pearson correlation coefficient (R)
−0.5 0 0.5
B
Correlation in CoGAPS gene amplitudes
1
2
3
3
1
2
Expression Amplitudes
DNA Methylation Amplitudes
Pearson correlation coefficient (R)
−0.02 0 0.02


PBS
Cetuximab
CTX resistant clones
0
1
2
3
4
5
6
7
8
9
10
11
1
2
3
4
5
6
7
8
9
10
11
SCC25
CTXR1
CTXR2
CTXR3
CTXR4
CTXR5
CTXR7
CTXR8
CTXR9
CTXR10
CTXR11
CTXR12
Weeks of treatment
Clone ID
Row Z-score
−4
0
4


PBS
Cetuximab
CTX resistant clones
0 1 2 3 4 5 6 7 8 9 10 11
1 2 3 4 5 6 7 8 9 10 11
SCC25
CTXR1
CTXR2
CTXR3
CTXR4
CTXR5
CTXR7
CTXR8
CTXR9
CTXR10
CTXR11
CTXR12
Weeks of treatment
Clone ID
Row Z-score (β)
−4
0
4



SCC25



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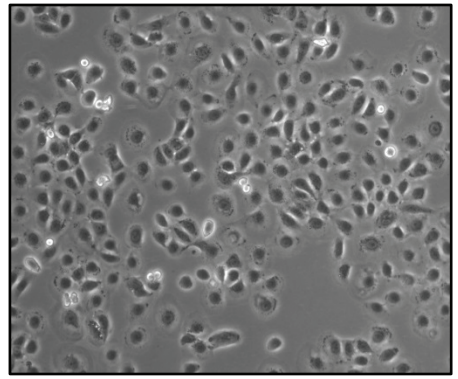

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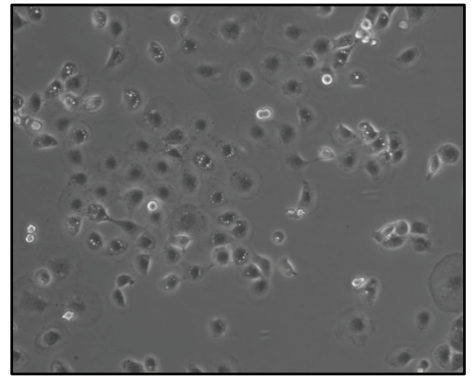

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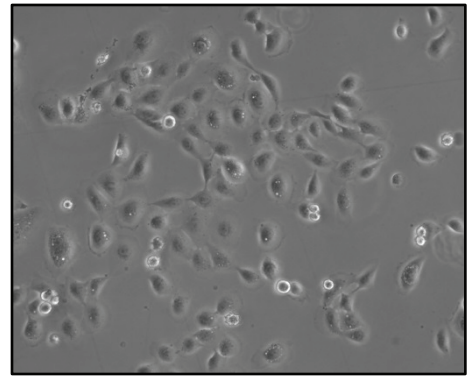

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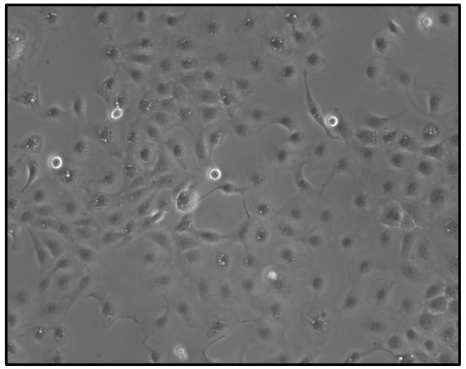

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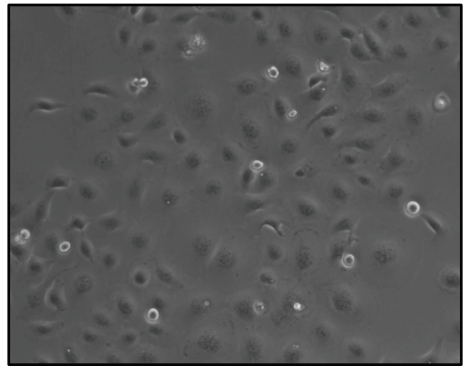

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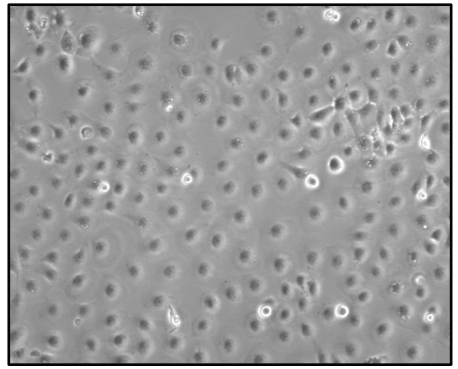

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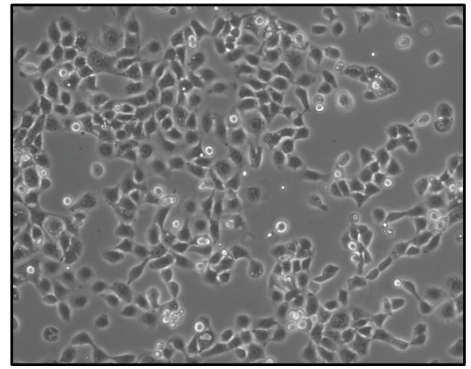

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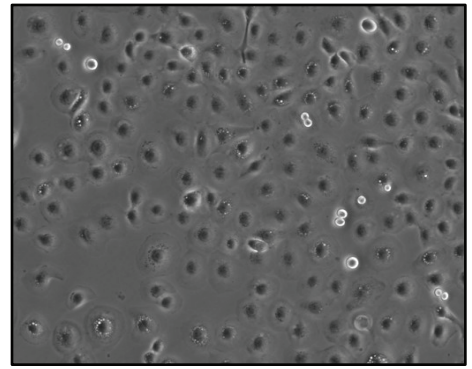

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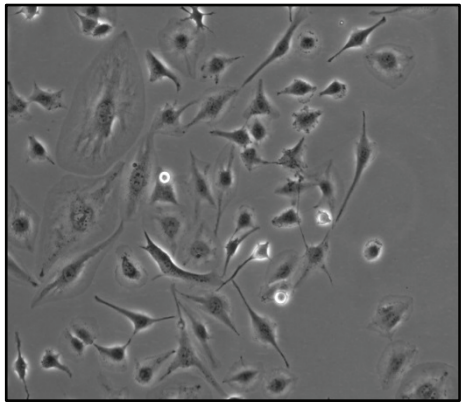
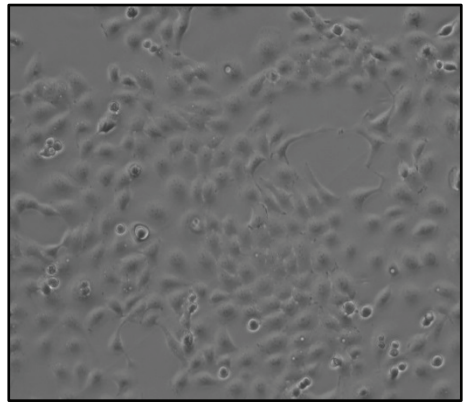


CTXR10



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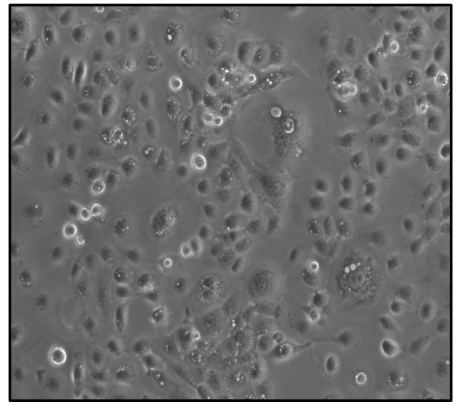

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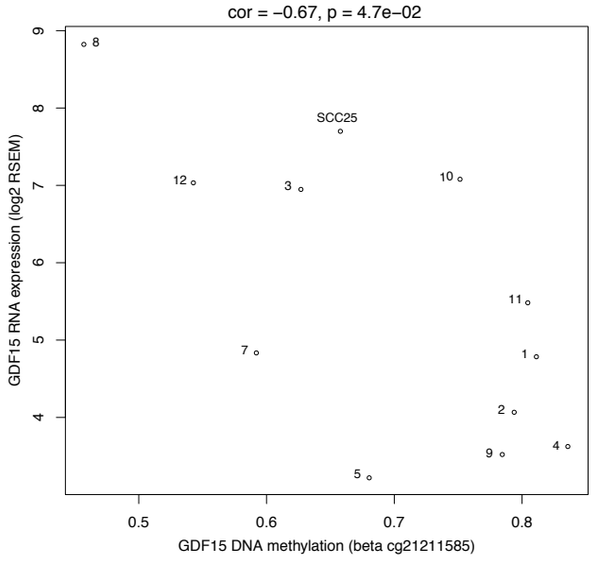
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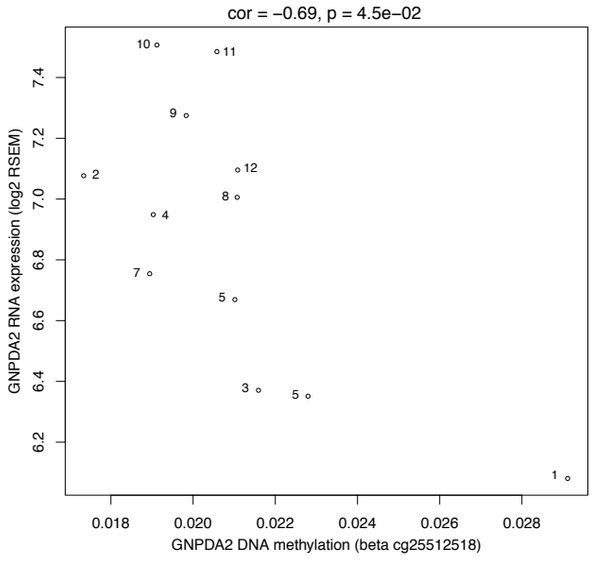
GDF15 DNA methylation (beta cg21211585)
SCC25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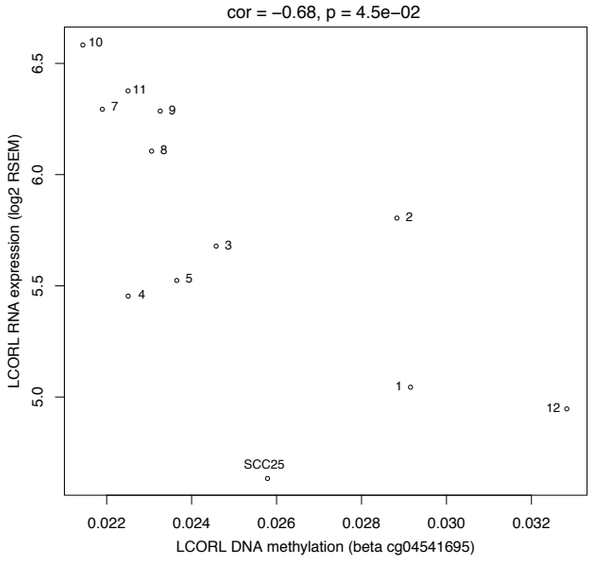
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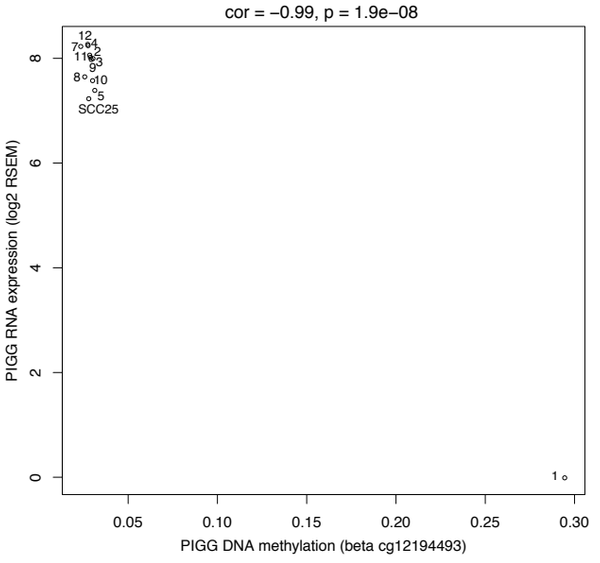
SCC25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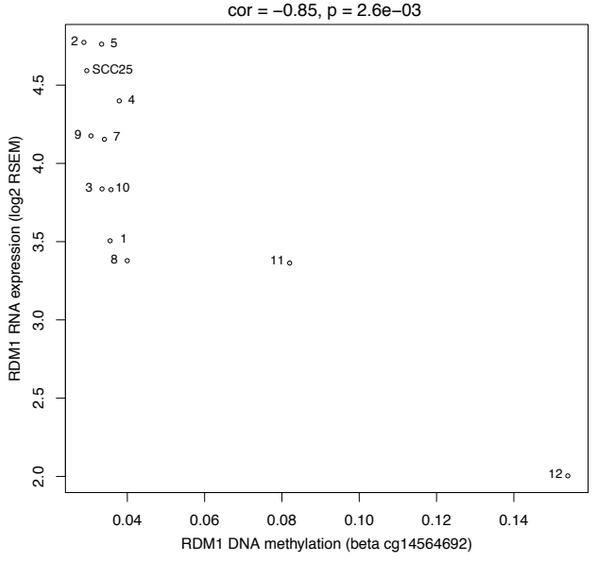
SCC25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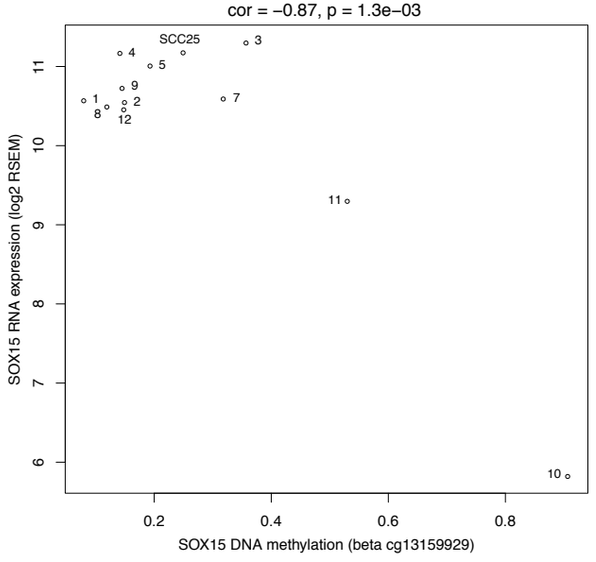
SCC25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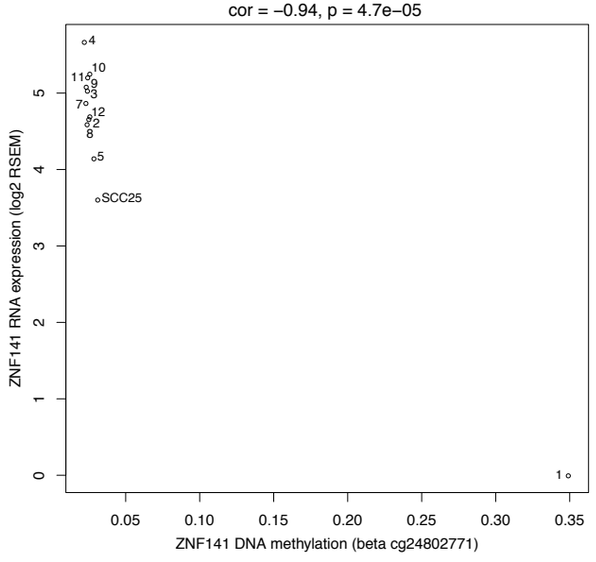
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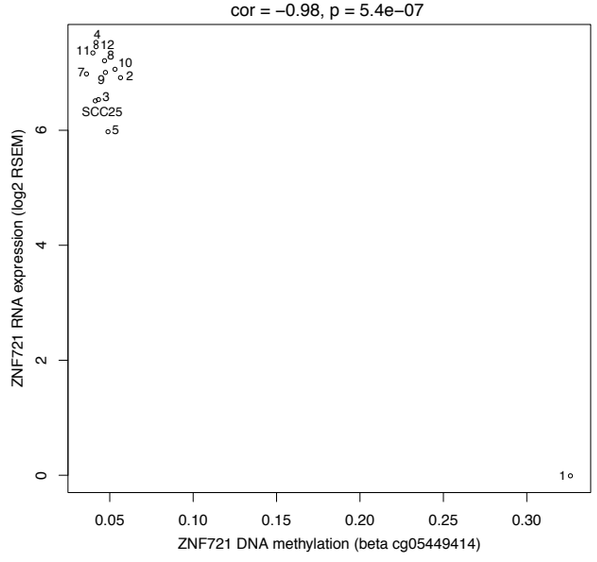
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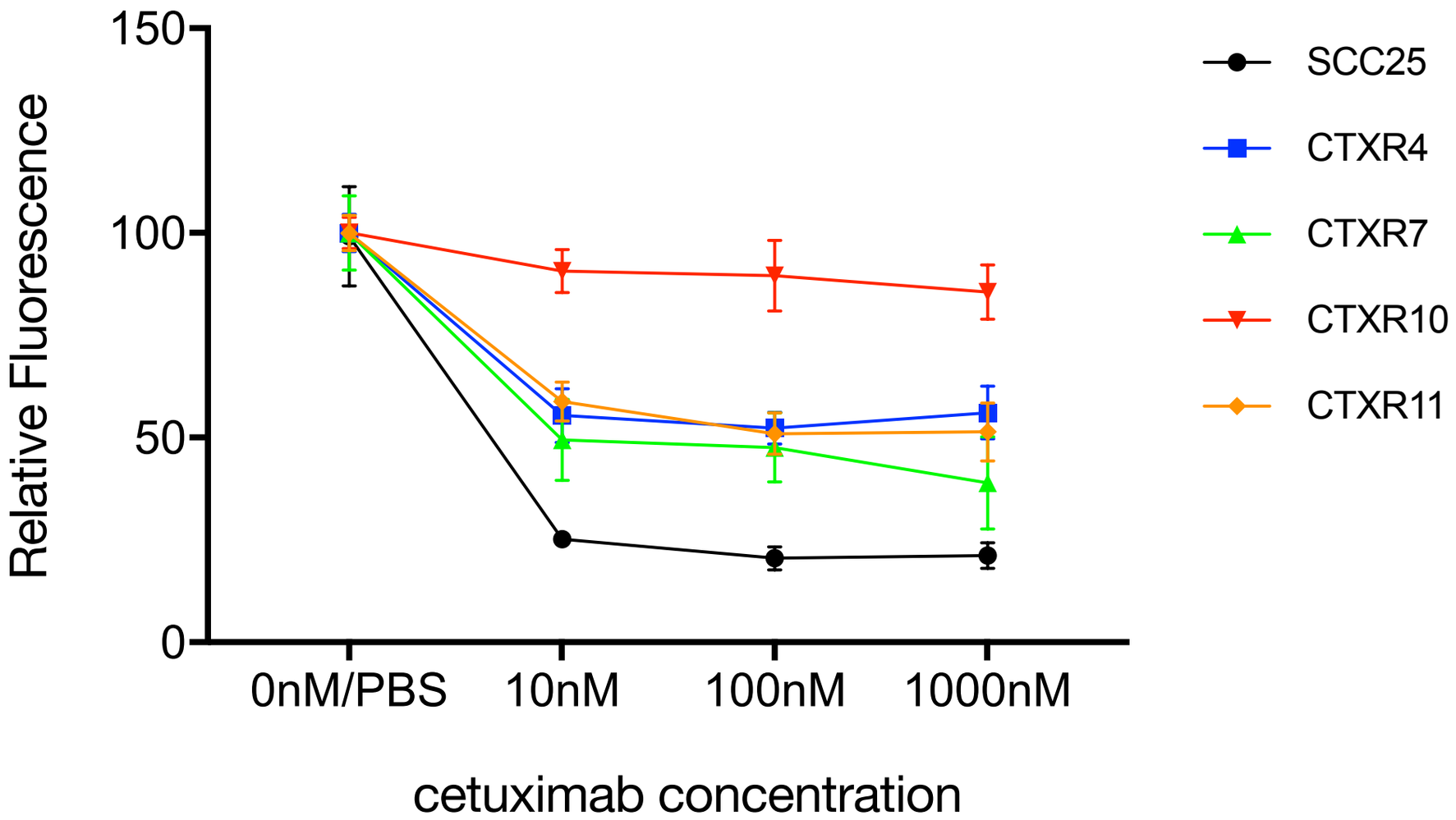
150
100
50
0
Relative Fluorescence
0nM/PBS
10nM
100nM
1000nM
cetuximab concentration
SCC25
CTXR4
CTXR7
CTXR10
CTXR11