

Supplementary Figures

Figure S1. Data overview and quality control. **A.** Summary table indicating the number of quantified proteins, phosphosites, and transcripts. **B.** Phosphoenrichment performed on samples of each of the six culture conditions shows expression value of reproducible identifications (i.e. ~80 % across all treatments).

Figure S2. No evidence for off-target effect. Scaled expression of phosphosites that are substrates of ARAF (ARAF S582, MAP2K2 S226, PBK S59, PRKCD S304, RBM10 S89, TBC1D25 S506, UBA1 S4, UBA1 S46) and MAP2K5 (MAP3K2 S153, MAP3K2 S164, MAP3K2 S239, PBRM1 S948, PRKCI S247, SQSTM1 S366). Solid lines indicate average expressions, while shaded areas standard errors.

Figure S3. Effect of EGFR inhibition or PTPN11 KO to BRAFⁱ. **A.** Correlation plot of log₂-fold changes in BRAFⁱ and BRAFⁱ+EGFRⁱ compared to Control in mRNA expression in CRC cell lines SNU-C5, VACO432 and KM-20. Log₂-fold-changes were obtained from a differential expression analysis of all three cell lines combined and can thus be interpreted as the average effect size over three cell lines. The 95% confidence interval of the regression $\log_2(\text{BRAF}^i+\text{EGFR}^i/\text{Control}) \sim 0 + \log_2(\text{BRAF}^i/\text{Control})$ (blue line) is 1.44-1.50. **B.** Correlation plots of log₂-fold-changes in BRAFⁱ and BRAFⁱ in PTPN11 KO compared to Control, for each dataset in WiDr cell line. The gene/protein/phosphosites-time point pairs selected are significantly differentially expressed for at least one of the two treatments. The 95% confidence interval regressions of $\log_2(\text{BRAF}^i \text{ in PTPN11 KO}/\text{Control}) \sim 0 + \log_2(\text{BRAF}^i)$ (blue lines) are 1.16-1.17, 0.93-1.01 and 1.08-1.19 for the transcriptomic, protein and phosphoproteomic data, respectively.

Figure S4. Examples of differential regulation at the mRNA and protein level upon WiDr PTPN11 KO. For each gene, the protein expression is provided in the top panel and the mRNA expression in the bottom panel: **A.** Creatine Kinase B (CKB). **B.** Proteasome subunit beta type-8 (PSMB8). **C.** 2-5-oligoadenylate synthase 1 (OAS1). **D.** Signal transducer and activator of transcription 1 (STAT1).

Figure S5. Correlation clustering at all three omics levels. A distance measure based on the Pearson correlation was used to generate 8 clusters for the **(A)** phosphoproteomics, **(B)** proteomics and **(C)** transcriptomics data. MAPK and PI3K-AKT regulated processes are enriched in clusters 3 (A) and 2 (C), while cell cycle process is enriched in clusters 4 (A) and 1 (C). Interferon alpha and gamma response genes, and targets of the transcription factors IRF-1, STAT1 are enriched in cluster 6 (B) and cluster 4 (C). Metabolic processes are enriched in clusters 5 (A), and 5 and 7 (C).

Figure S6. Expression profiles found to be enriched in specific clusters. Early response clusters exhibit immediate downregulation of MAPK and PI3K-AKT pathways as shown by decreased phosphorylation of key residues after 2-6 h upon treatment: **A.** MAPK3 Y204 and **B.** RPS6KB1 S247. As a consequence, transcription factors downstream of these pathways are deactivated, as shown by the reduction in: **C.** c-FOS mRNA. Late response clusters exhibit downregulation of the CDK pathway as further indicated by its downregulation at the protein level at the late stage: **D.** CDK1 and **E.** CDK2. **F.** The protein expression of retinoblastoma-associated protein RB1 remains constant.

Figure S7. Western blots validation on multiple protein and phospho-specific targets. Validation of selected target regulations by Western blots analysis on the same WiDr PTPN11 WT (**A.** R2; **B.** R3) and WiDr PTPN11 KO (**C.** R2; **D.** R3) cell lysates used in our omics analysis.

EGFR, ERK, P90 proteins and phosphoproteins are involved in the MAPK pathway, whereas AKT and p-AKT are involved in PI3K-AKT signaling. ERBB2 and ERBB3 were identified in RTK clustering together with INSR. IGF1R expression was also measured as it is considered to be closely related to INSR. The data showed consistent reduction of p-EGFR, p-ERBB2, p-ERBB3 and p-IGF1R expression at 48 h in BRAFⁱ⁺EGFRⁱ. Nevertheless, ERBB2 and ERBB3 showed a higher expression than the Control at the latest time point concurrent with a decrease in EGFR. This is more evident in WiDr PTPN 11 WT than WiDr PTPN11 KO.

Figure S8. Observed changes in metabolic processes in the BRAF(V600E) WiDr cells upon combined BRAFⁱ and EGFRⁱ treatment. Enrichment analysis revealed the TCA cycle, the peroxisome and lipid metabolism among the most upregulated processes upon BRAFⁱ⁺EGFRⁱ treatment at 48 h. Upregulation of the TCA cycle may depend on activity of PDHA1 which catalyzes oxidative decarboxylation of pyruvate and on fatty acid β -oxidation. Indirect inhibition of PDHA1 (via PDKs) by DCA or direct inhibition of CPT1 by etomoxir may enhance the effect of BRAFⁱ and EGFRⁱ treatment.

Figure S9. Upregulation of metabolic enzymes is stronger and more consistent on protein than on mRNA level. For each gene, the protein expression is provided in the top panel and the mRNA expression in the bottom panel: **A.** Isocitrate Dehydrogenase (NADP(+)) 1 Cytoplasmatic (IDH-1) **B.** Succinate-CoA ligase, GDP-forming, beta subunit (SUCLG2) **C.** Carnitine palmitoyltransferase 2 (CPT2) **D.** Hydroxyacyl-CoA Dehydrogenase Trifunctional Multienzyme Complex Subunit Beta (HADHB) **E.** mRNA expression of these genes in SNU-C5, VACO432 and KM-20 cells.

Figure S10. Half-life mRNA 48h in WiDr and additional BRAF(V600E) CRC cell lines. Log₂-fold-changes compared to T=0 h of regulators of the major signaling pathways again their

mRNA half-life. **A.** WiDr cell line. **B** KM20, SNUC5 and VaCo cell lines. Log₂-fold-changes were calculated using differential expression analysis of all three cell lines combined.

Figure S11. Proteins involved in energy metabolism. PDHA1 is a key metabolic enzyme, converting cytosolic pyruvate to mitochondrial acetyl-CoA, the substrate for the TCA cycle. Activity of PDHA1 is negatively regulated by pyruvate dehydrogenase kinase PDKs. Upon BRAFⁱ+EGFRⁱ treatment at 48 h, PDHA1 shows an inverse pattern of phosphorylation levels on its residues **A.** S232 and **B.** S293, while **C.** PDHA1 total protein expression remains constant. **D.** PDK1, **E.** PDK2 and **F.** PDK4 are detected only at mRNA level. Both PDK2 and PDK4 show significant upregulation of their transcripts at 24 and 48 h inverse to the PDK1 profile. From this observed inverse response our interpretation of PDHA1 modulation remains inconclusive.

Figure S12. Cell proliferation assays for drug treatment. **A.** Cell proliferation curves of WiDr cells upon treatments with either PLX4032 (BRAFⁱ) or etomoxir (CPT1 inhibitor) or DAC (PDKs inhibitor) show similar profiles. All three curves confirm the WiDr cell line is resistant to each monotherapy. **B.** Cell proliferation curves of WiDr cells upon triple combination treatment. A fixed concentration of a metabolic drug (DCA or etomoxir) is added to PLX4032 (BRAFⁱ) and 3 μM gefitinib (EGFRⁱ) at the same time (T=0 h). Resulting viability curves show similar profiles, with no evidence of additional benefit from triple treatment with any of the two metabolic drugs.

Figure S13. Combination treatment induces temporary cell cycle arrest. **A.** Prolonged exposure to BRAFⁱ+EGFRⁱ treatment (78 d) causes the formation appearance of resistant colonies in WiDr cell line. Interruption of drug treatment (“drug off”) induces cells to resume proliferation. **B.** A shorter period of “drug off” (72 h) further confirms the presence of reversibly arrested WiDr cells by showing a rapid resumption of cell proliferation after “drug on” (5 d). The difference in confluence between long- and short-term may depend on the ability of cells to

leave the quiescent adaptive state after continuous treatment.

Figure S14. Whole genome sequencing based karyotypes. A. WiDr B. WiDr PTPN11 KO.

These are the estimated copy number over the different chromosomes based on GC corrected read depth. These profiles confirms the similarity of these cell lines, fulfilling what is known from previous studies.

A**Phosphoproteomics***Modification Localization*

Class I (>0.75)	9055	62%
Class II (>0.5, ≤ 0.75)	2571	18%
Class III (>0.25, ≤ 0.5)	2923	20%

Modified amino acids

Serine (S)	7834	87%
Threonine (T)	1145	13%
Tyrosine (Y)	76	1%

Identifications

Phosphoproteins	3402
Phosphopeptides	14,878
Phosphosites (Class I)	9055
Phosphosites (Class I*)	7141

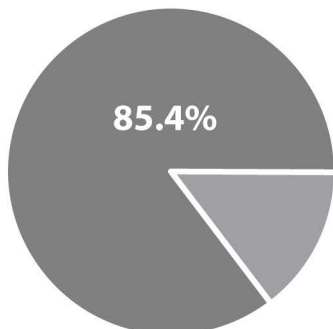
Proteomics

Proteins	8774
Unique peptides	83,508
Protein groups	6638
Protein groups*	5692

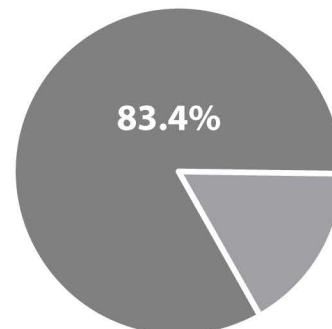
Transcriptomics

Genes	21,446
Missense variants (including X)	3969
Unique protein sequence variants	2387

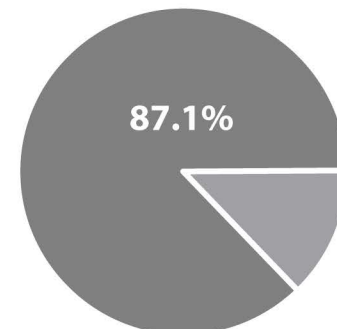
* = measured in at least one timepoint of any of the six cell culture conditions

B

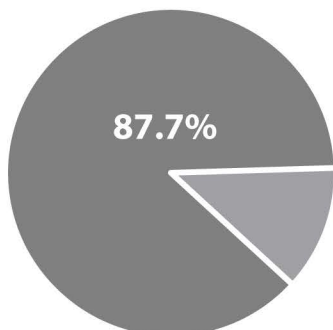
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Control



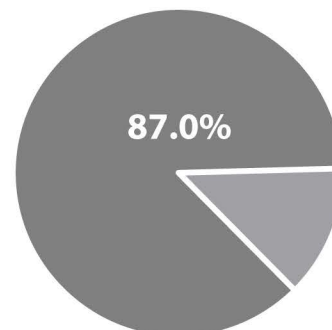
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BRAFi



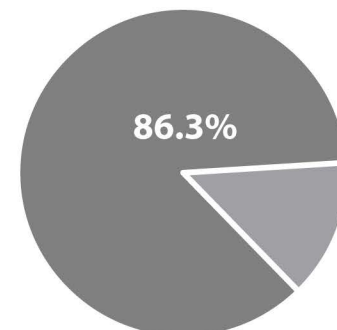
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WiDr
EGFRi



WiDr
BRAFi



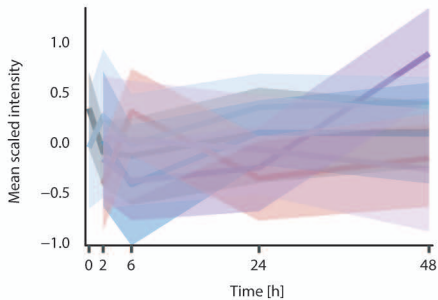
WiDr
BRAFi+EGFRi

peptides ■ phosphopeptides ■

Figure S1

ARAF substrates

N = 8



MAP2K5 substrates

N = 6

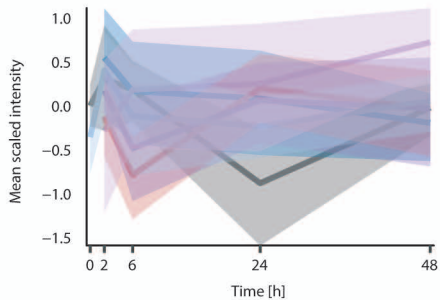


Figure S2

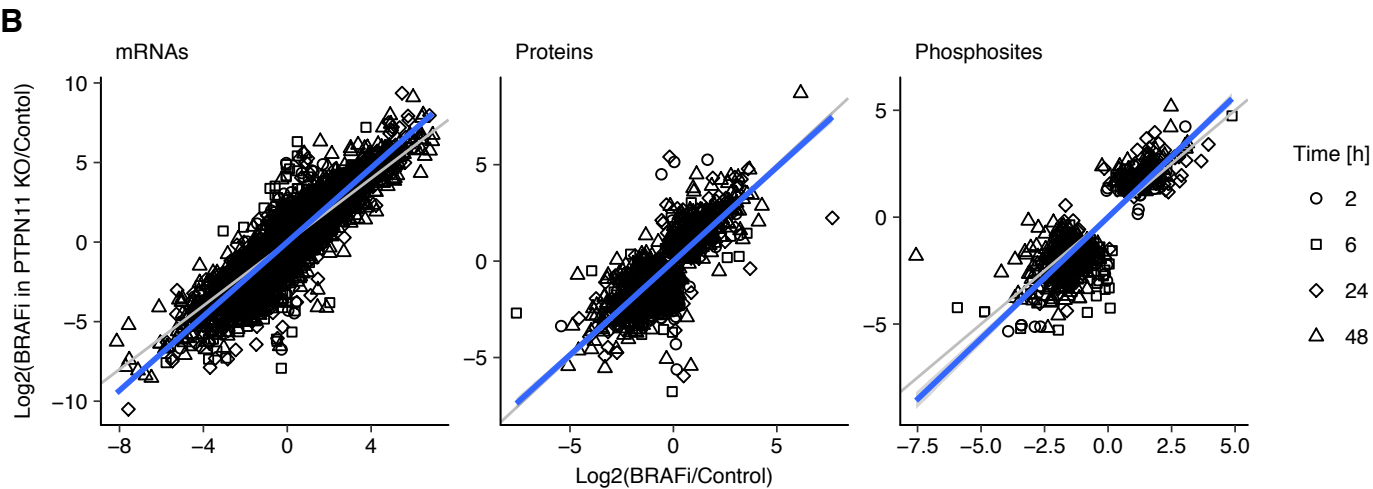
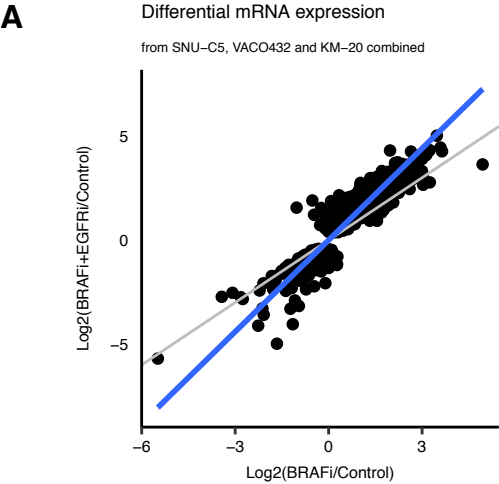


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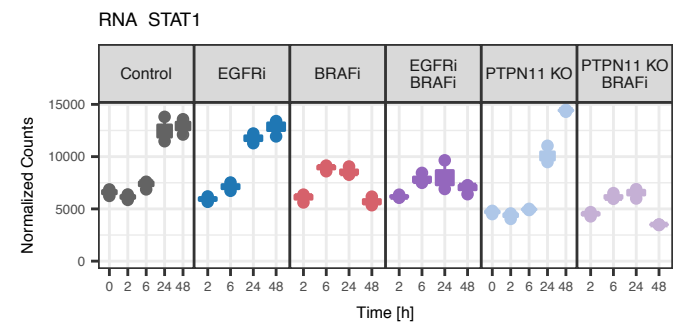
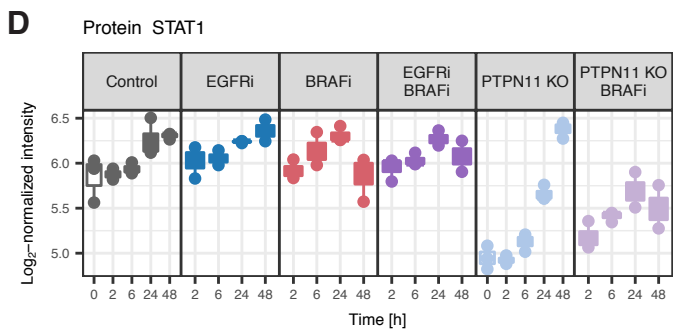
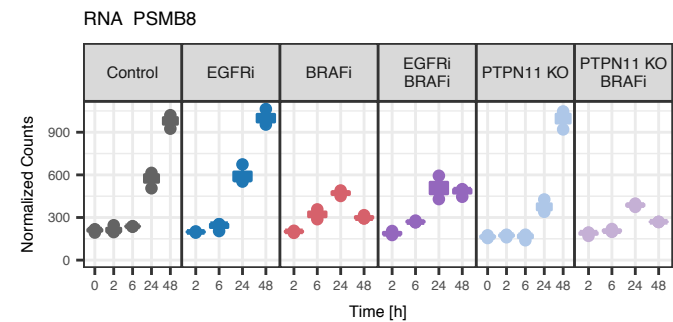
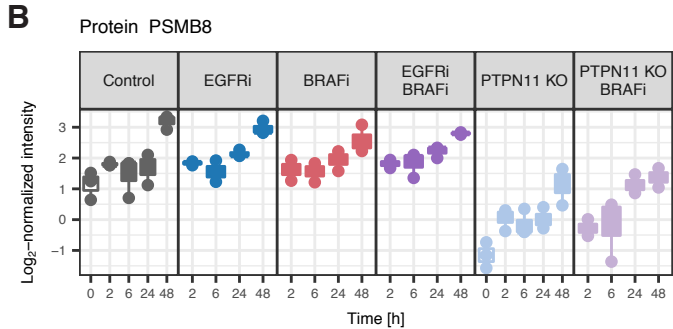
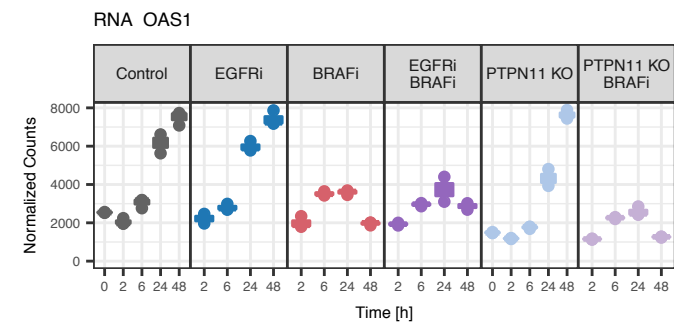
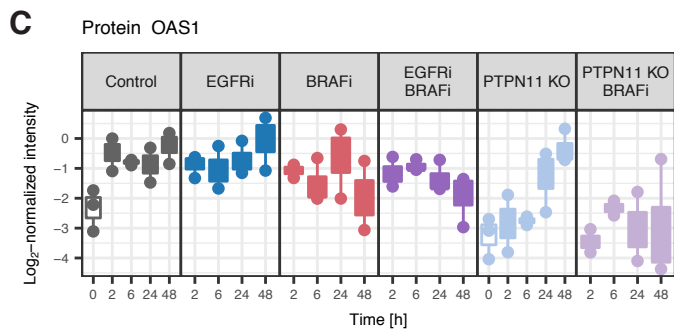
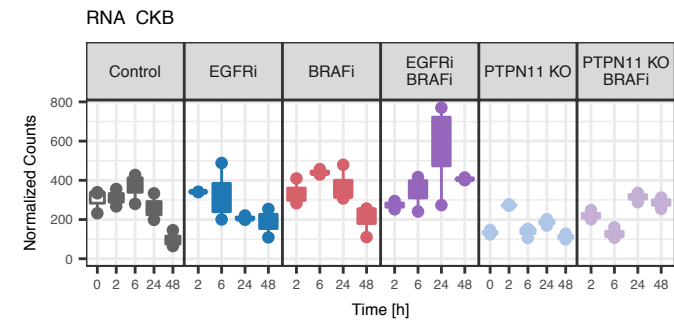
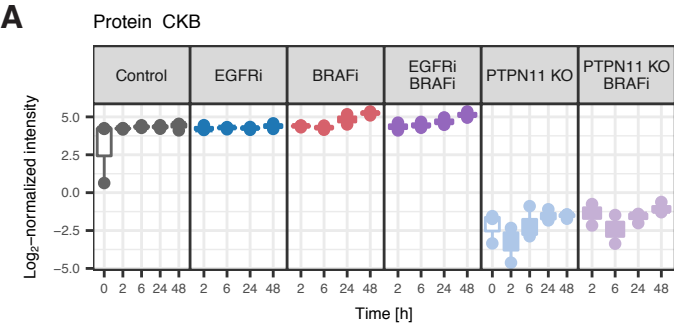
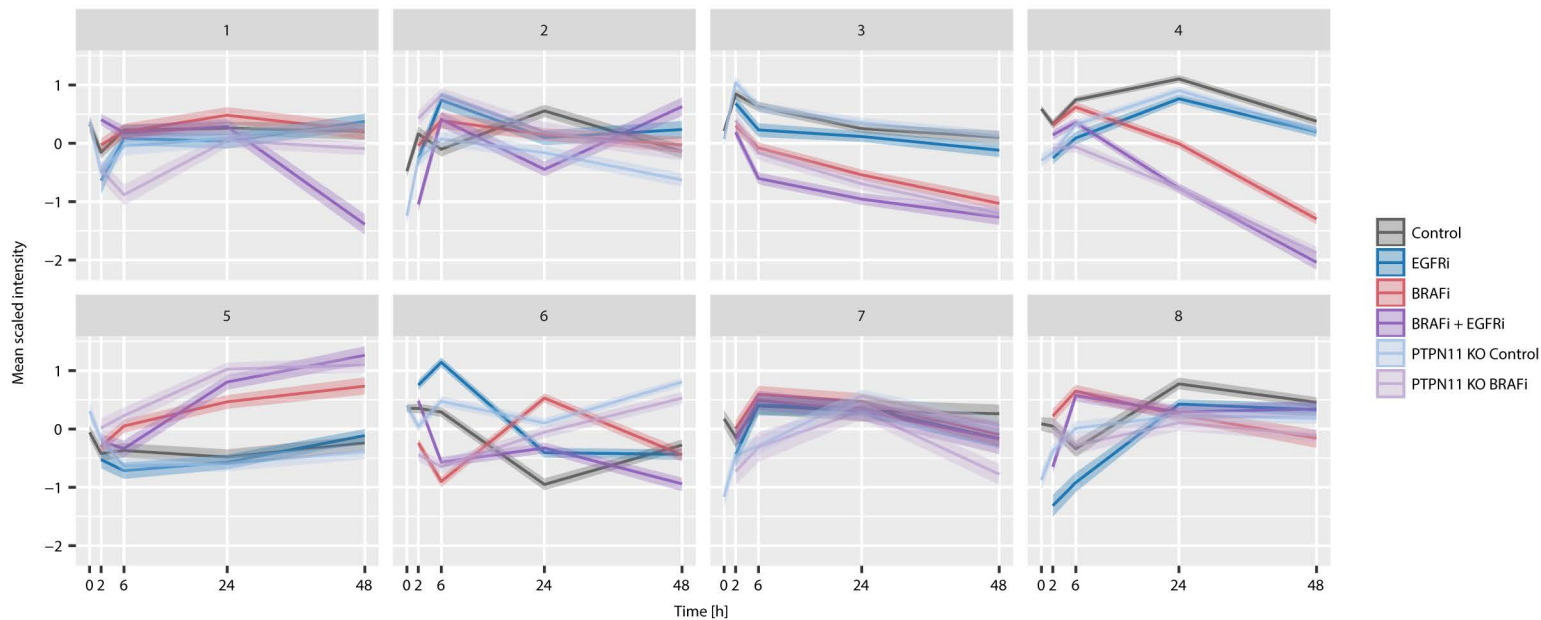
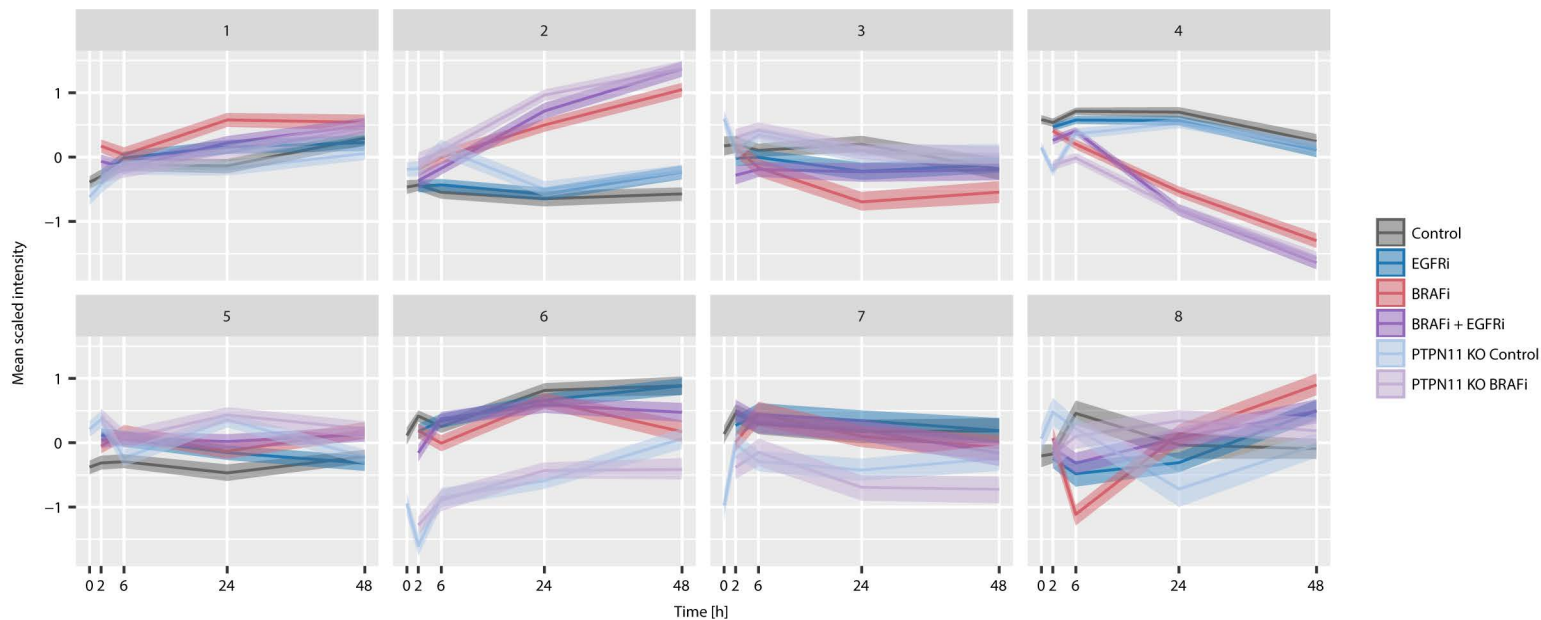


Figure S4

Phosphoproteomics



Proteomics



Transcriptomics

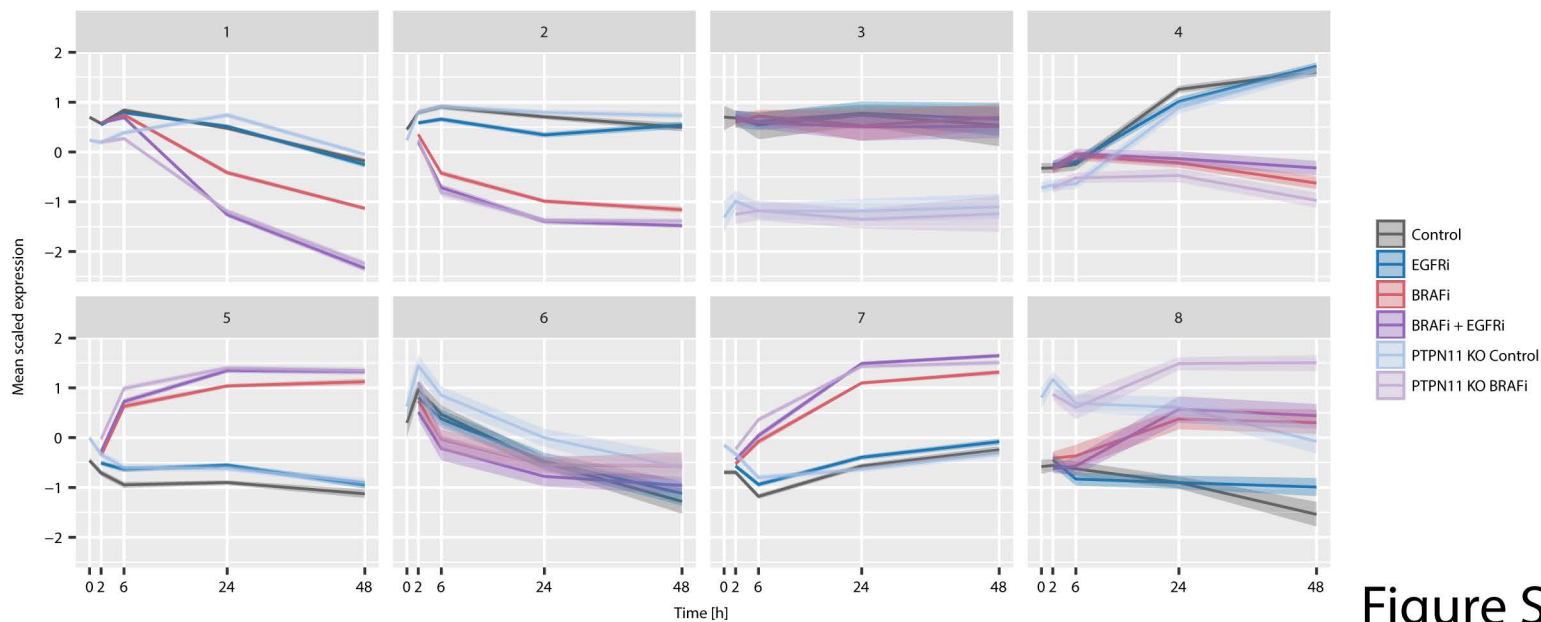


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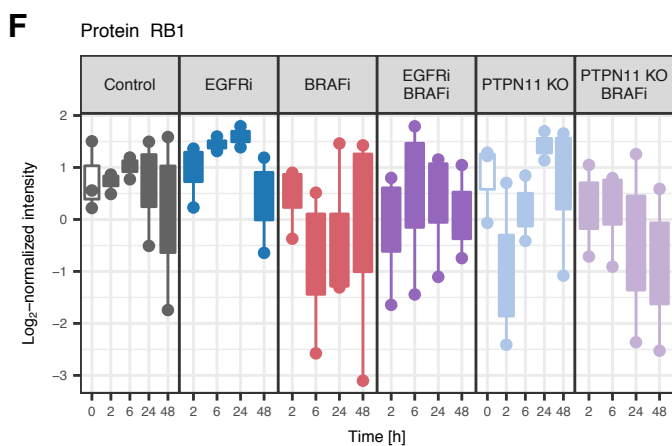
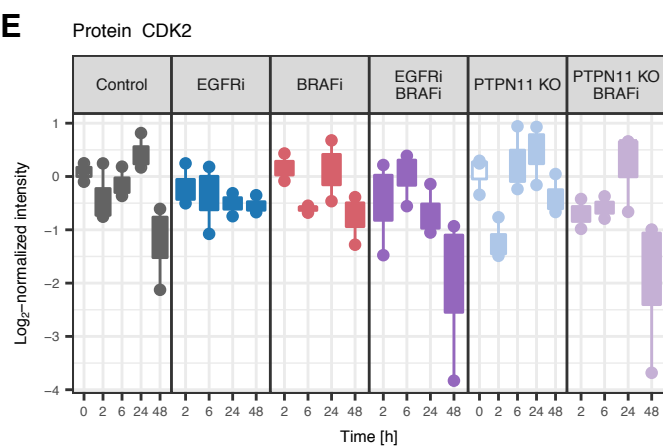
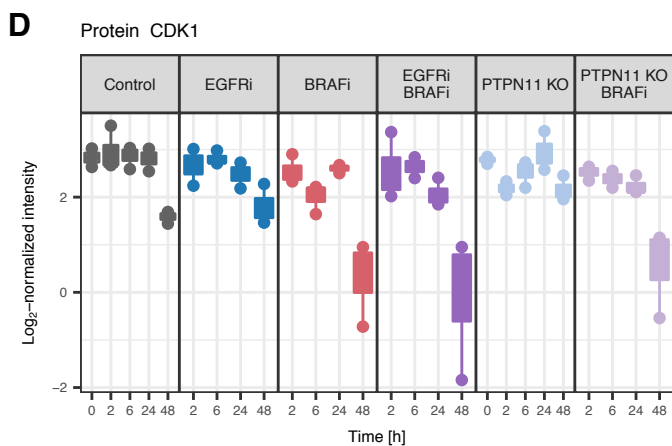
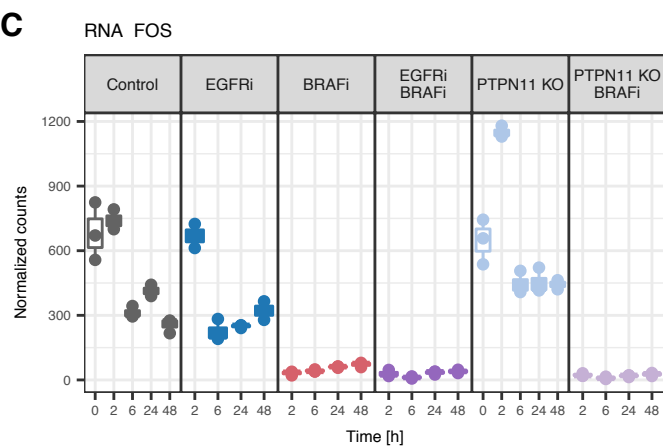
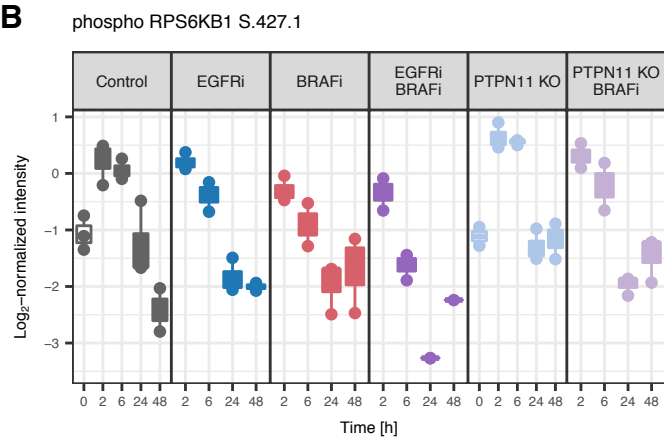
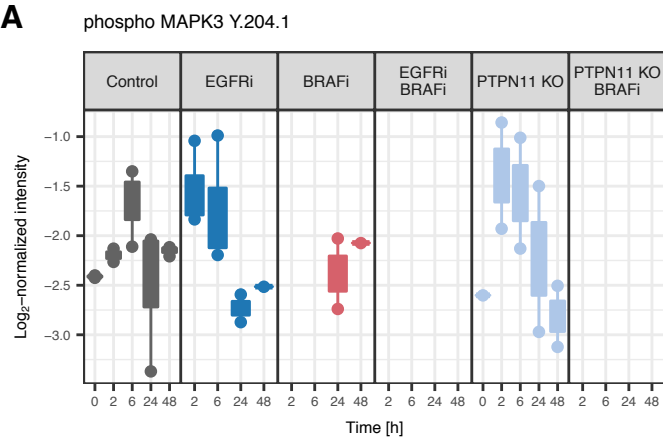


Figure S6

A

WiDr PTPN11 WT (R2)

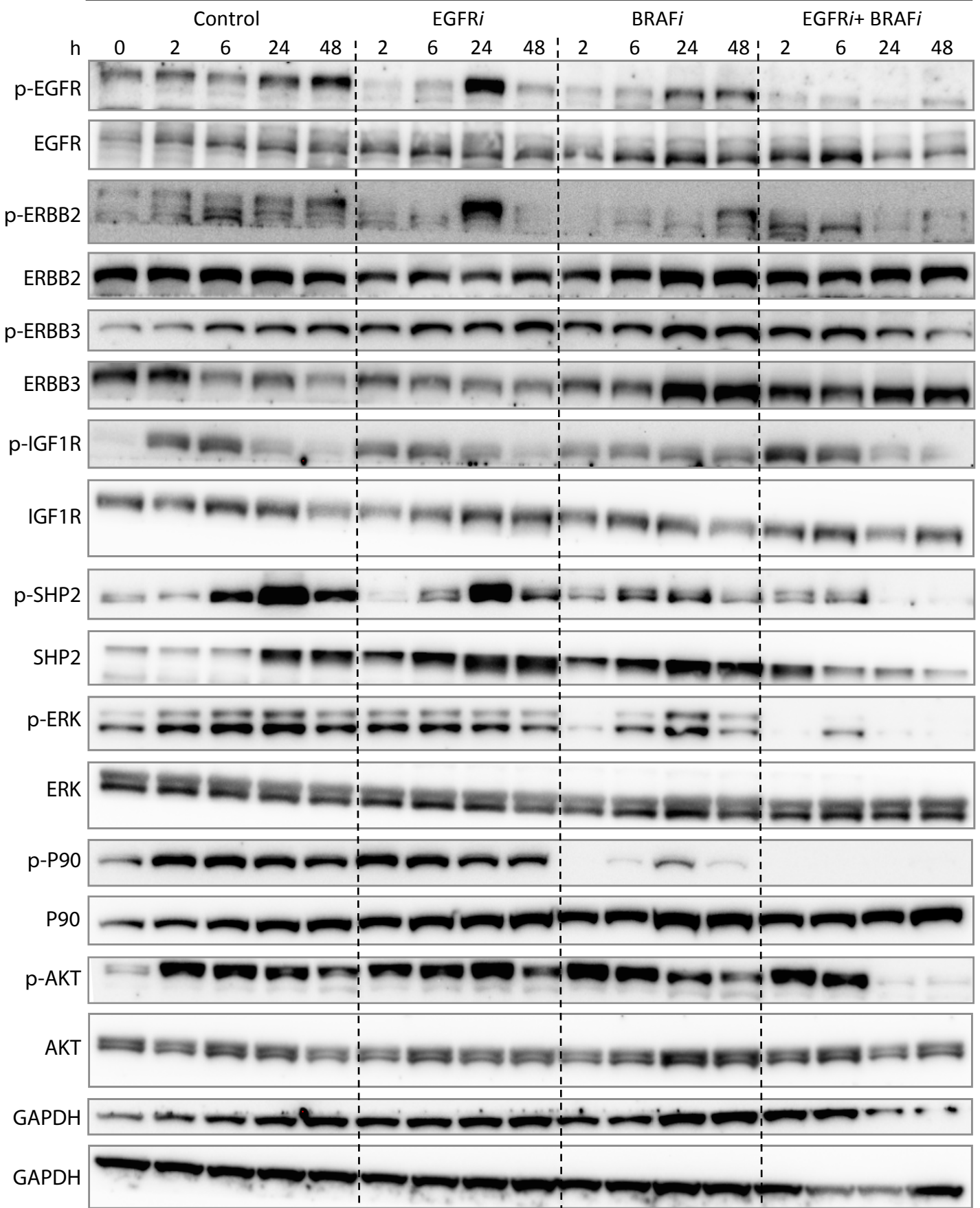
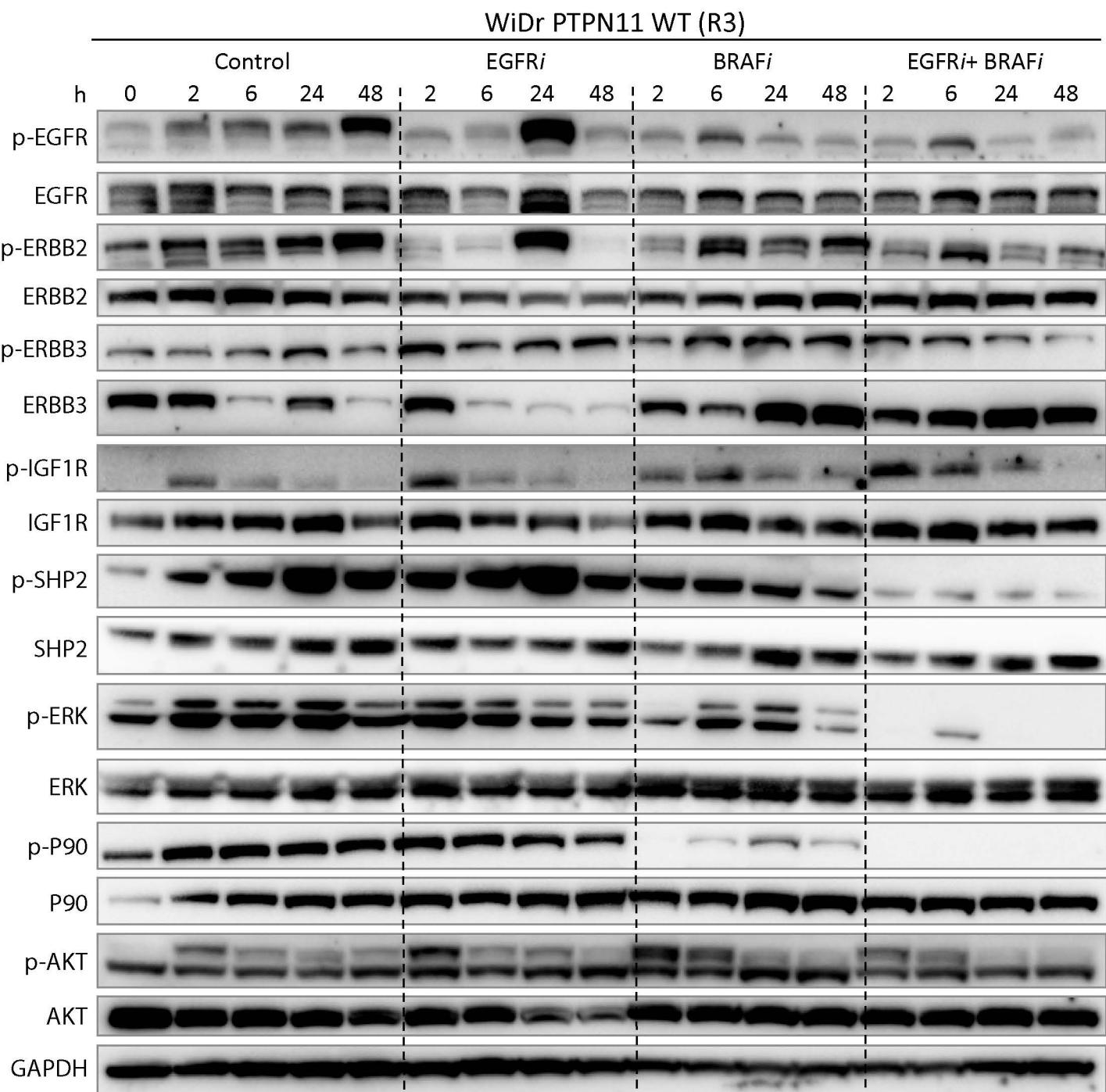


Figure S7

B**Figure S7**

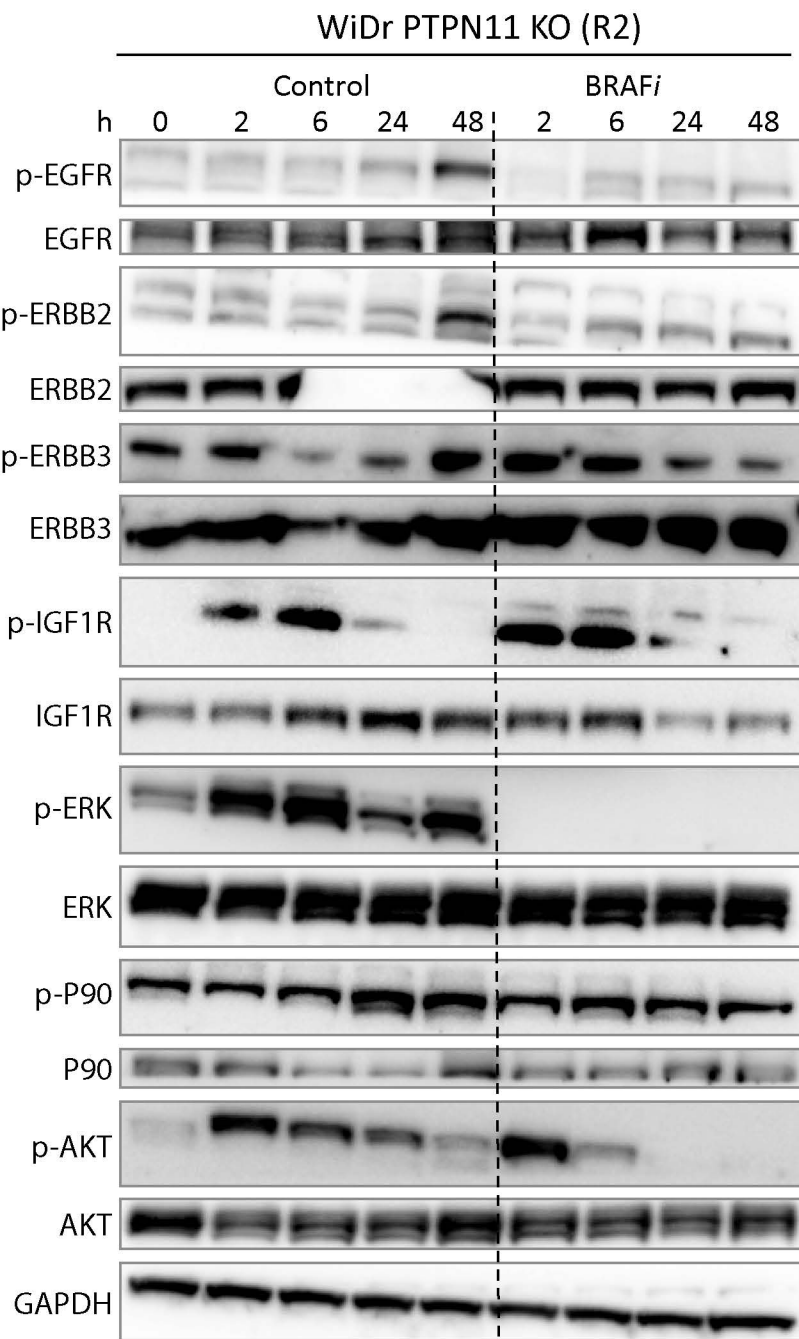
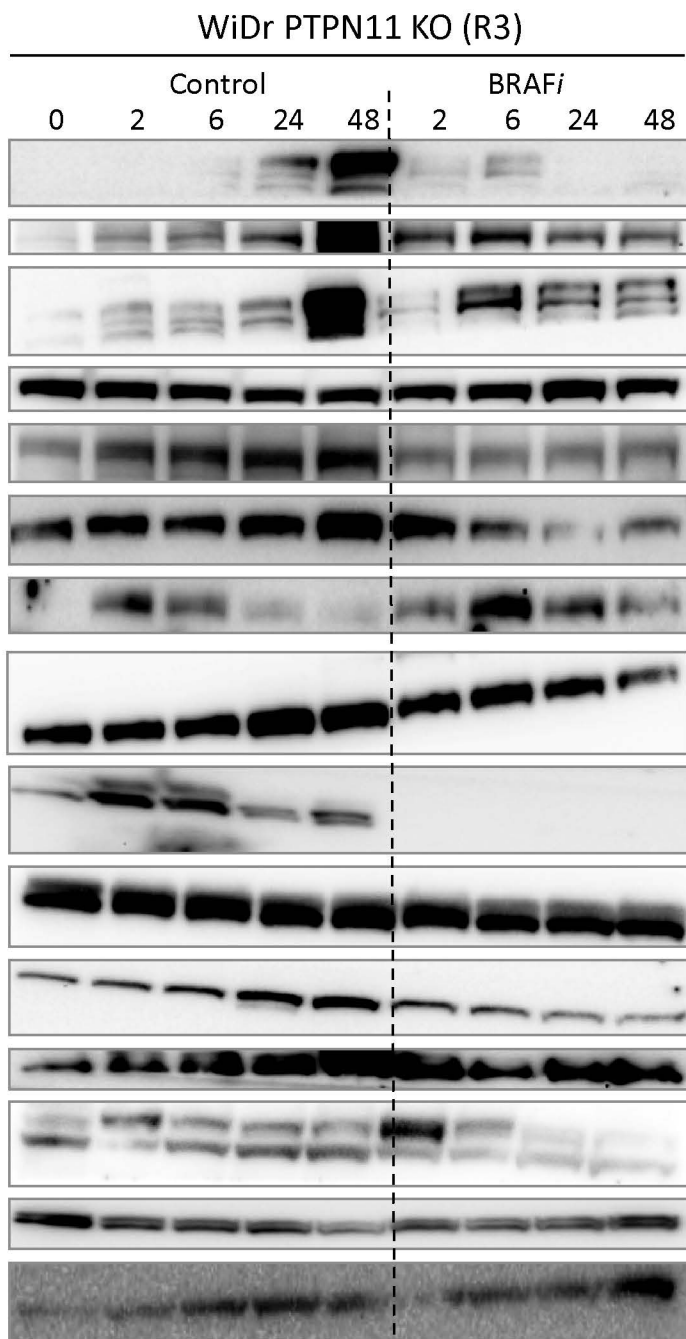
C**D**

Figure S7

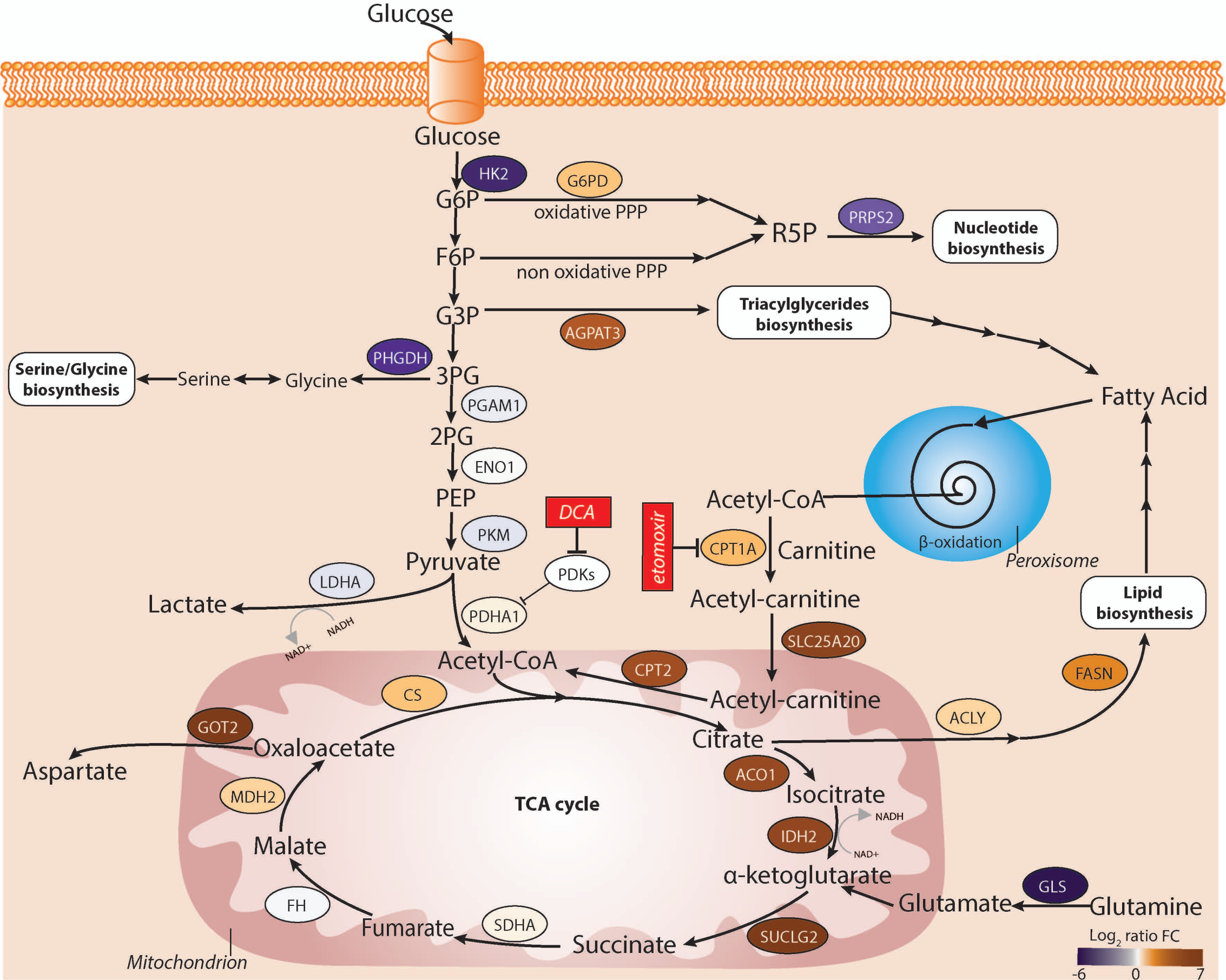


Figure S8

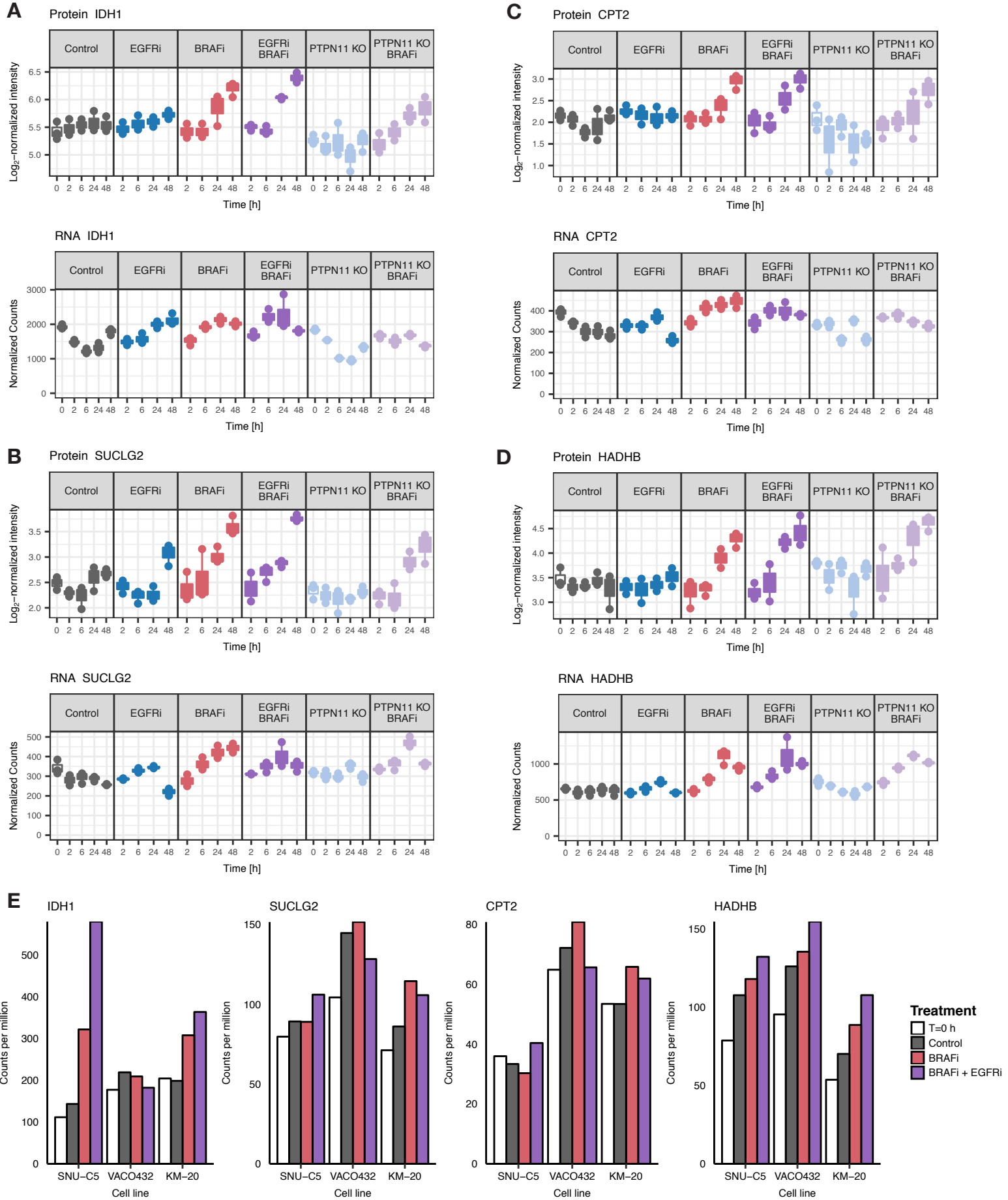


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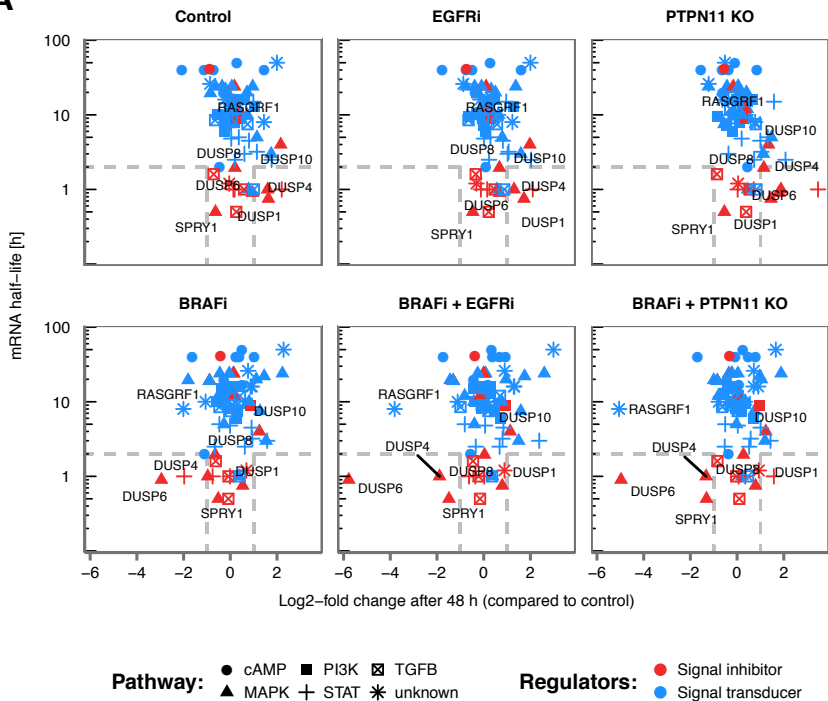
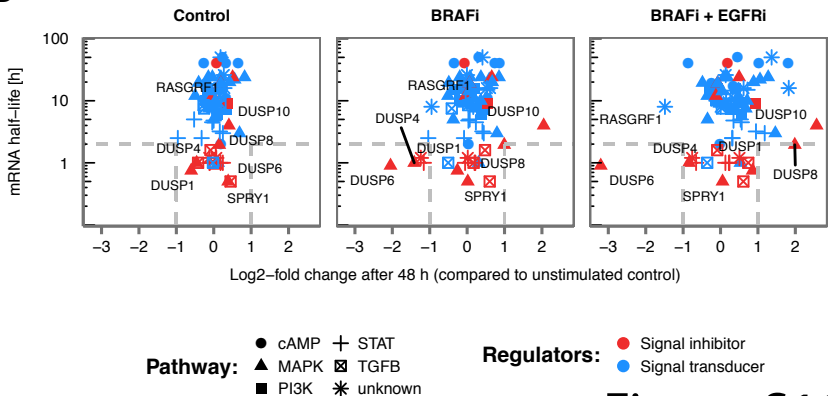
A**B**

Figure S10

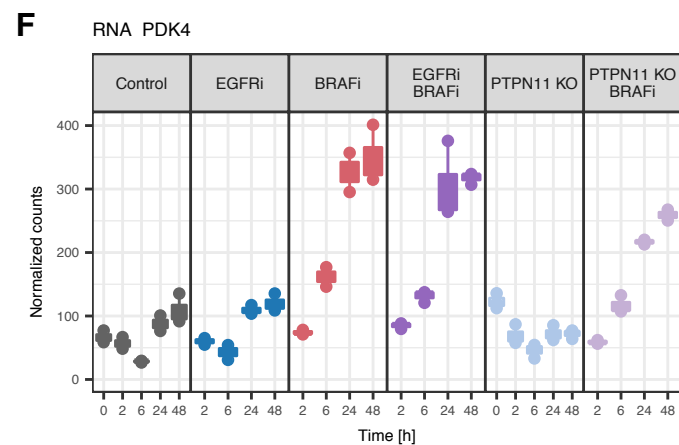
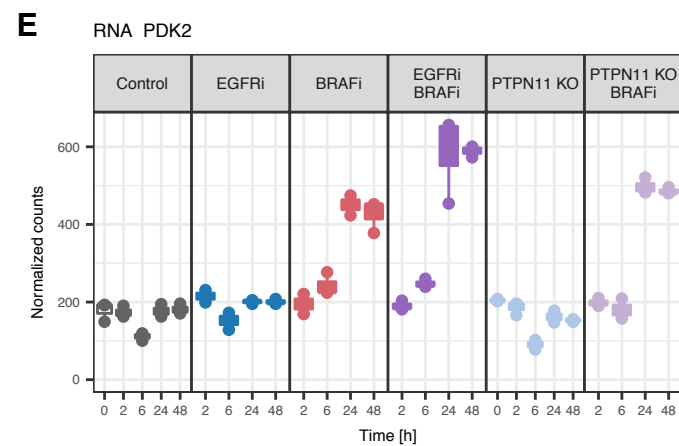
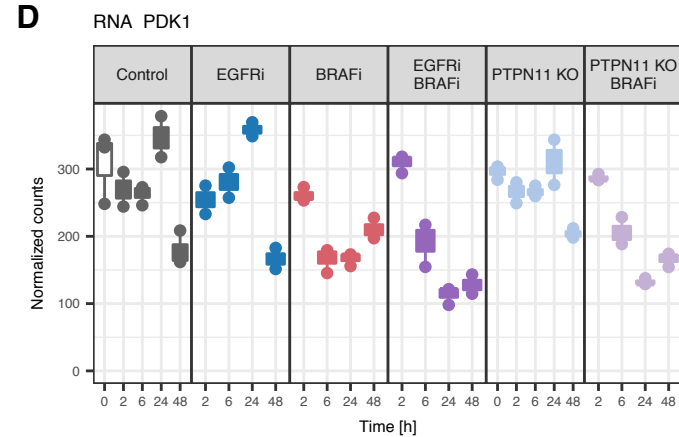
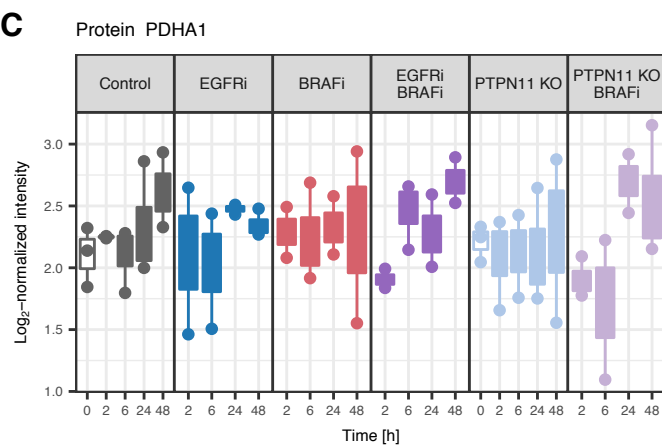
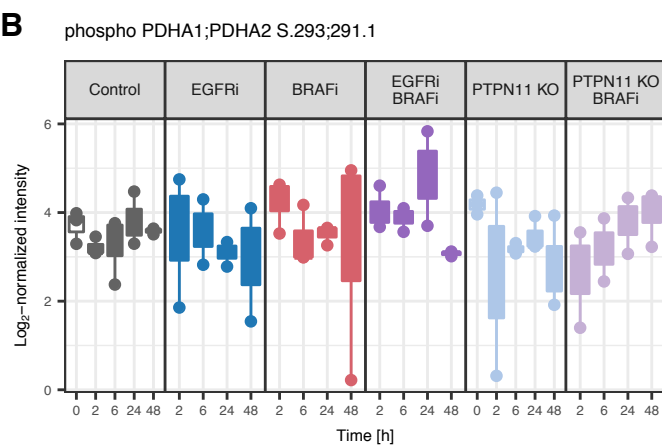
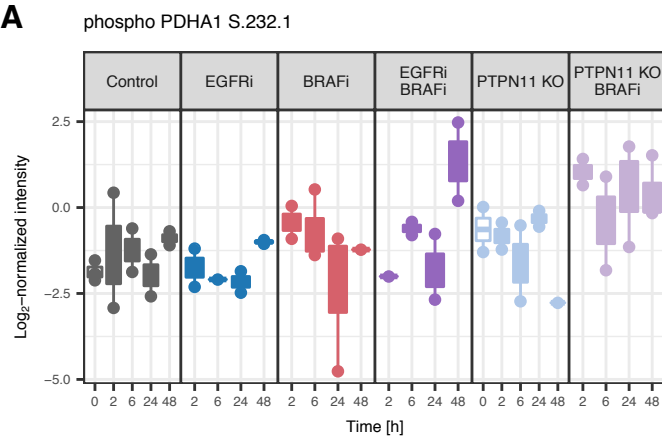


Figure S11

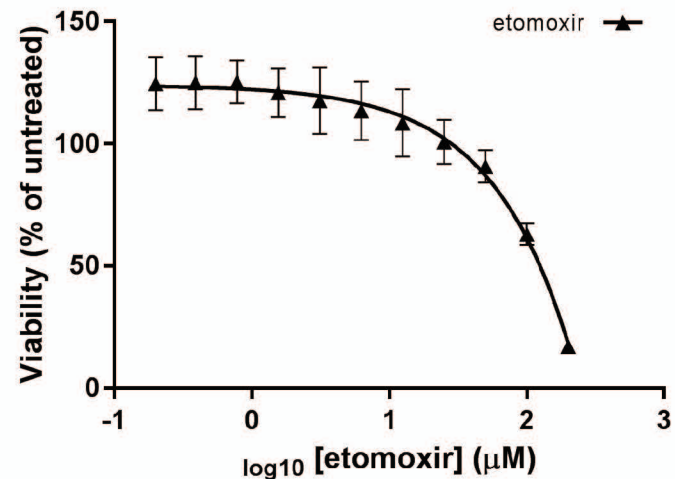
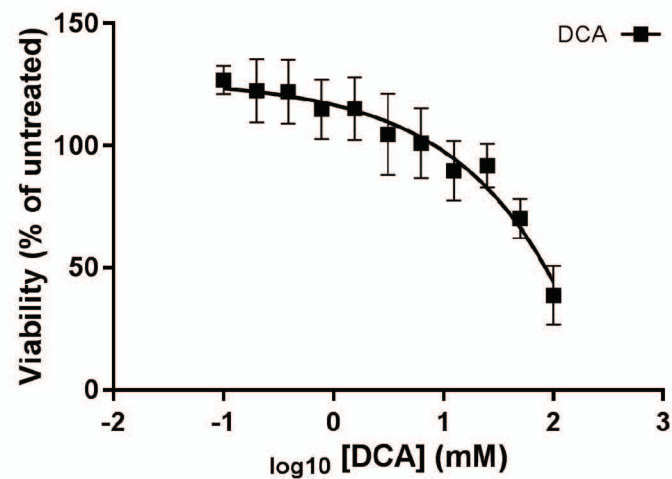
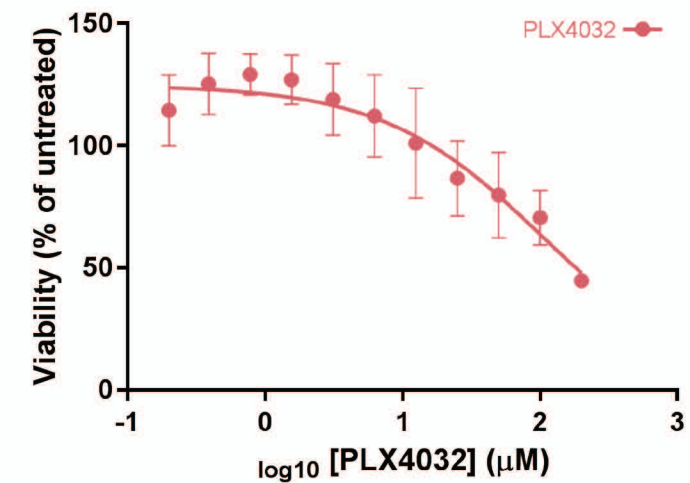
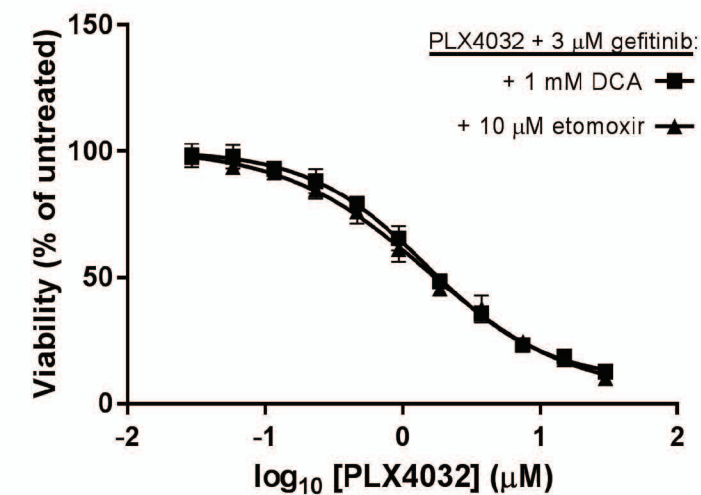
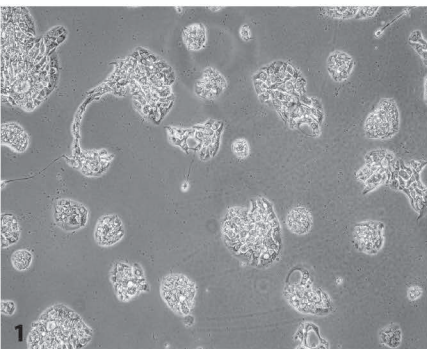
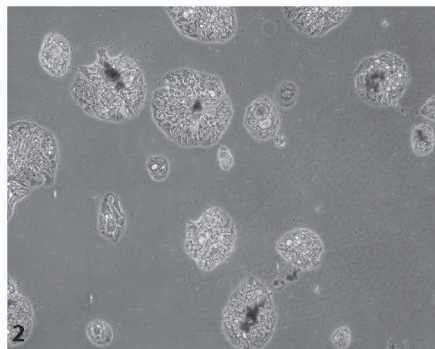
A**B**

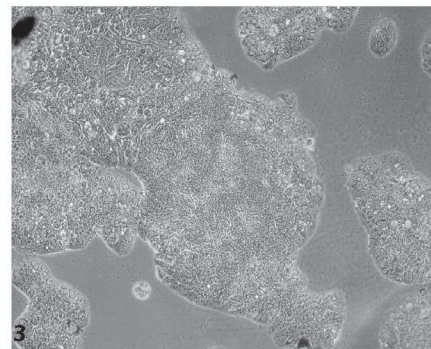
Figure S12

A

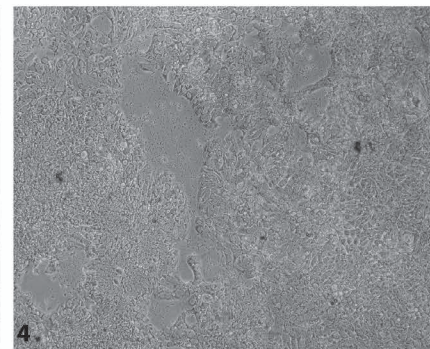
drug treatment (T= 78 d)



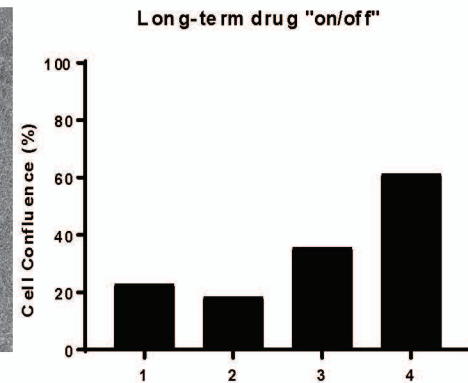
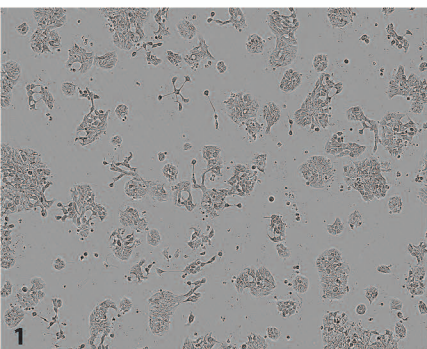
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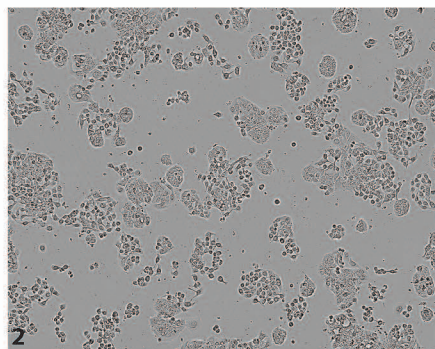
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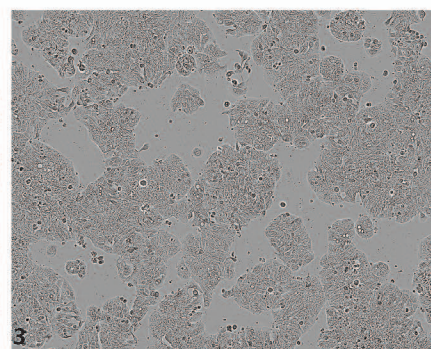
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**B**

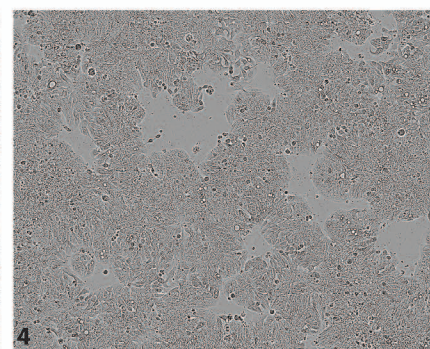
drug treatment (T= 5 d)



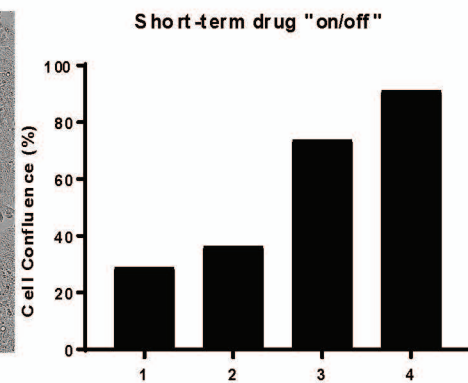
2-day "drug off" (T= 7 d)



3-day "drug off" (T= 8 d)



5-day "drug off" (T= 10 d)

**Figure S13**

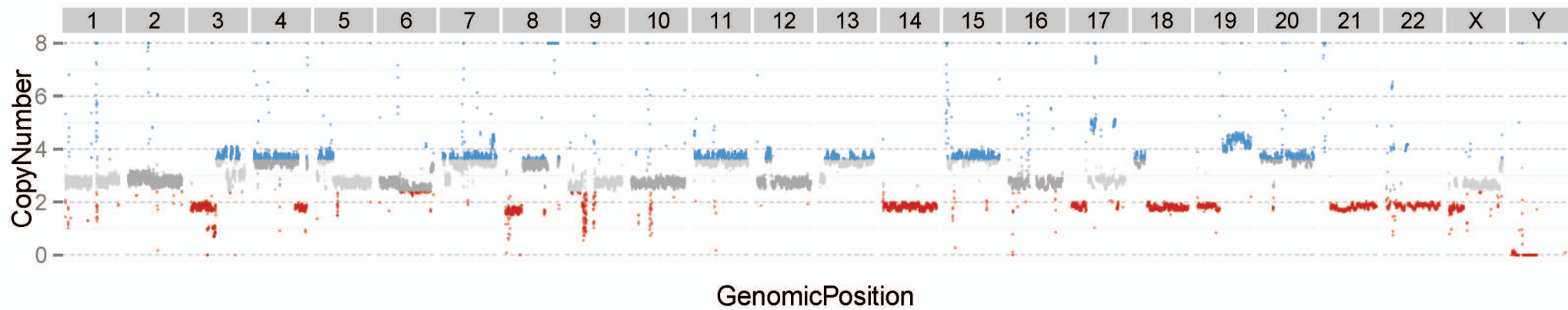
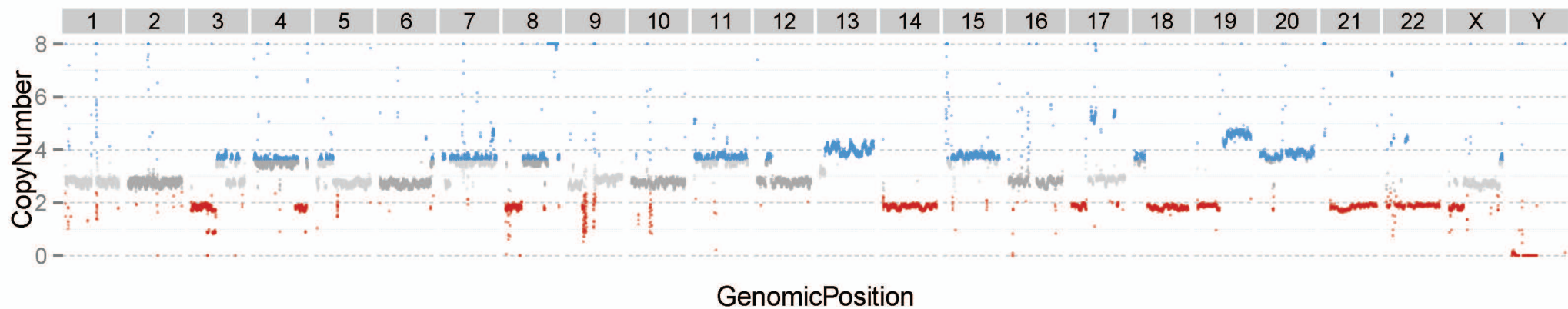
A**B**

Figure S14