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Phosphoproteomics of patient-derived xenographs identifies targets and markers associated with sensitivity and resistance to EGFR blockade in colorectal cancer

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1 **Title: Phosphoproteomics of PDXs identifies targets and markers associated**
2 **with sensitivity and resistance to EGFR blockade in colorectal cancer**

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20 **One Sentence Summary:** Phosphoproteomics on patient-derived xenografts of metastatic
21 colorectal cancer provided insight on the primary response to EGFR blockade.

22 **Abstract:** Epidermal growth factor receptor (EGFR) is a well-exploited therapeutic target in
23 metastatic colorectal cancer (mCRC). Unfortunately, not all patients benefit from current EGFR
24 inhibitors . Mass spectrometry-based proteomics and phosphoproteomics were performed on 30
25 genomically and pharmacologically characterized mCRC patient-derived xenografts (PDXs) to
26 investigate the molecular basis of response to EGFR blockade and identify alternative drug targets
27 to overcome resistance. Both the tyrosine and global phosphoproteome as well as the proteome
28 harbored distinctive response signatures. We found increased pathway activity related to MAPK
29 inhibition and abundant tyrosine phosphorylation of cell junction proteins, such as CXADR and
30 CLDN1/3, in sensitive tumors, whereas epithelial-mesenchymal transition and increased MAPK
31 and AKT signaling were more prevalent in resistant tumors. Furthermore, the ranking of kinase
32 activities in single samples confirmed driver activity of ERBB2, EGFR, and MET in cetuximab-
33 resistant tumors. This analysis also revealed high kinase activity of several members of the SRC
34 and Ephrin kinase family in 2 CRC PDX models with genomically unexplained resistance.
35 Inhibition of these hyperactive kinases, alone or in combination with cetuximab, resulted in growth
36 inhibition of ex vivo PDX-derived organoids and in vivo PDXs. Together, these findings highlight
37 the potential value of phosphoproteomics to improve our understanding of anti-EGFR treatment
38 and response prediction in mCRC and bring to the forefront alternative drug targets in cetuximab-
39 resistant tumors.

41 **Main Text:**

42 **INTRODUCTION**

43 Epidermal Growth Factor Receptor (EGFR) blocking monoclonal antibodies (mAb)
44 cetuximab and panitumumab belong to the standard therapeutic arsenal for patients with metastatic
45 colorectal cancer (mCRC). Administration of these drugs, regardless of treatment line or
46 chemotherapeutic backbone, has improved response rates and overall survival in this patient
47 population (1–4). Resistance to cetuximab and panitumumab has been partly attributed to
48 oncogenic mutations downstream of EGFR in KRAS proto-oncogene (*KRAS*) exon 2-4, NRAS
49 proto-oncogene (*NRAS*) exon 2-4, or B-Raf proto-oncogene (*BRAF*) V600E. According to the
50 European Society for Medical Oncology (ESMO) and the National Comprehensive Cancer
51 Network (NCCN) guidelines (5, 6) patients with these mutations are excluded for treatment with
52 cetuximab or panitumumab. Despite overall treatment benefit in patients without oncogenic
53 mutations in KRAS, NRAS or BRAF (RAS/RAF wild-type), 30% of patients do not receive
54 clinical benefit from anti-EGFR mAb treatment (7) due to the high molecular complexity and
55 heterogeneity of these tumors. In addition, patients with RAS/RAF wild-type (WT) tumors located
56 in the right side of the colon respond less to anti-EGFR antibodies compared with patients suffering
57 from left-sided CRC tumors, which are usually more dependent on EGFR signaling due to the
58 different embryological origin of the hindgut (8).

59 In recent years, genomics has identified several resistance mechanisms and predictive
60 biomarkers in RAS/RAF WT patient-derived xenograft (PDX) models, including MET proto-
61 oncogene (*MET*) and erb-b2 receptor tyrosine kinase 2 (*ERBB2*) amplification and mutations in
62 *ERBB2*, *EGFR*, fibroblast growth factor receptor 1 (*FGFR1*), platelet derived growth factor
63 receptor alpha (*PDGFRA*), and mitogen-activated protein kinase kinase 1 (MAP2K1) (9–11). This
64 study extends these findings, using proteomics and phospho-proteomics as a complementary
65 approach to capturing protein expression and activation states globally. Kinases control protein
66 activity and signaling through phosphorylation (12). Thus, unbiased profiling of protein
67 phosphorylation by mass spectrometry (phosphoproteomics) may uncover predictive markers and
68 drug targets (13–15). Specifically, *phosphotyrosine*-based (pTyr) phosphoproteomics provides
69 detailed quantification of tyrosine-phosphorylated proteins that are crucial for cancer proliferation
70 signaling and thereby may be advantageous to understand tyrosine kinase inhibitor responses.
71 Underscoring feasibility of such an approach in the clinical setting, we have previously shown that
72 down-scaling of the pTyr enrichment protocol is accomplishable and can be successfully deployed
73 to uncover patient-specific and drug-associated profiles in small tumor needle biopsies (16, 17).

74 Large-scale proteomics and global phosphoproteomics studies applied to CRC in a multi-
75 omics context have contributed to describing the molecular landscape of primary (18) and
76 metastatic CRC (19) and suggested new therapeutic opportunities for patients with tumors that do
77 not harbor druggable mutations (19). A recent proteomic study in two CRC PDX models that had
78 been rendered resistant to cetuximab by continuous antibody treatment highlighted changes in the
79 abundance of EGFR ligands and enrichment of proliferative kinase signatures as correlates of
80 acquired resistance (20). Whereas these analyses emphasized the importance of kinase-substrate
81 correlation networks for prediction of drug sensitivity in patients with mCRC, they did not address
82 the (phospho)proteomic underpinnings of innate sensitivity and resistance to EGFR inhibition on
83 a systematic basis.

84 In this study, we combined mass-spectrometry-based proteomics with global (TiO₂) and
85 pTyr-based phospho-proteomics, to analyze a unique panel of 30 genomically characterized
86 mCRC-PDX tumors annotated for response to cetuximab as seen in the clinic and confirmed in
87 the mouse setting (10). Our findings improve understanding of the mechanisms dictating
88 sensitivity and resistance to EGFR-blockade in mCRC (9, 10) and pinpoint actionable kinase
89 activities in individual tumors that provide new treatment options.

90 **RESULTS**

91 **Phosphoproteomics profiling of patient-derived xenografts**

92 To explore biological processes associated with sensitivity and resistance to EGFR blockade and
93 to identify candidate markers and alternative drug targets to overcome resistance, mass
94 spectrometry-based phospho-proteomics was performed on a cohort of 10 cetuximab-sensitive and
95 20 cetuximab-resistant xenograft tumors, as assessed in a mouse clinical trial that recapitulated the
96 clinical treatment outcomes (9, 10, 21) (table S1). Out of twenty resistant tumors, three did not
97 display genetic alterations known to affect responsiveness to EGFR blockade in mCRC (fig. S1A).
98 Mass spectrometry-based proteomics, global phosphoproteomics (TiO₂), and pTyr-based
99 phosphoproteomics (fig. S1B) were performed on each tumor tissue sample. Data analysis
100 consisted of comparative group-based analysis of cetuximab-sensitive (CS) versus (vs) cetuximab-
101 resistant (CR) tumors and kinase activity ranking analysis of individual resistant tumors to find
102 potential drug targets (fig. S1C).

103 Histological assessment of hematoxylin and eosin-stained sections indicated an average
104 percentage of 65% epithelial cancer cells, 15% stroma, and 20% necrosis (fig. S2A). Based on this
105 assessment, three samples (CRC0343, CRC0490, CRC1138_Repl1) showed more than 50%
106 necrosis in the histological assessment. Further proteomic analysis of the samples revealed that
107 two conventional markers for necrosis, namely high mobility group box 1 (HMGB1) and
108 peptidylprolyl isomerase A (PPIA), had only medium-to-low protein expression in the three samples
109 scored as necrotic. Therefore, no samples were excluded based on histological assessment (fig.
110 S2B).

111 Seven samples were excluded from TiO₂ data. Five samples (CRC0177, CRC0196_Repl1,
112 CRC0254, CRC1138_Repl1, CRC1147_Repl1) were excluded due to technical failure, and two
113 samples (CRC0166_Repl1, CRC0219_Repl1) were excluded due to high variation between
114 replicates and low peptide yield (Suppl. Table. 1, fig. S3). In general, biological replicates of PDX
115 tumors (pTyr, 13 replicates; TiO₂, 10 replicates; expression, 12 replicates) clustered together in
116 correlation heatmap (fig. S3).

117 The mCRC-PDX proteome dataset consisted of 5287 identified proteins and the
118 phosphoproteome dataset consisted of 13,110 class-I phospho-sites (8973 pSer, 1066 pThr, and
119 3073 pTyr) on 15,095 phospho-peptides from 5207 phosphoproteins (1669 pTyr and 3538 global
120 TiO₂ capture) including 255 kinases, of which 53 tyrosine kinases, including EGFR (fig. S4 and
121 Suppl. Tables 2-6). Unsupervised clustering using all (phospho-)proteome data did not show sub-
122 clustering of CS and CR tumors (fig. S5), underscoring the heterogeneity and the minor impact of
123 resistance to cetuximab on the profiles.

124 **Differential (phospho)proteome profiles of cetuximab-sensitive and resistant PDX tumors** 125 **provide insight into molecular determinants of response to cetuximab**

126 Group-based statistics were performed between CS and CR tumors to find discriminative
127 molecular determinants of response (Fig. 1 A, see Suppl. Table 7 for all comparisons for all data
128 types). These comparative analyses revealed 53 (12 pTyr, 41 TiO₂) differentially phosphorylated
129 phospho-sites and 53 proteins with differential abundance in CS tumors versus all resistant tumors
130 (CR-ALL); 75 (8 pTyr, 67 TiO₂) differentially phosphorylated phospho-sites and 49 proteins in
131 CS versus CR tumors with oncogenic mutations in KRAS, NRAS or BRAF (CR-MUT); and 17
132 (4 pTyr, 13 TiO₂) differentially phosphorylated phospho-sites and 72 proteins in CS tumors versus
133 resistant RAS/RAF WT tumors (CR-WT) (Fig. 1A).

134 Comparing significant phospho-sites (p-value <0.01, FC > 1.5) from the tree group
135 comparisons (CS vs. CR-ALL, CS vs. CR-MUT, CS vs. CR-WT) shows an overlap of phospho-
136 sites that either fall into a general response signature (CS1/2/3, CR1/2/3) or a signature for either
137 RAS/RAF mutated (CS4, CR4) or wild-type tumors (CS5, CR5) (Fig. 1A, Suppl. Table 8). (Fig.
138 1A, Suppl. Table 8). Combining this phospho-site signature for clustering showed clear
139 segregation between cetuximab-sensitive and resistant tumors. Although pTyr, global
140 phosphoproteome, and proteome analyses enabled separation according to drug response, they
141 yielded different layers of information: the pTyr signature predominantly consisted of phospho-
142 sites with increased phosphorylation in sensitive tumors, whereas the global TiO₂-based signatures
143 contained almost only phospho-sites that were more phosphorylated in resistant tumors. Moreover,
144 the global TiO₂ phosphorylation signatures additionally separated CR-MUT from CR-WT tumors.
145 Finally, the protein expression data were analyzed similarly, and they as well separated sensitive
146 tumors from resistant ones, providing a balanced number of proteins with higher expression in
147 either one of the two response classes (Fig. 1B, fig. S6A-C). Suppl. Table 9 summarizes top
148 discriminative proteins; the top 10 proteins for CS tumors include RAB11 family interacting
149 protein 5 (RAB11FIP5), claudin 3 (CLDN3), solute carrier family 16 member 1 (SLC16A1),
150 claudin 1 (CLDN1), SATB homeobox 2 (SATB2), 4-hydroxyphenylpyruvate dioxygenase like
151 (HPDL), serine incorporator 5 (SERINC5), chromodomain helicase DNA binding protein 7
152 (CHD7), sorting nexin 33 (SNX33), and CXADR Ig-like cell adhesion molecule (CXADR),
153 whereas the top 10 proteins for CR tumors include absent in melanoma 1 like (AIM1L),
154 asparaginase and isoaspartyl peptidase 1 (ASRGL1), claudin 2 (CLDN2), myelin expression factor
155 2 (MYEF2), TSC22 domain family member 2 (TSC22D2), torsin family 4 member A (TOR4A),
156 ATP binding cassette subfamily C member 3 (ABCC3), LIM domain and actin binding 1
157 (LIMA1), neural precursor cell expressed developmentally down-regulated 9 (NEDD9), and tight
158 junction protein 1 (TJP1).

159 Murine proteins from the host mice in PDX (phospho) samples may influence
160 protein/peptide quantification when doing a human-only database search, especially when tumor
161 cell percentage is not high. The samples from the PDX models described here have a relatively
162 high average of 65% epithelial cell content. Still, to underscore the validity of introducing less
163 complexity in the analysis of the phosphoproteomics PDX results by using a human-only database
164 search, we cross-checked results obtained for the differential phosphosites associated with
165 cetuximab sensitivity and resistance against a database search using the combined human and
166 mouse sequences. Suppl. Table 10 shows that the spectrum identifications based on the combined
167 search resulted in precisely the same underlying set of peptide sequences for each phosphosite.
168 However, the quantification of the phosphosites sites was almost wholly derived from the same
169 precursor signals as in the human-only database search. This additional human-mouse combined
170 database search underscored the validity of the differential phosphosites obtained from analyzing
171 PDX phosphoproteomics data search against the human-only database.

172 Differential (phospho) proteins of all three comparisons (CS compared to either CR-ALL,
173 CR-MUT, or CR-WT) were combined in a protein-protein interaction network (Fig. 2), showing
174 results from the comparison with the largest fold change in case of overlap between comparisons.
175 mRNA expression of both the complete PDX cohort (157 CS and 246 CR previously described
176 PDX tumors, referred to hereafter as RNA400) (22), and the subset of models used in this study
177 (RNAsub), was used to annotate proteins further (fig. S7). Markov clustering combined with gene
178 ontology analysis revealed eight biologically relevant protein clusters (fig. S8). Clusters associated
179 with cetuximab sensitivity were the “cell-cell junction organization” cluster, containing the cell-
180 cell adhesion molecules CXADR and claudin 1 and 3, and the “ribonucleoprotein complex
181 biogenesis” cluster with proteins POU class 2 homeobox associating factor 1 (BOB1), DEAD-box
182 helicase 27 (DDX27), DEAD-box helicase 28 (DDX28), ribosome biogenesis regulator 1 homolog
183 (RRS1), dyskerin pseudouridine synthase 1 (DKC1), RNA terminal phosphate cyclase like 1
184 (RCL1), nucleophosmin 1 (NPM1), and ribosomal L1 domain containing 1 (RSL1D1). Of note,
185 both RNA400 and RNAsub comparisons showed significant higher expression of CXADR in CS
186 models (p-value <0.05). The clusters “enzyme-linked receptor protein signaling” and “type 1
187 interferon signaling” were more associated with cetuximab resistance. The cluster “enzyme-linked
188 receptor protein signaling” included proteins involved in AKT serine/threonine kinase 1 (AKT1)
189 signaling, where the PI3K-PTEN-AKT signaling axis is known to be involved in resistance to
190 EGFR inhibitors (23). One other cetuximab resistance protein that stands out in Fig. 2 is
191 KIAA1522. Although KIAA1522 is an uncharacterized protein with unknown function, high
192 mRNA expression of KIAA1522 has been linked to non-small cell lung cancer as a marker of poor
193 prognosis (24).

194 Gene-set enrichment analysis (GSEA) on protein expression and RNAsub data revealed
195 oxidative phosphorylation (OXPHOS) as one of the most enriched processes (adj. *P*-value < 0.05)
196 in CS tumors, along with MYC targets and adipogenesis (Fig. 3, fig. S9). Although the latter two
197 processes were also captured at the RNA level, enrichment of OXPHOS was revealed at the protein
198 level only. In addition, enrichment of these processes was more pronounced (in the case of
199 OXPHOS) or unique (in the case of adipogenesis) in the CR-MUT compared to the CR-WT
200 comparison. CR tumors showed strong enrichment of processes associated with epithelial-
201 mesenchymal transition (EMT) at the RNA and protein levels and interferon response-related
202 biology at the protein level only. (Fig. 3, fig. S9).

203 Post-translational modification signature enrichment analysis (PTM-SEA) of pTyr data
204 revealed enrichment of the fibroblast growth factor 1 (FGF1) pathway (FDR p-value <0.25) in
205 cetuximab-sensitive tumors. In contrast, resistant tumors showed enrichment (FDR p-value <0.05)
206 of the thymic stromal lymphopoietin pathway (TSLP) and ABL proto-oncogene 1 (ABL1) (FDR
207 p-value <0.05). Also, neuroblastoma (FDR p-value <0.25) and anti-CD3 perturbation-related
208 biology (FDR p-value <0.25) were enriched more prominently in CR-MUT tumors (Fig. 4). TiO2
209 data indicated enrichment of AKT serine/threonine kinase 1 (AKT1) in CR tumors that correlates
210 with the earlier findings in the protein-protein interaction of CS versus CR (Fig. 2). In agreement
211 with expectations, comparative analysis of CS versus CR-MUT showed reduced enrichment for
212 many signaling signatures downstream in the Ras/Raf pathway, including mitogen-activated protein
213 kinase 1 (MAPK1) (FDR p-value <0.25), mitogen-activated protein kinase 3 (MAPK3) (FDR p-
214 value <0.25), and MAPK activated protein kinase 2 (MAPKAPK2) (FDR p-value <0.05) in CS
215 samples (Fig. 4). Conversely, CS tumors showed a positive enrichment for phosphosite-driven
216 signatures related to the mitogen-activated protein kinase (MEK)1/2 inhibitor U0126 and the p38
217 MAPK inhibitor losmapimod (Fig. 4). Additionally, enrichment of the TEK receptor tyrosine

218 kinase (TIE2) pathway, protein kinase C alpha (PRKCA), and, as in the pTyr signature, anti-CD3
219 perturbation was related to cetuximab resistance (Fig. 4).

220 **In-depth analysis of resistant tumors reveals hyperactive kinases**

221 To investigate whether hyperactive kinases can act as alternative targets for treatment in
222 cetuximab-resistant tumors, a single sample Integrative inferred Kinase Activity (INKA) analysis
223 was performed on all PDX tumors (15) (Suppl. Fig 9 and fig. S11). Figure 6A shows the pTyr
224 kinase activities with an overall 25% higher INKA score comparing CS to CR models, including
225 for CS the kinases; EGFR, EPH receptor B2 (EPHB2), EPH receptor B3 (EPHB3), and fyn related
226 Src family tyrosine kinase (FRK) and in CR tumors; cyclin dependent kinase 5 (CDK5), EPH
227 receptor A3 (EPHA3), insulin like growth factor 1 receptor (IGF1R), spleen associated tyrosine
228 kinase (SYK), mitogen-activated protein kinase 14 (MAPK14), ERBB2, erb-b2 receptor tyrosine
229 kinase 3 (ERBB3), and MET. Comparing the INKA score per kinase across all tumors revealed
230 that some tumors had outlier kinase activity (Fig. 5A, fig. S12). These high INKA scores were
231 found in models that harbored previously identified gene amplifications (10) in *EGFR* (CRC0098),
232 *MET* (CRC0196), or *ERBB2* (CRC0080, CRC0176). Further underscoring a critical oncogenic
233 driver function for these kinases is the high (number 1) INKA rank number relative to other
234 identified kinases in the amplified PDX models (Fig. 5B, fig. S11, and fig. S12). Previous work
235 showed that these cetuximab-resistant PDX models with gene amplifications respond to specific
236 inhibition of MET (CRC0196) (25) or ERBB2 (CRC0080, CRC0176) (10, 26) especially in
237 combination with cetuximab, highlighting their potential as alternative targets for combination
238 treatment (fig. S13). Finally, kinase activity analysis of the TiO₂ phosphoproteomics data revealed
239 high activity of AKT1 and MAPK3 in resistant models (fig. S11 and fig. S12). These results are
240 in line with previous work showing that AKT inhibition potentiates the effect of cetuximab
241 treatment (27). Altogether, these results indicate the value of phosphoproteomics coupled with
242 INKA analysis of individual cetuximab-resistant tumors to identify hyper-active kinases as targets
243 for treatment.

244 **Hyper-active kinases highlight potential treatment targets**

245 Previous genomic analysis of PDX models CRC0161, displaying EGFR outlier activity (Fig. 5A)
246 and CRC0166 did not identify oncogenic driver alterations that can explain resistance to
247 cetuximab. Therefore, these two models were investigated in functional experiments with drug
248 selection based on their INKA profile. In both models, INKA analysis pinpointed high activity for
249 EGFR, EPHA2, several other ephrin receptors, as well as SRC family tyrosine kinases (Fig. 6A).
250 EPHA2 has been previously implicated in resistance to EGFR inhibition in gefitinib-resistant
251 HCC827 cells (28) and high expression of EPHA2 has been correlated with worse clinical
252 prognosis in patients with mCRC treated with cetuximab (29, 30).

253 Dasatinib is a potent inhibitor of ephrin family kinases, especially EPHA2, as well as SRC
254 family kinases, and inhibits EGFR when used in the high nanomolar range (31–33) (Fig. 6B).
255 Therefore, dasatinib was selected to test the potential of these kinases as alternative treatment
256 targets. Viability upon treatment was tested in organoid cultures derived from PDX-model
257 CRC0161, which showed INKA profiles analogous to those of its matched PDX counterpart (Fig.
258 7A). CRC0196 and CRC0254 organoids were included as negative controls since both models did
259 not show high INKA scores for dasatinib targets (fig. S14).

260 Treatment with dasatinib reduced cell viability in the micro to nano molar range in
261 CRC0161 but not in control models CRC0196 and CRC0254 with low dasatinib target activity
262 (Fig. 7A). In addition, the combination of low doses of both cetuximab [0,7 µg/ml (~ 5nM)] and
263 dasatinib (5nM) impaired cell viability in CRC0161, whereas either treatment alone was
264 ineffective (Fig. 7B). A 200-fold dose increase of the inhibitors [140 µg/ml (~ 1000nM) for
265 cetuximab and 1000nM for dasatinib] confirmed the relatively poor responsiveness of model
266 CRC0161 to EGFR inhibition (only approximately 40% reduction of cell viability) and its
267 sensitivity to dasatinib (about 80% reduction). Combining cetuximab and dasatinib did not
268 significantly reduce cell viability in CRC0161 compared to dasatinib alone, likely because
269 monotherapy with high-dose dasatinib also blocked EGFR and was sufficient to reach the
270 inhibitory plateau (Fig. 7B). Furthermore, as expected, treatment of CRC0161 organoids with the
271 EGFR inhibitor afatinib as an additional control did not affect viability, confirming the finding
272 that CRC0161 is resistant to EGFR blockade (fig. S15). Finally, JAK was chosen as another
273 negative control since CRC0161 did not show high INKA scoring of JAK. In line with the absence
274 of JAK target activity, treatment of CRC0161 organoids with the JAK inhibitor ruxolitinib did not
275 reduce viability (fig. S15).

276 Unfortunately, CRC0161 proved unable to re-engage in mice after thawing, which
277 prevented in vivo validation experiments. Conversely, the other predicted dasatinib sensitive
278 model CRC0166 was hard to grow as organoids but could be tested in vivo in a PDX assay. After
279 three weeks of treatment, dasatinib alone was ineffective in controlling tumor growth, and
280 cetuximab alone only retarded tumor growth. Tumors volume increased 40% on average, in
281 agreement with our historical data in which response of this model to cetuximab was categorized
282 as progressive disease (table 1). Conversely, the combination of dasatinib and cetuximab
283 completely blocked tumor growth, with a significant advantage (p-value = 0.0377) in tumor
284 volumetric reduction compared with single-agent cetuximab (Fig. 7C, Suppl. Table 11).
285 Altogether, these experiments show that phosphoproteomics coupled to INKA analysis may
286 provide a relevant read-out of kinase activities for individualized (combination) treatment.

287 **DISCUSSION**

288 This study analyzed the phospho-proteome and proteome profiles of 30 mCRC patient-derived
289 xenografts, genomically characterized and annotated for response to cetuximab treatment, to shed
290 light onto the signaling events associated with sensitivity and resistance to EGFR blockade in
291 patients with mCRC. Mass spectrometry-based analysis revealed distinctive phospho-sites and
292 proteins between cetuximab-sensitive and resistant tumors; identified kinase driver hyperactivity
293 sustained by underlying genomics aberrations; and yielded potential kinase targets to treat
294 genomically unexplained resistant models. The phosphoproteome and proteome provided
295 complementary insights, and combined interpretation aided a deeper understanding of cetuximab
296 response in mCRC tumor biology. More specifically, the tyrosine-based phosphorylation data
297 provided insight into upstream tyrosine kinase signaling pathways more enriched in cetuximab-
298 sensitive tumors. In contrast, the global phosphoproteome, dominated by pSer/pThr phosphosites,
299 was enriched in downstream signaling events more associated with resistant tumors (Fig. 1). In
300 line with our results, Rivera et al., observed positive enrichment of canonical EGFR and EGF
301 pathway signatures in their cetuximab sensitive CRC PDX models (20). Our findings support the
302 idea that there is no single factor that can accurately predict how tumors will respond to treatment.
303 Positive outcomes for patients are not solely determined by one factor, but rather by a combination

304 of multiple molecular characteristics evident in multi-omics data. Therefore, it is important to
305 consider all factors when predicting treatment outcomes. (19).

306 The distinctive phospho-sites and proteins combined in a network diagram, annotated with
307 mRNA data (Fig. 2), provided more insight into the biological processes involved in cetuximab
308 response. Sensitive tumors revealed higher phosphorylation of proteins functionally related to cell-
309 cell contact and cellular tight junction organization, such as CXADR, CLDN1, CLDN3. Loss of
310 junction proteins and disruption of overall cell-cell organization has historically been implicated
311 with an early invasive and metastatic phenotype (34). However, more recent studies have
312 documented that increased expression and activity of essential proteins involved in the tight-
313 junction organization also correlates with tumor progression, likely due to their role as signaling
314 substrates (35). For example, Pike et al. (36) described that CXADR potentiates EGFR signaling
315 by delaying receptor internalization. This suggests that CXADR overexpression and
316 phosphorylation, as observed in cetuximab-sensitive tumors, may enhance EGFR pathway
317 activity, hence contributing to EGFR dependency. Accordingly, analysis of global gene expression
318 data from 403 CRC PDXs for which annotation of response to cetuximab was available (22)
319 revealed higher RNA expression of CXADR in cetuximab-sensitive than in cetuximab-resistant
320 PDX models, making a solid case for CXADR as a potential predictor of response to cetuximab in
321 metastatic colorectal cancer. Previous proteomics and phosphoproteomics analysis of acquired
322 resistance to cetuximab in two isogenic CRC PDX models revealed multiple pathways
323 downstream of EGFR and found endocytosis, cell-cell adhesion, tight and adherence junctions
324 related terms enriched in the upregulated proteins and phosphosites of the sensitive model (20).

325 GSEA revealed an association between EMT related signaling and resistance to cetuximab.
326 EMT signaling has been previously linked to resistance to EGFR blockade (20) by prompting a
327 switch to alternative kinase signaling pathways (33), including the AKT1 pathway. In addition, a
328 recent study has found that CXADR acts as a negative regulator of EMT by providing an AKT-
329 inhibitory signalosome at the tight junction (34). The role of CXADR as an EMT gate-keeper,
330 combined with its ability to potentiate EGFR signaling and constrain AKT signaling, suggests that
331 CXADR and cell-cell adhesion locks CRC cells into an epithelial phenotype dependent on EGFR-
332 induced growth, making tumors susceptible to EGFR inhibition. Conversely, cells transitioning
333 toward a mesenchymal phenotype become more dependent on pathways that are parallel or
334 downstream to EGFR signaling, such as the AKT1 pathway, and are thus less vulnerable to EGFR
335 inhibition (18). This assumption is consistent with our results, whereby low CXADR expression
336 and high AKT signaling, as evidenced by both INKA and PTM-SEA analyses, typify cetuximab-
337 resistant tumors. In a complementary perspective, activation of AKT and its downstream signaling
338 effector mTOR has been documented to stimulate EMT (35). The relevance of AKT in cetuximab
339 resistance is supported by the observation that AKT inhibition enhances the depth of response to
340 cetuximab in CRC PDX models (27).

341 OXPHOS was found to be enriched in CS tumors. Evidence that tyrosine kinase signaling
342 can regulate mitochondrial oxidative phosphorylation function (37) combined with the finding that
343 cetuximab may have a role in impairing mitochondrial function in CRC (38), could suggest that
344 tumors with a more abundant OXPHOS expression profile may be more susceptible to EGFR
345 inhibition.

346 Kinase activity analysis using INKA was employed to highlight essential kinases and
347 identified potential targets for single and combination treatment for individual tumors (15). This
348 analysis pinpointed high signaling activity of ERBB2, EGFR, and MET in resistant PDX models

349 in line with the corresponding gene amplifications. Moreover, INKA revealed high activity of
350 ephrin kinases, and in particular EPHA2, in models CRC0161 and CRC0166, with genomically
351 unexplained mechanisms of resistance. The potential of these kinases as targets was evaluated by
352 treating organoid cultures of CRC0161 and mice harboring CRC0166 PDXs with dasatinib, a
353 potent inhibitor of EPHA2 (low nanomolar range), several other members of the ephrin kinase
354 family, as well SRC family kinases (31–33) that were also active in these models. Dasatinib is also
355 a weak inhibitor of EGFR. When used at near-micromolar concentrations, dasatinib reduced cell
356 viability in CRC0161 organoids compared to organoid models that did not share the high ephrin
357 INKA profile. At low nanomolar concentrations that do not inhibit EGFR, the combination with
358 cetuximab increased the effect of dasatinib. Likewise, mice bearing CRC0166 PDXs responded to
359 combination therapy with cetuximab and dasatinib with disease stabilization, whereas they
360 experienced disease progression when exposed to either monotherapy. These functional
361 experiments show the potential of phosphoproteomics combined with INKA analysis to select a
362 suitable treatment strategy for tumor models with previously unexplained resistance to cetuximab.

363 Our findings have certain limitations. For example, we have focused our analyses on the
364 static interrogation of phosphoprotein and protein biomarkers in treatment naïve PDX tumors and
365 correlated results with the outcome of cetuximab administration. Assessing the proteomic and
366 signaling changes that occur over the course of therapy is expected to provide useful information
367 about the dynamic mechanisms of tumor adaptation to antibody pressure and will contribute to
368 identifying reactive pathways that likely compensate for target blockade. Moreover, we
369 acknowledge that the association between resistance to cetuximab and high EPHA2 and SRC
370 family kinase activity has not been validated in clinical samples from therapeutically annotated
371 patients with mCRC, and thus requires further study. Regrettably, we were unable to confirm the
372 organoid results for CRC0161 *in vivo* because it was unsuccessful in re-engrafting as PDX. On
373 the other hand, although it was unable to cultivate as organoids, the projected dasatinib-sensitive
374 model CRC0166 was viable for testing *in vivo* in a PDX assay.

375 In conclusion, this research highlights the added value of phospho-proteomics and
376 proteomics in studying the bio-molecular basis of responses to targeted treatment in cancer. It
377 provides insight into the biology of the primary response to treatment with cetuximab in metastatic
378 colorectal cancer and advocates that CXADR in relation to cetuximab sensitivity deserves further
379 study for its potential use as a biomarker for response. Additionally, this work confirmed the
380 potential of single sample kinase activity analysis using INKA for the selection of potential
381 treatment strategies (15). Our study extends our previous analysis of the genomically unexplained
382 resistant PDX model CRC0177 in which INSR/IGF1R activity was identified and validated as co-
383 target for cetuximab combination treatment (15). Using a down-scaled protocol that utilizes only
384 small amounts of tumor tissues (16), we recently uncovered drug-specific signatures in needle
385 biopsies (17). This underscores the feasibility of potential clinical application of pTyr
386 phosphoproteomics. The sequential phosphopeptide capture strategy as employed here allows
387 pTyr-phospho-proteomics to be performed together with global phosphoproteomics and
388 proteomics on the same samples. Recent developments enable phosphoproteomics and proteomics
389 in conjunction with other omics analyses on the same samples with further streamlining of
390 protocols (16, 17, 39). Together, these integrative approaches will further motivate the
391 development of phosphoproteomics-based companion diagnostics for more informed patient
392 stratification and treatment decisions, further contributing to the realization of personalized
393 anticancer medicine.

394

395 MATERIALS AND METHODS

396 Study Design

397 This study used proteomics and phosphoproteomics to understand the biological processes that
398 lead to sensitivity and resistance to EGFR blockade in mCRC and to identify alternative drug
399 targets for resistant tumors. Tissue samples from 10 cetuximab-sensitive and 20 cetuximab-
400 resistant PDXs were analyzed using label-free Liquid Chromatography with tandem mass
401 spectrometry (LC-MS/MS) proteomics and phospho-proteomics. Biological and technical
402 replicates of representative models were measured for reproducibility. To comprehensively
403 investigate global phosphorylation, tyrosine-specific phosphorylation, and protein expression,
404 pTyr-immunoprecipitation, general phosphopeptide enrichment using titanium dioxide, and
405 global protein expression proteomics were performed in all samples. The samples were measured
406 in 5 cohorts, and LC-MS/MS measurement reliability was assessed by including HCT116 lysates
407 with known performance profiles in each measurement cohort. The PDX tissue samples were
408 processed blindly, without considering molecular characteristics, and underwent histological and
409 technical assessments as standard quality checks. Group-based statistics were performed between
410 CS and CR tumors to find discriminative molecular determinants of response, and single-sample
411 Integrative Inferred Kinase Activity (INKA) analysis was used to explore the potential
412 hyperactive kinases as alternative targets. Functional validation of hyperactive kinases was
413 performed using organoids and PDX models.

414 Patient-derived xenografts

415 Tumors were obtained from patients treated by liver metastasectomy at the Candiolo Cancer
416 Institute (Candiolo, Torino, Italy), Mauriziano Umberto I (Torino, Italy), and San Giovanni
417 Battista (Torino, Italy). All patients provided informed consent. Samples were procured, and the
418 study was conducted under the approval of the Review Boards of the Institutions. The cohort
419 studied here contains ten models sensitive to cetuximab, nine cetuximab resistant models that lack
420 mutations in RAS or RAF genes, and 11 cetuximab resistant models that harbor a mutation in
421 KRAS ($n = 4$), NRAS ($n = 3$) or BRAF ($n = 4$). Of the resistant models that lacked RAS/RAF
422 mutations, some models did harbor a genomic aberration relevant to cetuximab resistance,
423 including 2 models with an amplification of ERBB2, 1 model with an amplification of MET, 1
424 model with a mutation in ERBB2 (V777L), 1 model with a mutation in MAP2K1 (K57N) and 1
425 model with a mutation in EGFR (G465R) (Suppl. Table 1). For a subset of model's representative
426 of the whole cohort in this study, biological and or technical replicates were available to assess
427 reproducibility (Suppl. Table 1). In addition, representative tumor slices were assessed by
428 pathology for percentage tumor, stromal tissue, and necrosis. Pathology report was not used as an
429 upfront exclusion criterion for analysis.

430 Organoid cultures and cell viability

431 Organoids were established from colorectal cancer patient-derived xenografts CRC0161,
432 CRC0196 and CRC0254 and cultured in extracellular matrix hydrogel (Cultrex pathClear,
433 Reduced Growth Factor Basement Membrane Extract, type 2) and Dulbecco's Modified Eagle's
434 Medium (DMEM) F12 culture medium supplemented with 1% penicillin/streptomycin, 1% B27,
435 1% N2, 2 mM L-glutamine, 1nM N-acetyl-cysteine and 0.02 $\mu\text{g/ml}$ EGF. For testing viability,
436 organoids were seeded as single cells on a coating of BME hydrogel in the above culture medium
437 depleted of EGF growth factor. After seven days, CellTiter-Glo luminescent cell viability assay
438 (Promega) was used, as described before (40), to measure viability. Results were analyzed using

439 PRISM GraphPad software and statistical analysis was performed using ordinary one-way
440 ANOVA. Error bars were calculated based on SEM.

441 **In vivo treatments**

442 Tumor implantation and expansion were performed as previously described (41). Briefly, tumor
443 material not required for histopathologic analysis was collected and placed in medium 199
444 supplemented with 200 U/mL penicillin, 200 µg/mL streptomycin, and 100 µg/mL levofloxacin.
445 Each sample was cut into 25- to 30-mm³ pieces in antibiotic-containing medium; some of the
446 pieces were snap-frozen in liquid nitrogen for phospho-proteomics, and some others were
447 incubated overnight in RNeasy lysis buffer and then frozen at -80°C for DNA and RNA analyses; 2 other
448 pieces were coated in Matrigel (BD Biosciences) and implanted in 2 different 4- to 6-week-old
449 male or female NOD (nonobese diabetic)/SCID (severe combined immunodeficient) mice. After
450 mass formation, the tumors were passaged and expanded for 2 generations until production of a
451 cohort of 12 or 24 mice, depending on the amount of the original material. Established tumors
452 (average volume 400 mm³) were treated for three weeks with the following regimens, either
453 single-agent or in combination: cetuximab (Merck) 20 mg/kg by intraperitoneal injection, twice-
454 weekly (vehicle: physiological saline); dasatinib (Carbosynth) 50 mg/kg, daily by oral gavage
455 (vehicle: 80 mM sodium citrate, pH 3.1). Tumor size was evaluated once- weekly by caliper
456 measurements and the approximate volume of the mass was calculated using the formula
457 $4/3\pi \cdot (d/2)^2 \cdot D/2$, where d is the minor tumor axis and D is the major tumor axis. For assessment
458 of tumor response to therapy, we adopted a classification loosely inspired by clinical criteria (9,
459 10, 16, 27): (i) tumor regression was defined as a decrease of at least 50% in the volume of target
460 lesions, taking as reference the baseline tumor volume; (ii) at least a 35% increase in tumor volume
461 was categorized as disease progression; and (iii) responses that were neither sufficient reduction
462 to qualify for shrinkage nor sufficient increase to qualify for progression were considered as
463 disease stabilization. Animal procedures were approved by the Italian Ministry of Health
464 (authorization 806/2016-PR).

465 **Tissue lysis and phosphopeptide enrichment**

466 Tissue from patient-derived xenograft was cut on a cryotome in 20µM slices and lysed in lysis
467 buffer (9 M urea, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) pH 8.0, 1
468 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1mM β-glycerophosphate) in a 1:40
469 wet-weight to lysis buffer ratio, sonicated (3 cycles of 30 s) and extracts were stored at -80 °C.

470 For phosphoproteomics, lysate aliquots equivalent to 5.5 mg total protein were used as
471 described before (42, 43). Proteins were reduced by incubation in 4.5 mM dithiothreitol for 30 min
472 at 55 °C, alkylated in 10 mM iodoacetamide for 15 min at room temperature in the dark, and
473 digested overnight at room temperature with 10 µg/ml trypsin after fourfold dilution with 20 mM
474 HEPES pH 8.0, to reduce the urea concentration. After acidification (trifluoroacetic acid to 1%
475 final concentration), tryptic digests were desalted on Sep-Pak C18 cartridges (Waters
476 Chromatography), divided in aliquots for immunoprecipitation (5mg) or affinity enrichment
477 (500µg) and lyophilized and stored at -80 °C.

478 For immunoprecipitation of tyrosine-phosphorylated peptides, peptides were dissolved in
479 350 µl immunoprecipitation buffer (50 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH
480 7.2, 10 mM sodium phosphate, 50 mM NaCl) and transferred at 4 °C to a microcentrifuge tube
481 containing 20 µl of a 50% (v/v) slurry of agarose beads harboring P-Tyr-1000 anti-
482 phosphotyrosine monoclonal antibodies (Cell Signaling Technologies) that had been washed and

483 taken up in PBS. After 2-h of incubation at 4 °C on a head-over-tail rotator, beads were washed
484 twice with cold PBS and three times with cold High-performance liquid chromatography (HPLC)
485 grade water. Bound peptides were eluted with a total of 50 µl 0.15% trifluoroacetic acid in two
486 steps. Phosphopeptides were desalted using 200 µl STAGE tips containing a 16G empore SDB-
487 XC membrane plug (3 M) using the same solvents as used for the Seppak cartridge (20 µl, 1000
488 ×g, 1 min). Desalted peptides were dried in a vacuum centrifuge at 45°C and solubilized in 20 µl
489 4% acetonitrile/0.5% trifluoroacetic acid, prior to LC-MS/MS analysis on the same day.

490 For global affinity enrichment of phosphopeptides, aliphatic hydroxy-acid modified metal
491 oxide chromatography using TiO₂ beads was performed (44). Briefly, 500 µg peptides (1 µg/µl)
492 were mixed with 500 µl washing buffer (80% ACN, 0.1%TFA containing 300 mg/ml lactic acid)
493 and applied to 2.5 mg TiO₂ beads (GL sciences, 10 µm) packed in a 200 µl STAGE tip containing
494 a 16G empore C8 membrane plug (3 M, St Paul, MN). The STAGE tip was washed with 200 µl
495 washing buffer, followed by 200 µl of 80% ACN and 0.1% TFA. Phosphopeptides were eluted in
496 two steps in 50 µl 0.5% and 5% piperidine (Fisher Scientific) and were quenched in 100 µl 20%
497 H₃PO₄. All steps were performed by centrifugation (1500 ×g, 4 min). Phosphopeptides were
498 desalted using SDB-XC STAGE tips as described above. Desalted phosphopeptides were dried in
499 a vacuum centrifuge and redissolved in 30 µl 4%ACN, 0.5%TFA;15 µl was injected on column

500 **Protein-expression profiling**

501 Protein lysates (50 µg) were separated on precast 4–12% gradient gels using the NuPAGE SDS-
502 PAGE system (Invitrogen, Carlsbad, CA). Following electrophoresis, gels were fixed in 50%
503 ethanol/3% phosphoric acid solution and stained with Coomassie R-250. Gel lanes were cut into
504 five bands, and each band was cut into ~1 mm³ cubes. Gel cubes were washed with 50 mM
505 ammonium bicarbonate/50% acetonitrile and were transferred to a 1.5 ml microcentrifuge tube,
506 vortexed in 400 µl 50 mM ammonium bicarbonate for 10 min, and pelleted. The supernatant was
507 removed, and the gel cubes were vortexed in 400 µl 50 mM ammonium bicarbonate/50%
508 acetonitrile for 10 min. After pelleting and removal of the supernatant, this wash step was repeated.
509 Subsequently, gel cubes were reduced in 50 mM ammonium bicarbonate supplemented with 10
510 mM DTT at 56°C for 1 h. The supernatant was removed, and gel cubes were alkylated in 50 mM
511 ammonium bicarbonate supplemented with 50 mM iodoacetamide for 45 min at room temperature
512 in the dark. Next, gel cubes were washed with 50 mM ammonium bicarbonate/50% acetonitrile
513 dried in a vacuum centrifuge at 50°C for 10 min and covered with trypsin solution (6.25 ng/µl in
514 50 mM ammonium bicarbonate). Following rehydration with trypsin solution and removing excess
515 trypsin, gel cubes were covered with 50 mM ammonium bicarbonate and incubated overnight at
516 25°C. Peptides were extracted from the gel cubes with 100 µl of 1% formic acid (once) and 100
517 µl of 5% formic acid/50% acetonitrile (twice). For each sample the three extracts were pooled and
518 stored at –20°C until use. Before LC-MS, the extracts were concentrated in a vacuum centrifuge
519 at 50°C, and volumes were adjusted to 50 µl by adding 0.05% formic acid, filtered through a 0.45
520 µm spin filter, and transferred to an LC autosampler vial.

521 **LC-MS/MS**

522 Peptides were separated on an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-Packings)
523 equipped with a 20-cm, 75-µm inner diameter fused silica column custom packed with 1.9-µm
524 ReproSil-Pur C18-AQ silica beads (120-Å pore size; Dr. Maisch). After injection, peptides were
525 trapped at 6 µl/min on a 10-mm, 100-µm inner diameter trap column packed with 5-µm ReproSil-
526 Pur C18-AQ silica beads (120-Å pore size) in buffer A (buffer A: 0.5% acetic acid, buffer B: 80%

527 acetonitrile, 0.5% acetic acid), and separated at 300 ml/min with a 10–40% buffer B gradient in
528 90 min (120 min inject-to-inject). Eluting peptides were ionized at a potential of +2 kV and
529 introduced into a Q Exactive mass spectrometer (Thermo Fisher). Intact masses were measured in
530 the orbitrap with a resolution of 70,000 (at m/z 200) using an automatic gain control (AGC) target
531 value of 3×10^6 charges. Peptides with the top 10 highest signals (charge states 2+ and higher)
532 were submitted to MS/MS in the higher-energy collision cell (4-Da isolation width, 25%
533 normalized collision energy). MS/MS spectra were acquired in the orbitrap with a resolution of
534 17,500 (at m/z 200) using an AGC target value of 2×10^5 charges and an underfill ratio of 0.1%.
535 Dynamic exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

536 **Peptide identification**

537 MS/MS spectra of both phosphopeptide enrichment experiments (TiO₂ and pTyr IP) were searched
538 against the UniProt human reference proteome FASTA file (downloaded August 2015, no
539 fragments; 62447 entries) using MaxQuant 1.5.2.8 software. To cross-check the
540 phosphoproteome results from the database search against the human genome, we also searched
541 the phosphoproteomics data against the same combined human and mouse sequences as we did
542 for the proteome expression dataset. MS/MS spectra of the protein expression experiment were
543 searched against the same human FASTA file and the Uniprot mouse reference FASTA file
544 (downloaded June 2015, no fragments, canonical and isoforms; 42296 entries) Enzyme specificity
545 was set to trypsin, and up to two missed cleavages were allowed. Cysteine
546 carboxamidomethylation (+57.021464 Da) was treated as fixed modification and serine, threonine,
547 and tyrosine phosphorylation (+79.966330 Da), methionine oxidation (+15.994915 Da), and N-
548 terminal acetylation (+42.010565 Da) as variable modifications. Peptide precursor ions were
549 searched with a maximum mass deviation of 4.5 ppm and fragment ions with a maximum mass
550 deviation of 20 ppm. Peptide and protein identifications were filtered at a false discovery rate of
551 1% using a decoy database strategy. The minimal peptide length was set at 7 amino acids, the
552 minimum Andromeda score for modified peptides was 40, and the corresponding minimum delta
553 score was 6. Proteins that could not be differentiated based on MS/MS spectra alone were clustered
554 into protein groups (default MaxQuant settings). Phosphopeptide identifications were propagated
555 across samples using the ‘match between runs’ option checked. In the protein expression search
556 match between runs was not applied. Phosphopeptide MS/MS spectral counts were calculated from
557 the MaxQuant evidence file using R.

558 **Organoid phosphoproteomics**

559 For organoids phosphoproteomics, lysate aliquots (1.6 mg total protein) were reduced, alkylated,
560 digested, and desalted as described. For pTyr immunoprecipitation, peptides were dissolved in 350
561 μ l IP buffer with 20 μ l 50% (v/v) P-Tyr-1000 agarose beads and pTyr phosphopeptides were
562 captured and desalted as described. The non-bound fraction was desalted as well and TiO₂ beads
563 were used for pSer/pThr phosphopeptide enrichment as described, using 500 μ g peptides as input.
564 For protein-expression profiling 1 μ g of the non-bound fraction of the pTyr IP was used for single-
565 shot analysis. Peptides were separated on an Ultimate 3000 nanoLC-MS/MS system (Dionex LC-
566 Packings) equipped with a 50-cm 75 μ m ID C18 Acclaim pepmap column (Thermo Scientific).
567 After injection, peptides were trapped at 3 μ l/min on a 10-mm, 75- μ m ID Acclaim Pepmap trap
568 column (Thermo Scientific) in buffer A (buffer A: 0.1% formic acid, buffer B: 80% acetonitrile,
569 0.1% formic acid), and separated at 300 ml/min with a 10–40% buffer B gradient in 90 min (120
570 min inject-to-inject). Eluting peptides were ionized at a potential of +2 kV and introduced into a
571 Q Exactive HF mass spectrometer (Thermo Fisher). Intact masses were measured in the orbitrap

572 with a resolution of 120,000 (at m/z 200) using an automatic gain control (AGC) target value of 3
573 $\times 10^6$ charges. Peptides with the top 15 highest signals (charge states 2+ and higher) were
574 submitted to MS/MS in the higher-energy collision cell (1.6-Da isolation width, 25% normalized
575 collision energy). MS/MS spectra were acquired in the Orbitrap with a resolution of 15,000 (at
576 m/z 200) using an AGC target value of 2×10^5 charges and an under fill ratio of 0.1%. Dynamic
577 exclusion was applied with a repeat count of 1 and an exclusion time of 30 s.

578 MS/MS spectra of both phosphopeptide enrichment experiments (TiO₂ and pTyr IP) were
579 searched against the Swissprot human_canonical_and_isoform.fasta (42258 entries)t FASTA file
580 (downloaded January 2018, canonical and isoforms; 42258 entries) using MaxQuant 1.6.0.16.
581 Search setting were the same as described for PDX models. Proteins that could not be differentiated
582 based on MS/MS spectra alone were clustered into protein groups (default MaxQuant settings).
583 Phosphopeptide identifications were propagated across samples using the ‘match between runs’
584 option checked. Phosphopeptide MS/MS spectral counts were calculated from the MaxQuant
585 evidence file using R. For phosphopeptide data, we used data from the MaxQuant
586 ‘modificationSpecificPeptides’ table. For phosphosite data, we used data from the MaxQuant
587 Phospho (STY) Sites’ table.

588 **Quantification**

589 Group-based comparisons were made with MS/MS spectral counts for protein expression data and
590 MS ion intensities (area under the MS1 extracted ion chromatogram) for the phosphosite data.
591 Moreover, INKA uses spectral counts as input.

592 **Data filtering and annotation.**

593 For phosphopeptide data, we used data from the MaxQuant ‘modificationSpecificPeptides’ table.
594 Table rows with data linked to multiple UniProt gene symbols were deconvoluted into separate
595 rows with a single gene symbol. For phosphosite data, we used data from the MaxQuant’ Phospho
596 (STY) Sites’ table, filtering for so-called class I sites (localization probability > 0.75). Table rows
597 with data linked to multiple UniProt accessions, and those linked to multiple phosphopeptides,
598 were deconvoluted into separate rows. Data from the web resources UniProt (UniProt Consortium,
599 2015) (for mapping attributes of UniProt accessions; www.uniprot.org, mapping date 8 June
600 2016), PhosphoSitePlus (45) (for experimentally observed phosphorylation sites and kinase-
601 substrate relationships; www.phosphosite.org, Phosphorylation_site_dataset, and
602 Kinase_Substrate_Dataset, versions of 3 July 2016) and KinBase (46) (for currently recognized
603 protein kinases; kinase.com/web/current/kinbase, mapping date 20 July 2016), and HGNC (47)
604 (for mapping to official gene symbols of the HUGO Gene Nomenclature Committee;
605 www.genenames.org) were used in combination with a UniProt human reference proteome
606 FASTA file derived from release 2014_01 filtered for “no fragments,” and containing 21849
607 TrEMBL entries and 39703 Swiss-Prot entries to prioritize rows linking the same phosphosite to
608 the same gene, only retaining the row with the best-annotated accession. Subsequently, the
609 phosphosite data were merged with pertinent phosphopeptide data in a single, non-redundant class-
610 I phosphosite-phosphopeptide table.

611 **2-group comparisons**

612 Group comparisons were made between all cetuximab sensitive and all resistant models (CS vs.
613 CR-ALL), between sensitive and resistant models lacking mutations in RAS/RAF (CS vs. CR-
614 WT), and between sensitive (CS-WT) and resistant RAS/RAF mutant models (CS vs. CR-MUT).

615 For phospho-proteomics data, phosphosite intensities were taken from the non-redundant class-I
616 phosphosite-phosphopeptide table and normalized using the sum of all intensities and median
617 centering of the values in each sample. Biological and technical replicates were averaged omitting
618 zero values from average and missing data points were imputed using the half-min method. Limma
619 was then performed for each group comparison using the R package “limma” (48). Nominal p-
620 values were not corrected for multiple tests. Results of each group comparison were filtered for p-
621 value (< 0.01), fold change ($< -1.5, > 1.5$), average intensity ($> 1 \times 10^7$), and data coverage of more
622 than 50% in at least one of the comparison groups. Analysis was performed separately for data
623 derived from pTyr-immunoprecipitation and TiO₂ affinity capture. For global protein expression,
624 protein spectral counts were normalized, and biological and technical replicates were averaged,
625 omitting zero values from average. A beta-binomial test was then performed for each group
626 comparison using the R package “ibb” as described before (49). Nominal p-values were not
627 corrected for multiple tests. Results of each group comparison were filtered for p-value (< 0.01),
628 fold change ($< -1.5, > 1.5$), and data coverage of more than 50% in at least one of the comparison
629 groups. Heatmaps were created with the R package ComplexHeatmap (50) utilizing z-score
630 normalization, euclidean distance, and ward.D2 linkage.

631 RNA expression data was analyzed where RNAsub refers to a comparison among the same
632 subset of PDX models also used for (phospho-)proteomics profiling, and RNA400 refers to a
633 comparison among an extensive collection of 157 CS and 246 CR models. Gene probes were kept
634 if considered expressed in Isella et al. (2017) (22) or excluded if probes cross-reacted with murine
635 genes. When there were multiple probes per gene, only the probe with the highest standard
636 deviation was picked. Two-group comparisons were made between CS vs. CR-ALL, CS vs. CR-
637 WT, and CS vs. CR-MUT using limma (48) on log₂-scaled expression values. Nominal p-values
638 were corrected for multiple tests using the Benjamini & Hochberg procedure (51). For the RNAsub
639 comparison, no RNA-sequencing data was available for 9 CR-MUT models. In addition, model
640 CRC0358 (CR-WT) was not considered for RNA analyses due to outlier behavior. The CS vs. CR-
641 MUT comparison on RNA level was excluded because it was unbalanced and underpowered.

642 **Expression-driven signature enrichment analysis**

643 Gene set enrichment analysis (GSEA) was conducted using the “fgsea” R package (52). The
644 analysis utilized ranked genes, including log-transformed and signed p-values obtained from
645 protein expression comparisons between CS and CR, as well as RNA400 CS versus CR-all. The
646 C5 ontology gene sets collection from the MSigDB v7.2 database served as input files. The
647 resulting GSEA outputs were visualized in R, employing the ggplot2 package (53).

648 **Post-translational modification signature enrichment analysis**

649 Phosphosite-specific signature analysis was performed with post-translational modification
650 signature enrichment analysis (PTM-SEA) (54) using the R-script ssgsea-gui.R
651 (<https://github.com/broadinstitute/ssGSEA2.0>). Ranked phosphosites (log-transformed and
652 signed p-values of the CS vs. CR comparisons) and the PTMsigDB v1.9.0 database was used as
653 input files. Visualization of results was performed in R using the ggplot2 package (53).

654 **Overlap analysis**

655 Using Venny 2.1.0 (55), the overlap of significant phospho-sites and proteins of the comparisons
656 CS vs. CR-ALL, CR-MUT, and CR-WT created three response signatures. The general response
657 signature consisted of significant phospho-sites and proteins exclusive in the comparison of CS

658 vs. CR-ALL (Venn part CR1 and CS1) combined with the overlap of all comparisons (Venn part
659 CR2 and CS2) and the overlap between CS vs. CR-MUT and CR-WT (Venn part CR3 and CS3).
660 The signatures for either RAS/RAF mutated or wild-type tumors contained significant phospho-
661 sites and proteins exclusive to CS vs. CR-MUT (Venn part CR4 and CS4) or CS vs. CR-WT (Venn
662 part CR5 and CS5). The significant phospho-sites and proteins of the overlap between CS vs. CR-
663 ALL and CS vs. CR-MUT or CS vs. CR-WT were excluded. These significant phospho-sites and
664 proteins could not be qualified as a general response or specific for RAS/RAF mutated or wild-
665 type tumors.

666 **Analysis of biological pathways and processes**

667 Phospho-sites and proteins from the general response signature, RAS/RAF mutated signature, and
668 the wild-type signature were combined in one table (179 proteins and 83 phosphosites). All
669 (phospho)proteins were used to retrieve protein-protein association data from the STRING
670 database v11 (56) to build a combined network in Cytoscape v3.7 (57). Statistical data
671 encompassed three comparisons: CS vs. CR-ALL, CR-MUT, and CR-WT. The maximum fold
672 change among these three comparisons was log₂-transformed and used to color-code a donut ring
673 around the pertinent network node for every protein or phosphosite. Using the Cytoscape Omics
674 Visualizer app (58), a separate donut ring was added for expression, pTyrIP, or TiOx.

675 To complement the protein data, we also analyzed RNAseq data on the models in the
676 present cohort (RNAsub) as well as on a larger cohort of 157 cetuximab sensitive and 246
677 cetuximab resistant PDX tumors (RNA400) (22). RNA features were tested in the three
678 comparisons mentioned above using the limma R package and filtered for a link to the
679 (phospho)proteins in the network. If any of the comparisons were significant ($p < 0.05$), the
680 associated network node was colored orange or blue, depending on the direction of change in the
681 CS vs. CR-ALL comparisons.

682 Protein clusters were identified using the MCL algorithm of the clusterMaker2 app (59)
683 inside Cytoscape, and gene ontology analysis was performed with the BiNGO app (60) using
684 ontology definitions of April 2020 to analyze biology covered in this network.

685 **INKA analysis**

686 Integrative Inferred Kinase Activity (INKA) based on both phosphorylated kinases and their
687 substrates was calculated for each sample as previously described (15). Mean INKA scores for CS
688 and CR tumors were calculated for each kinase, excluding kinases measured in less than five
689 tumors.

690 **Statistics**

691 Statistical analysis was conducted on the phosphosite intensities by normalizing them using the
692 sum of all intensities and median centering within each sample. Averages of biological and
693 technical replicates were calculated, excluding zero values, and missing data points were imputed
694 using the half-min method. Group comparisons for phosphosite intensities were performed using
695 the R package "limma," nominal p-values were obtained without correction for multiple tests. The
696 R package "ibb" was utilized with a beta-binomial test for group comparisons of global protein
697 expression. Furthermore, statistical analyses for organoid viability assays were carried out in Prism
698 7.0 software (GraphPad) using ordinary one-way ANOVA. Statistical significance was determined
699 for all experiments using the following criteria: n.s. (not significant), * $p < 0.05$, ** $p < 0.01$, *** p
700 < 0.001 .

701 **Supplementary Materials**

702 fig. S1. to S14.

703 Table S1. to S11

704 Data file S1

705 MDAR reproducibility checklist

706

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977 **Author contributions:**

978 L.T. and A.B. generated and provided patient-derived xenografts (PDX). R.B. and R.G.H.
979 performed tissue lysis and phosphopeptide enrichment. S.R.P. conducted LC-MS/MS
980 measurements. S.R.P., J.C.K., A.A.H., and T.V.P. analyzed and processed LC-MS/MS
981 data for peptide identification and quantification. The (phospho)proteomics data was
982 curated and annotated by R.B. and J.C.K. R.B. conducted 2-group comparisons, GSEA,
983 and INKA analysis. F.B. performed PTM-SEA analysis. A.A.H. cross-checked the
984 phosphoproteome results against combined human-mouse peptide identification and
985 quantification. M.V. and E.G. analyzed RNAseq data. V.V., F.C., and B.L. generated
986 organoids from PDX models. R.B. conducted organoid cell viability assays. E.R.Z. and
987 G.M. performed and analyzed in vivo PDX experiments. C.R.J., H.M.W.V., L.T., A.B.,
988 and M.L. conceived and supervised the project. C.R.J., L.T., and A.B. acquired funding.
989 R.B. wrote the manuscript. R.B., J.C.K., F.B., and A.A.H. created the figures. C.R.J.,
990 H.M.W.V., L.T., A.B., and M.L. reviewed and edited the manuscript. The final
991 manuscript was approved by all co-authors.

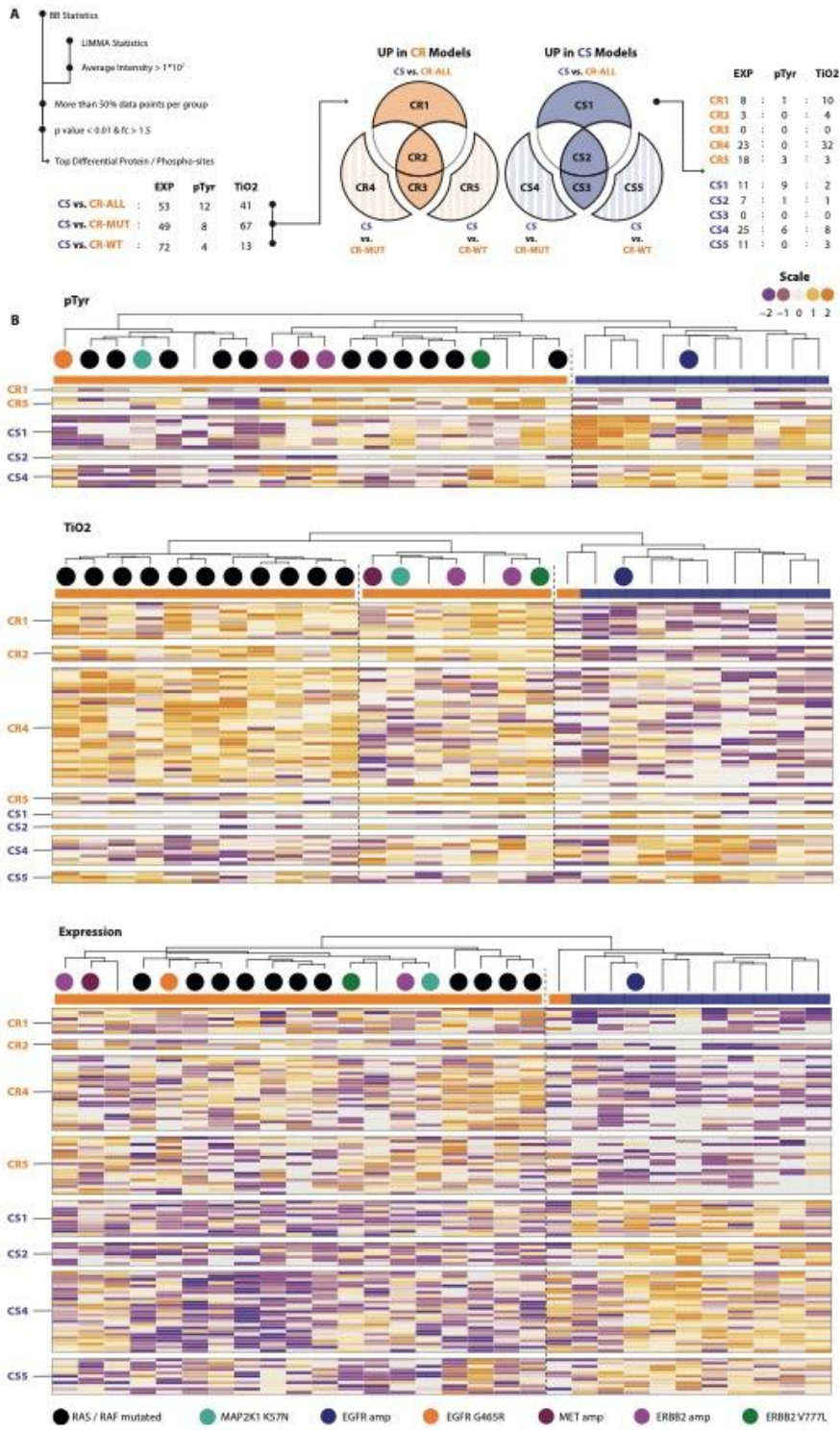
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993 **Competing interests:** Authors declare that they have no competing interests.

994 **Data and materials availability:**

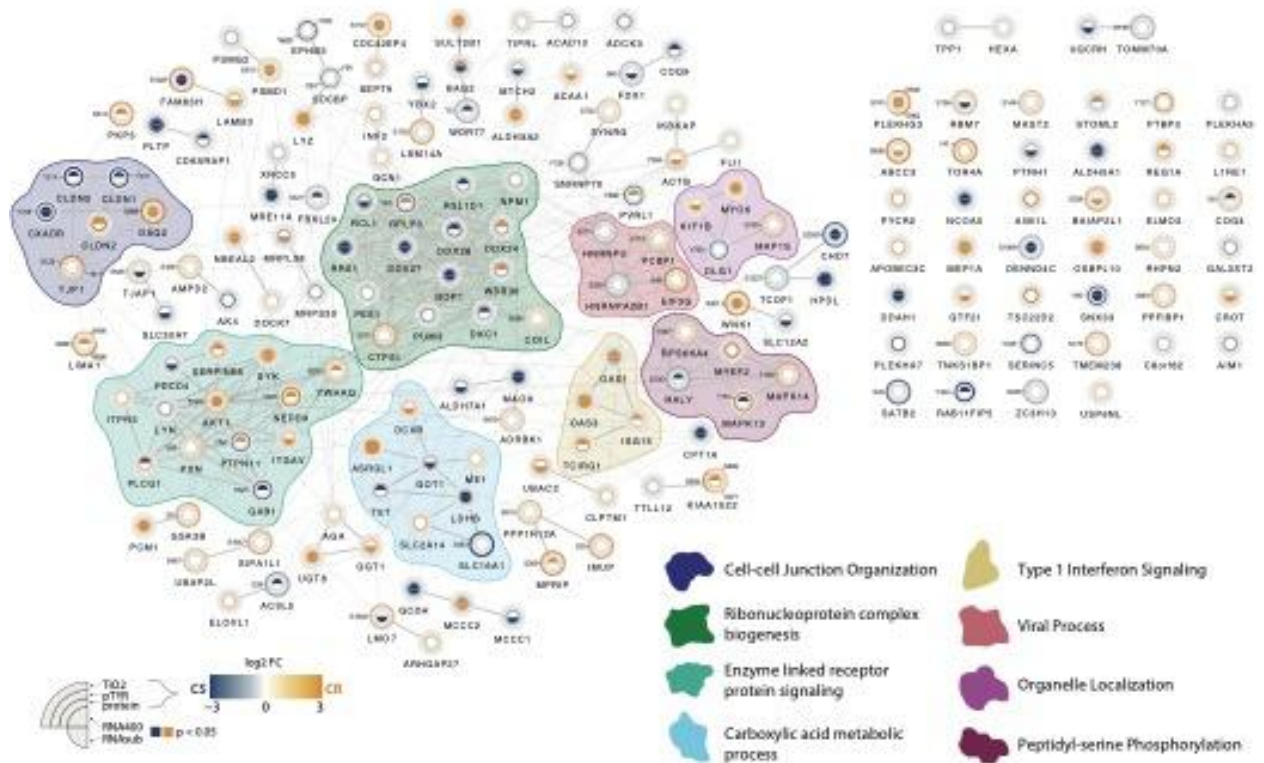
995 All data associated with this study are in the paper or supplementary materials. The mass
996 spectrometry proteomics data have been deposited to the ProteomeXchange Consortium
997 through the PRIDE (61) partner repository with the dataset identifier PXD029127 for the
998 main dataset and PXD029299 for the organoid dataset. RNAseq data have been deposited
999 in the European Genome-Phenome Archive (EGA) under accession code
1000 EGAS00001006492. Access to these data will be granted upon registration to EGA and
1001 request to access these studies. Processed expression levels and raw read counts are
1002 publicly available in GEO (GSE204805). PDX models and derivatives thereof are
1003 available for solely academic purposes from L.T. or A.B. under a material transfer
1004 agreement with the University of Torino.
1005

1006 **Figures**



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1008

1009 **Fig. 1. Comparative analysis of sensitive and resistant PDX models to cetuximab**
1010 **identifies a differential signature. (A)**, Overview of group comparison of identified
1011 phospho-sites using LIMMA statistics between cetuximab sensitive models (CS) versus all
1012 resistant models (CR ALL), versus the resistant models wild-type for mutations in RAS/RAF
1013 (CR WT) or versus the RAS/RAF-mutated resistant models (CR MUT). LIMMA results
1014 where filtered for each comparison. Overlap between these comparisons shows differential
1015 phospho-sites that are more distinctive for the general comparison CR ALL versus sensitive
1016 (S/R1, S/R2, S/R3) whereas parts without overlap (S/R4 and S/R5) are distinctive for
1017 respectively CR WT and CR MUT. **(B)**, Clustering of the combined signature of the top
1018 differential phospho-sites (rows) from the comparisons in the pTyr (top), TiO2 (middle) and
1019 Expression (bottom) datasets. Response is indicated for sensitive (blue) and resistant (orange)
1020 models. Genomic aberrations of models are indicated with colored circles below. Clustering
1021 shows separation between sensitive and resistant models in pTyr and near-complete
1022 separation in TiO2 and Expression with separate clusters for RES WT and RES MUT in
1023 TiO2.
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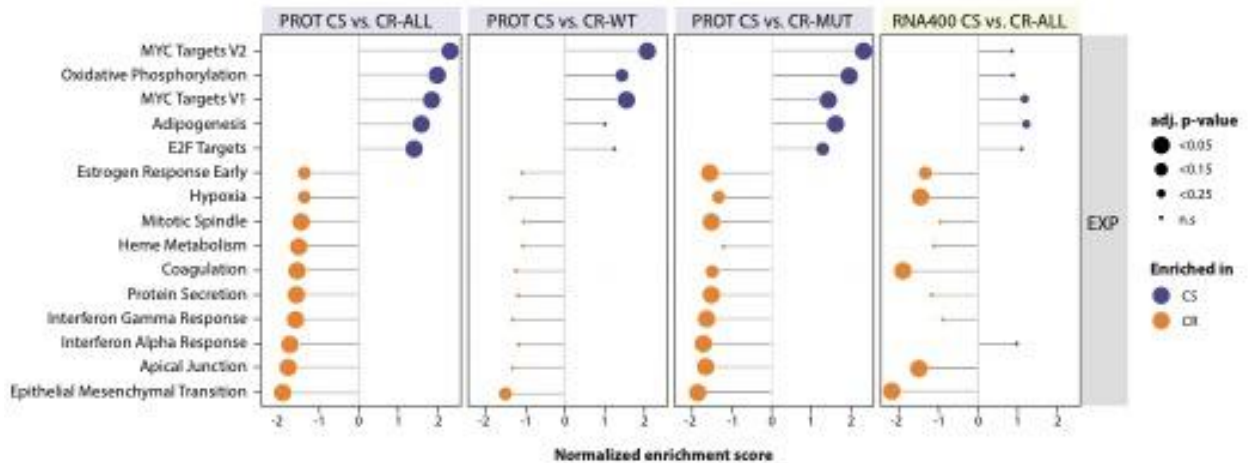
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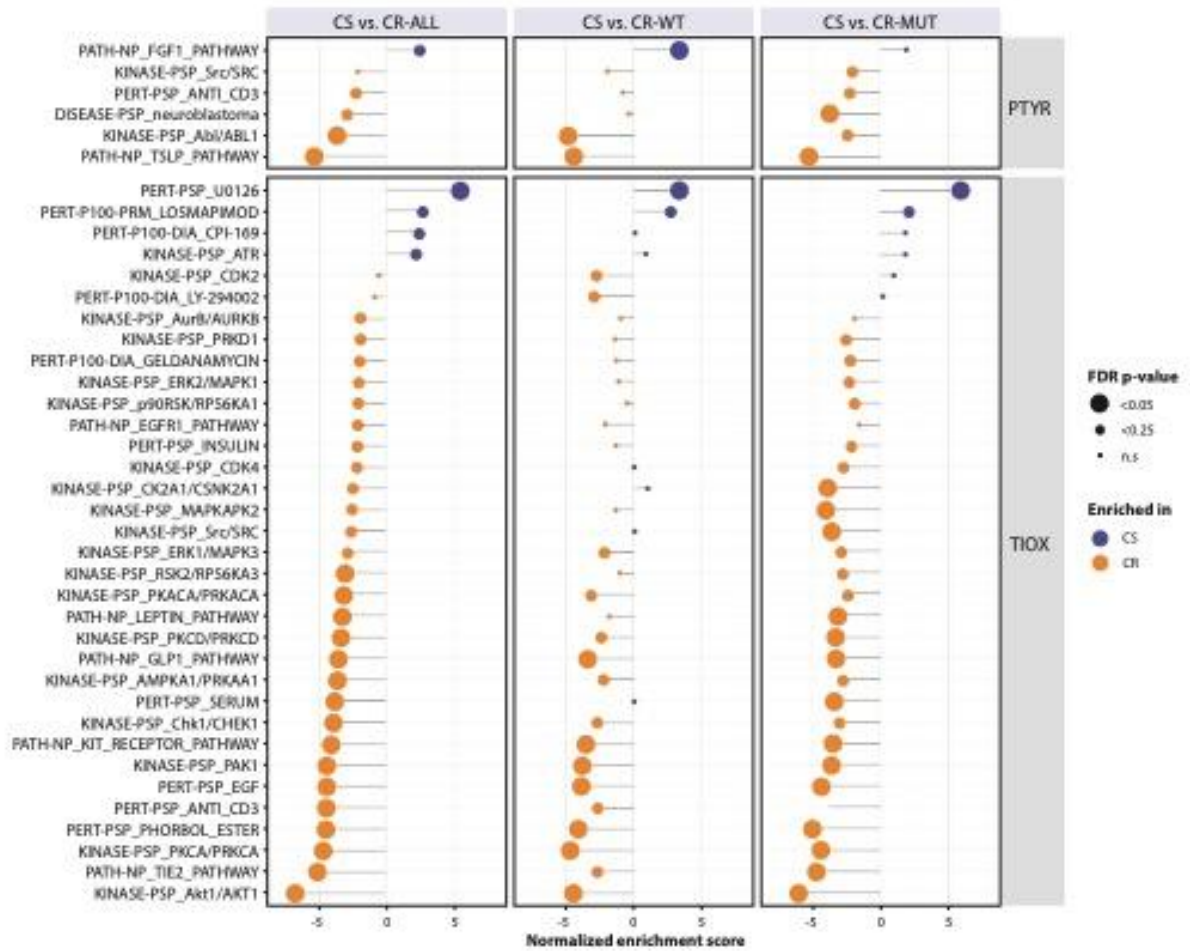
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Fig. 2. Interaction network of proteins associated with cetuximab sensitivity and resistance. Previous group comparisons of cetuximab sensitive versus resistant tumors (CS vs. either CR ALL, CR MUT, and CR WT) were merged on CS versus CR. The maximum fold change among these three comparisons was log₂-transformed and used to color-code a donut ring around the pertinent network node for every protein and a section of the ring for each phosphosite. Donut rings represent protein expression (inner ring), TiO₂ (middle ring), and pTyr (outer ring). If any of the comparisons were significant ($p < 0.05$), the associated ring was colored a hue of orange (CR) or blue (CS), depending on the direction and fold change in the CS vs. CR-ALL comparisons. The inner circle represents significant genes (p -value < 0.05) from mRNA analysis comparing the models in this cohort (RNAsub, bottom half of the inner circle) and a more extensive comparison between 157 cetuximab sensitive and 246 cetuximab resistant PDX tumors (RNA400, top half of the inner circle). All (phospho)proteins were used to retrieve protein-protein associations from the STRING database v11, proteins without association are shown in the top right corner. Protein clusters were identified using the MCL algorithm of the clusterMaker2 app inside Cytoscape, and gene ontology analysis was performed with the BiNGO app using ontology definitions of April 2020 to analyze biology covered in this network.



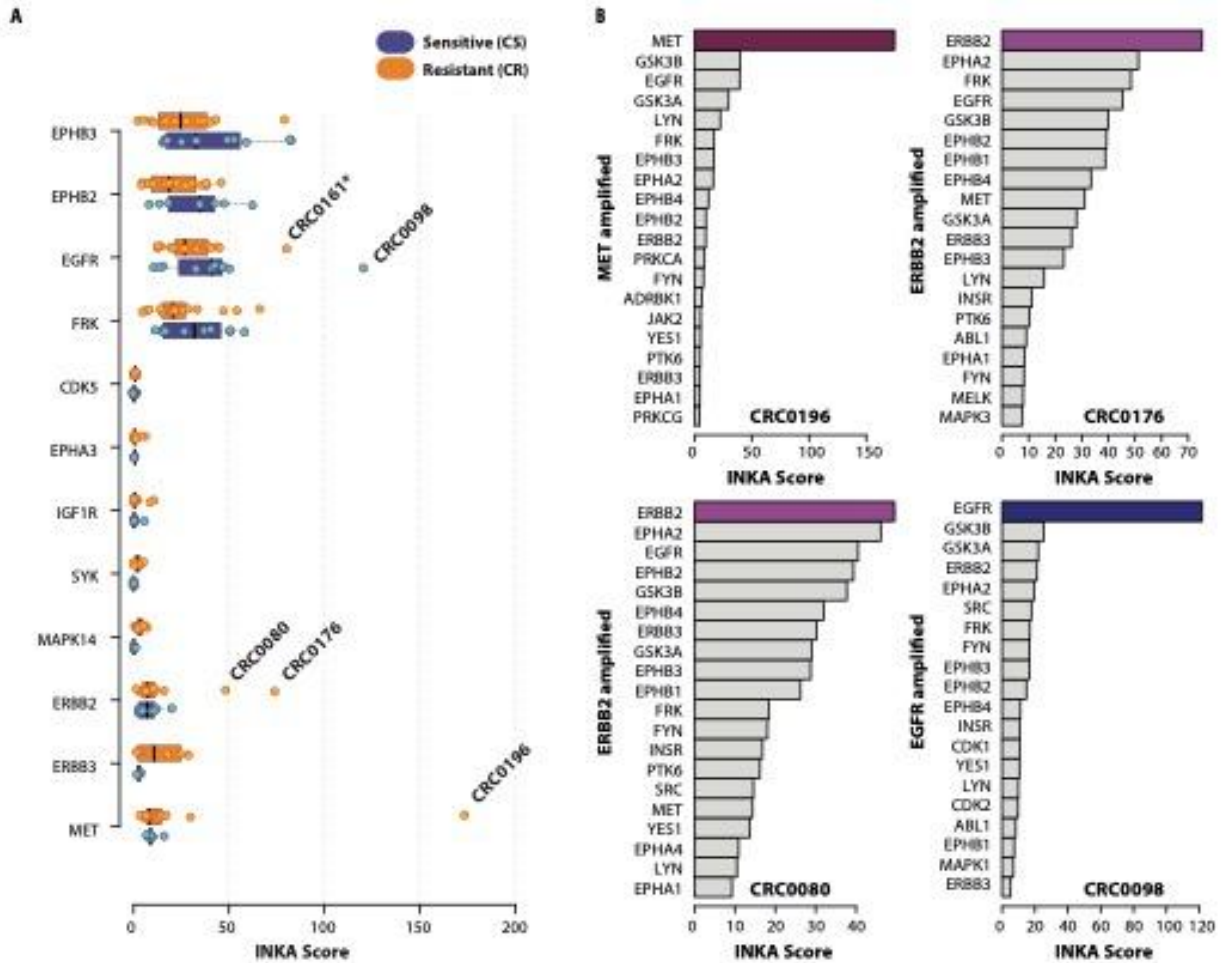
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Fig. 3. Protein expression-driven enrichment of cetuximab sensitive and cetuximab resistant biology. Lollipop plots showing amount of enrichment as the normalized enrichment scores on the x-axis of differentially regulated protein expression-driven HALLMARK signatures between cetuximab sensitive (CS) and cetuximab resistant (CR) PDX models as determined by gene set enrichment analysis (GSEA). The analysis utilized genes ranked according log-transformed and signed p-values obtained from all protein expression (PROT) comparisons between CS and CR, as well as the RNA400 CS versus CR-all comparison. The C5 ontology gene sets collection from the MSigDB v7.2 database served as input files. The size of the circles corresponds to significance of enrichment with blue indicating enrichment in CS and orange indicating enrichment in CR tumors. Shown are processes that have at least a adj. p-value less than 0.15 in protein expression (PROT) CS vs. CR-ALL comparison.



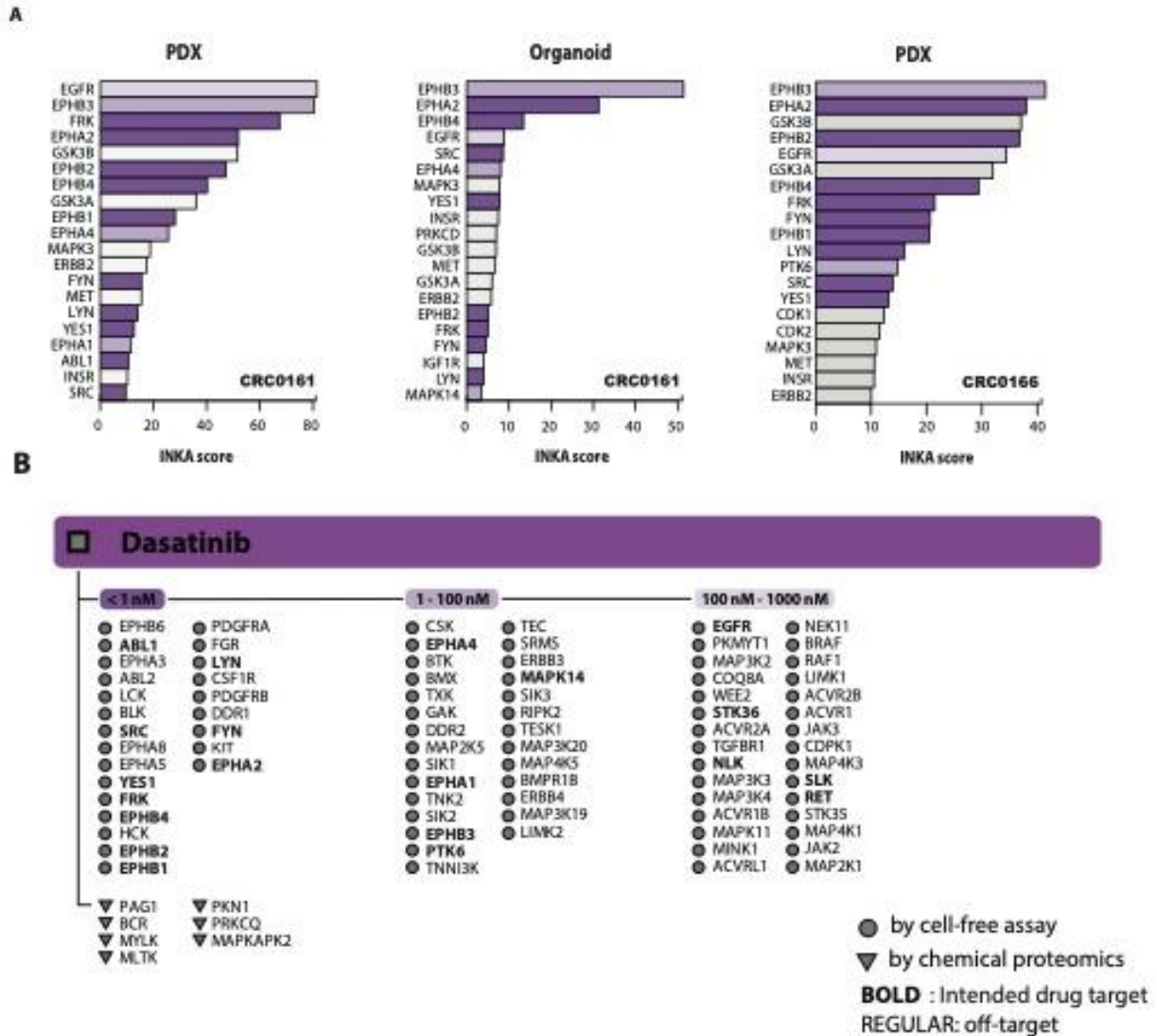
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Fig. 4. Post-translational modification signature enrichment analysis of cetuximab sensitive and cetuximab resistant biology. Lollipop plots showing amount of enrichment as the normalized enrichment scores on the x-axis of differentially regulated phosphosite-driven signatures between CS (blue) and CR (orange) PDX models as determined by post-translational modification signature enrichment analysis (PTM-SEA). The analysis utilized phosphosites ranked according log-transformed and signed p-values obtained from all pTyr and Tio2 comparisons. The size of the circles corresponds to significance of enrichment; shown are all signatures with FDR-adjusted P -value < 0.25 .



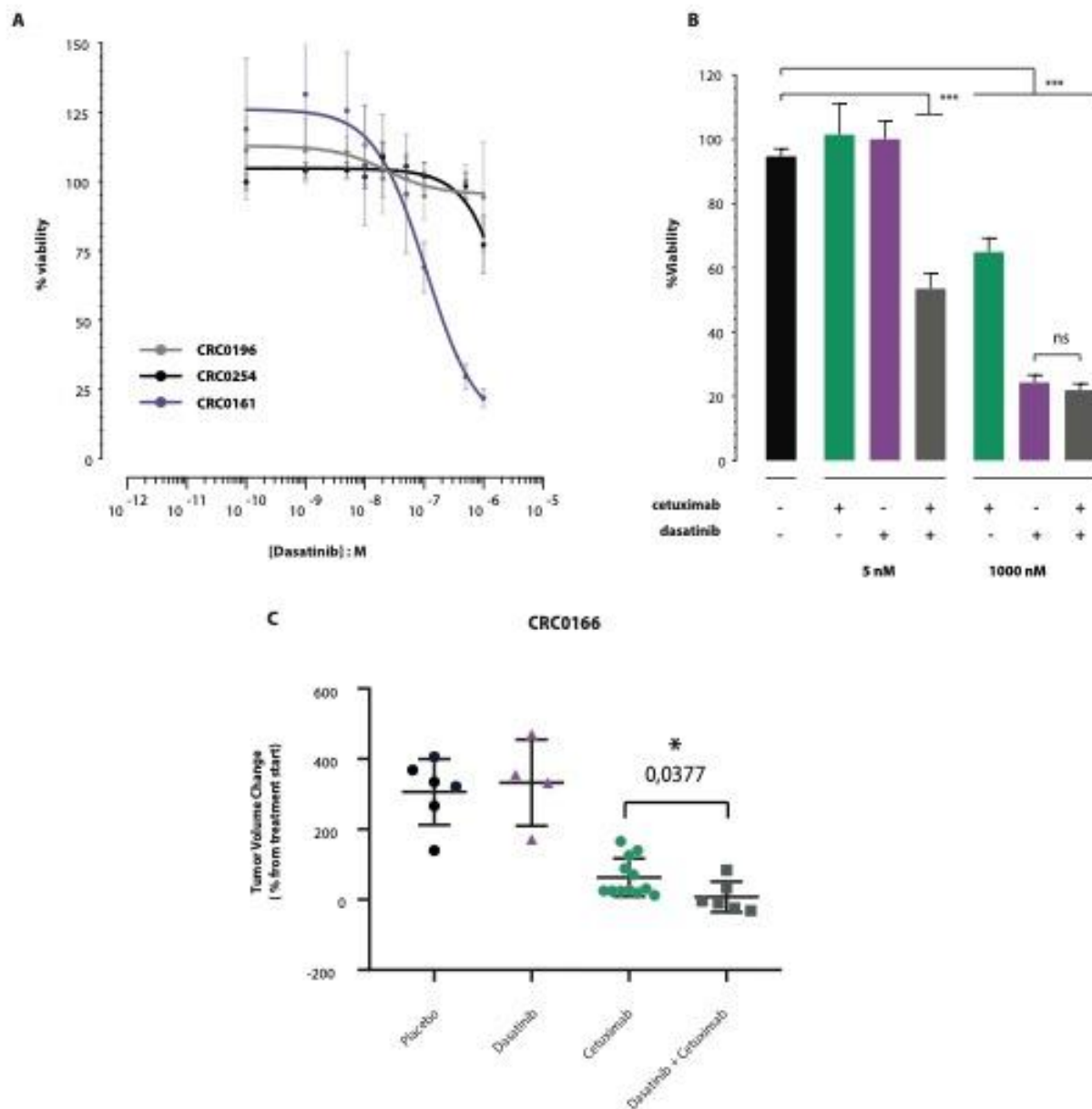
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Fig. 5. INKA analysis of resistant models reveals hyper-active kinases. (A), Boxplot depicting mean INKA score of CS (blue) and CR models (orange), only kinases with more than 25% difference between CS and CR and measured in more than 5 models are shown. Dots indicate individual PDX models. (*) model CRC161 with unknown mechanism of cetuximab resistance. (B) INKA bar plots of models CRC0196 (top left), CRC0176 (top right), CRC0080 (bottom left) and CRC0098 (bottom right) with hyper active kinases. Overview of all INKA bar plots including plots in this figure are shown in fig. S10 & fig. S11.



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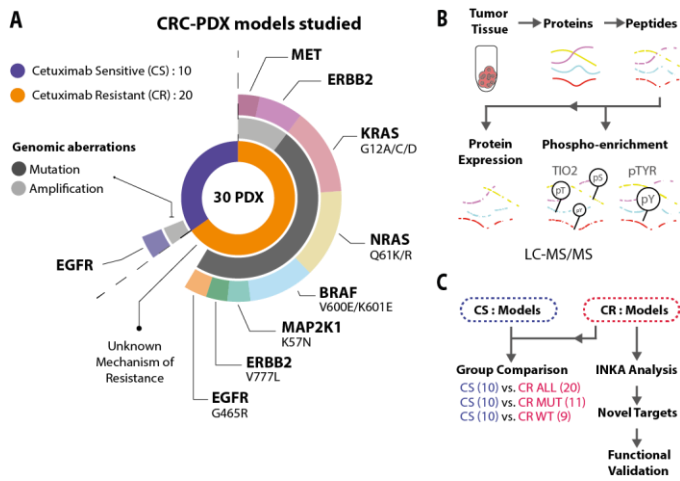
Fig. 6. INKA analysis of models with unknown mechanism of cetuximab resistance reveal dasatinib as a potential treatment. (A), INferred Kinase Activity (INKA) profiles of unexplained resistant PDX tumors CRC0161 (left) CRC0166 (right) and corresponding organoid culture of CRC0161 (middle). Targets of dasatinib are indicated in purple (dark purple: affinity < 1nM, medium: affinity 1 - 100nM, light: affinity 100 - 1000nM). All INKA bar plots including plots in this figure are shown in fig. S10 & fig. S11. (B) Overview of experimentally established targets of dasatinib with an affinity in the nanomolar range. Targets denoted by triangles were discovered using a chemical proteomics approach (32), and those denoted by circles were identified using cell-free assays (33, 62, 63).



1089
 1090 **Fig. 7. Organoid and PDX viability in response to dasatinib inhibition.** (A) Organoid
 1091 viability in response to dasatinib inhibition was tested in three organoids: CRC0196 (low
 1092 target expression, n = 6), CRC0254 (low target expression, n=6), and CRC0161 (high target
 1093 expression, n = 6). Error bars represent SEM. (B) Viability of CRC0161 organoids at 5nM
 1094 and 1000nM treated with Cetuximab (green, 5nM n = 2, 1000nM n = 6), Dasatinib (purple,
 1095 5nM n = 19, 1000nM n = 20), combination (grey, 5nM n = 4, 1000nM n = 6), no treatment
 1096 (black, n= 18). Statistical analysis was performed using ordinary one-way ANOVA. Error
 1097 bars represent SEM. Asterisks represent the level of significance (***)p-value < 0.001). (C)
 1098 Dot plot graph showing percentage tumor volume changes after three weeks of therapy with
 1099 placebo, dasatinib (50 mg/kg by oral gavage, daily), cetuximab (20 mg/kg by intraperitoneal
 1100 injection, twice-weekly) and the combination of dasatinib and cetuximab. NOD-SCID mice
 1101 were inoculated subcutaneously with CRC0166 tumor fragments and randomized to the

1102 different treatments when tumors reached an average volume of 400 mm³. $n = 4$ to 12
1103 animals per each treatment arm. Statistical analysis by two-tailed unpaired Welch's t-test.
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1106 **Supplementary Figures**



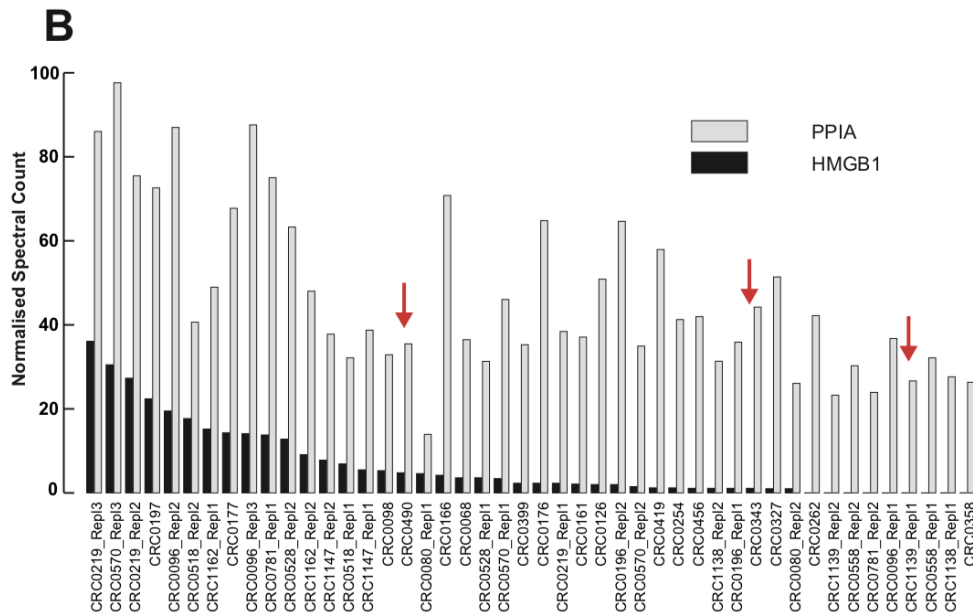
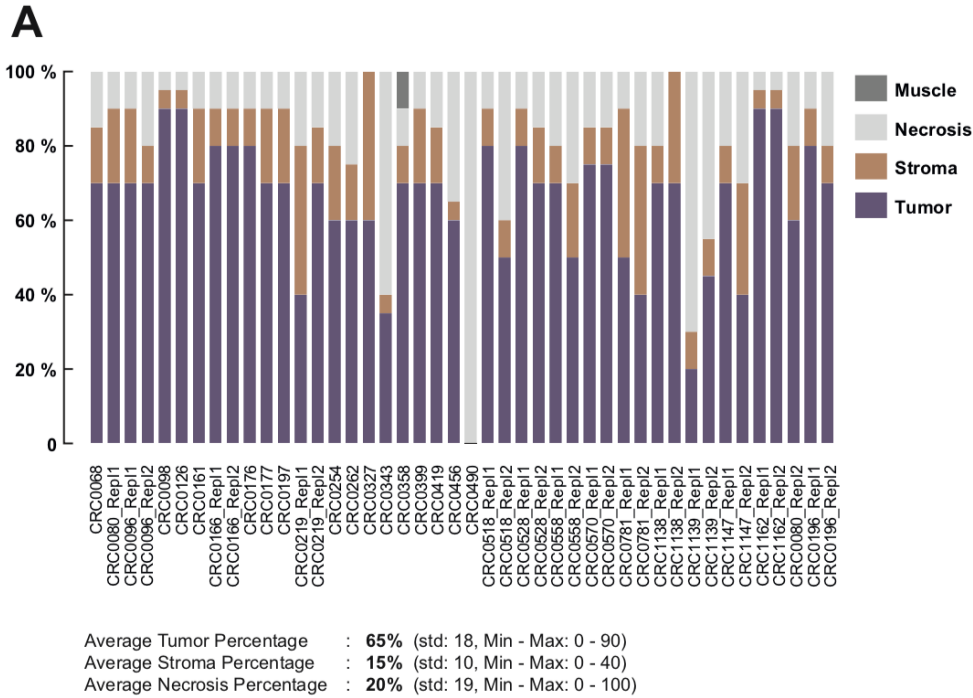
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1109 **Suppl. Fig. 1. Phosphoproteomics identifies targets and markers associated with**
 1110 **sensitivity and resistance to EGFR blockade in colorectal cancer. (A)**, Thirty mCRC-
 1111 PDX models were profiled including 10 cetuximab-sensitive (green, inner ring) and 19
 1112 cetuximab-resistant xenograft tumors (red, inner ring). Resistance-associated genomic
 1113 aberrations in resistant tumors included both mutations (black, middle ring) and
 1114 amplifications (gray, middle ring). The outside ring shows known genomic aberrations
 1115 associated with resistance to cetuximab **(B)**, LS-MS/MS phospho-proteomics and proteomics
 1116 were used to profile both the proteome and phosphoproteome. This enabled the collection of
 1117 three data sets: protein expression, global phosphoproteomics (TiO2), and phosphotyrosine-
 1118 enriched phosphoproteomics (pTyr) **(C)**, Proteome and phosphoproteome data were used to
 1119 make group-comparisons between cetuximab sensitive and resistant tumors to identify
 1120 biomarkers of response. INKA analysis was done on individual tumors to find potential
 1121 targets in cetuximab resistant tumors, followed by functional validation in PDX-derived
 1122 organoids.

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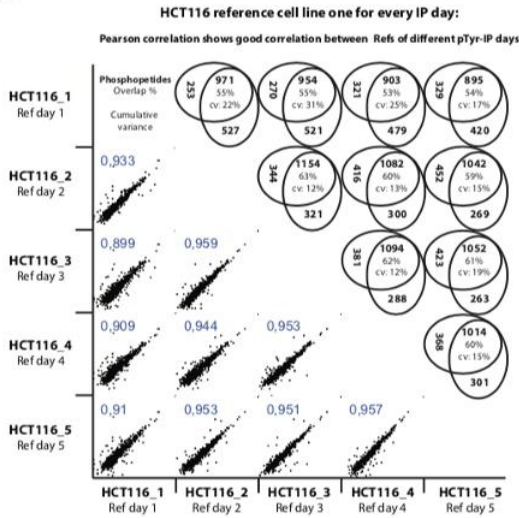
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 1126 **Suppl. Fig 2. Histological assessment of PDX tissue.** (A), Overview of pathology report on HE
 1127 stained representable sections of PDX tissue used for further (phospho) proteomics shows an
 1128 average percentage of 65% epithelial cancer cells, 15% stroma and 20% necrosis. (B), Bar graph
 1129 indicating expression data from proteomics analysis of two known protein markers for necrosis
 1130 (PPIA, HMGB1). Red arrows indicate samples that based on pathology report showed high
 1131 percentage of necrosis. Proteomic results show only average expression of necrosis markers in
 1132 these models.

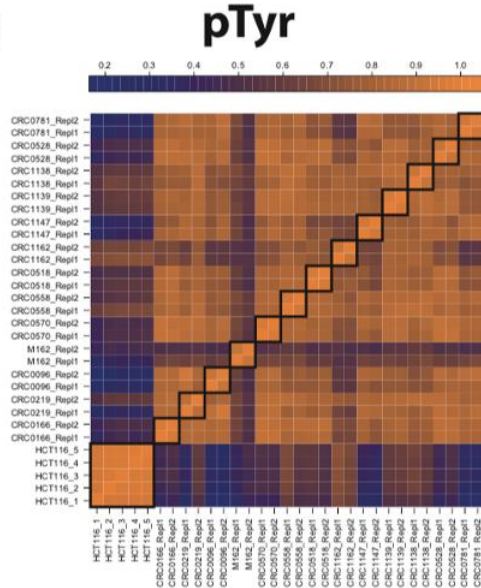
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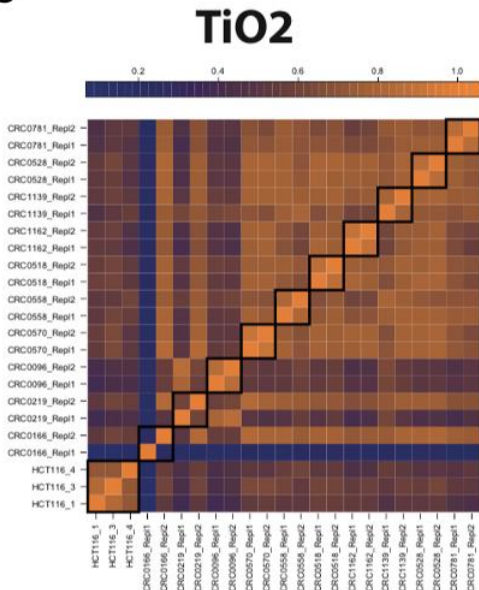
Median: 0.948, std: 0.021, min: 0.899, max: 0.959

B



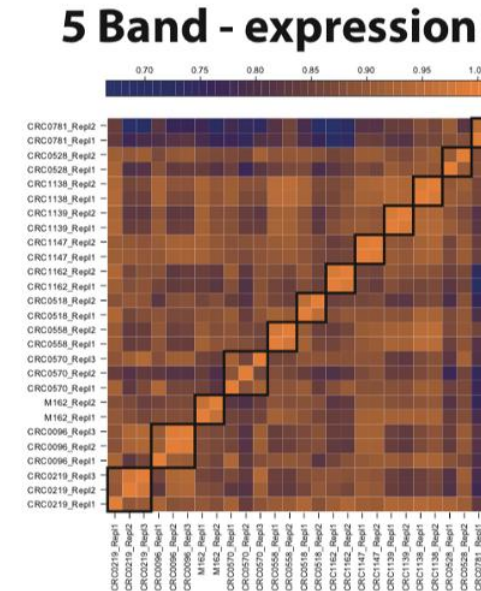
Average Correlation : **0.935**
(std: 0.021, Min - Max: 0.881 - 0.967)

C



Average Correlation : **0.770**
(std: 0.183, Min - Max: 0.253 - 0.916)

D



Average Correlation : **0.956**
(std: 0.029, Min - Max: 0.904 - 0.990)

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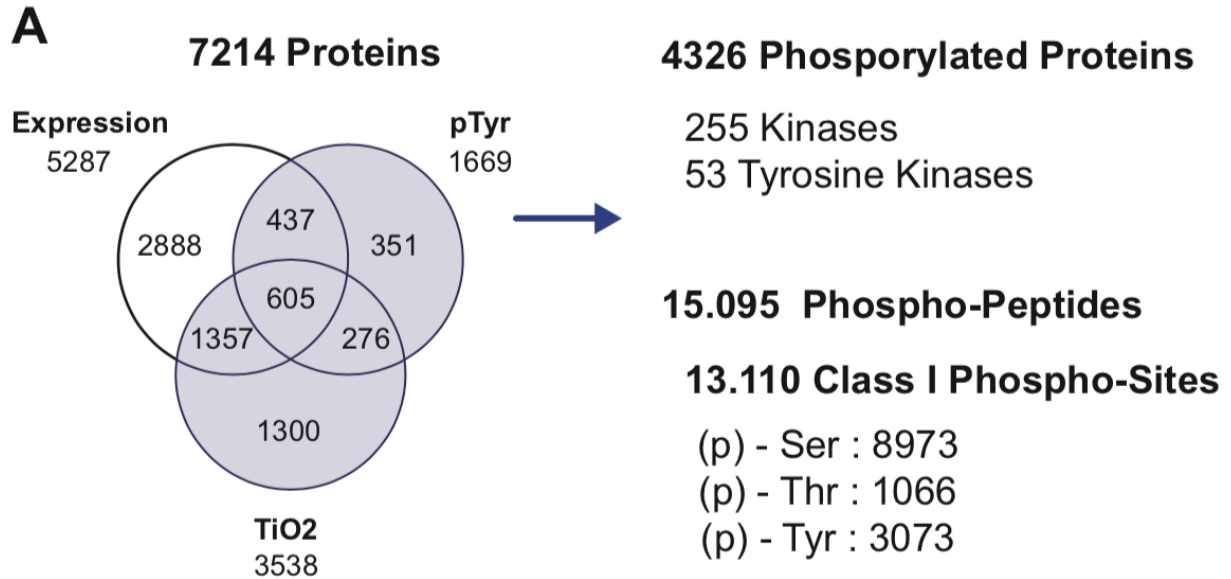
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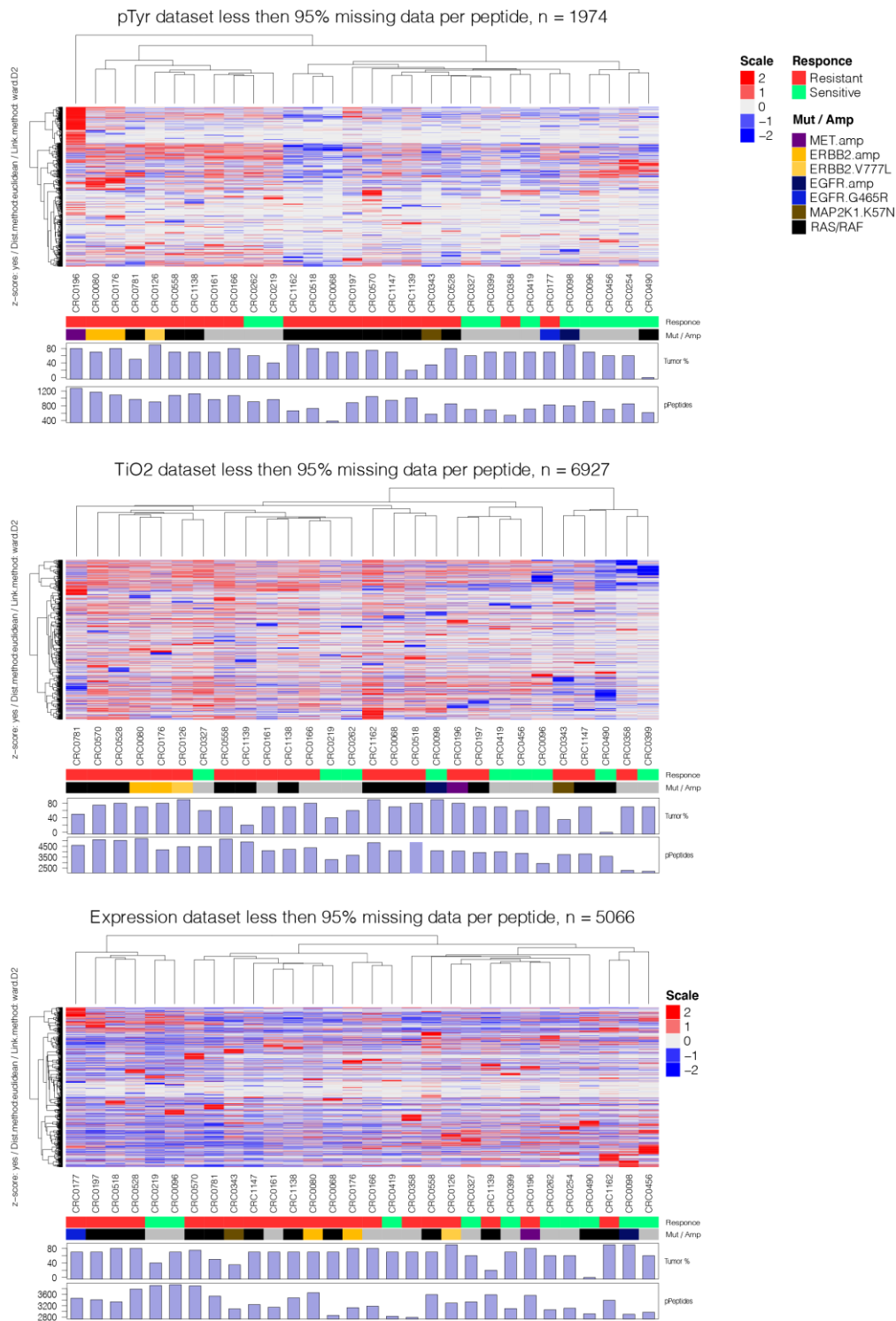
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Suppl. Fig 3. Data reproducibility. (A), Pearson correlation and cumulative variance show high reproducibility between technical replicates. (B, C and D) biological replicates of PDX tumors (pTyr: 13 replicates, TIO2: 10 replicates, Expression: 12 replicates) clustered together in correlation clustering.



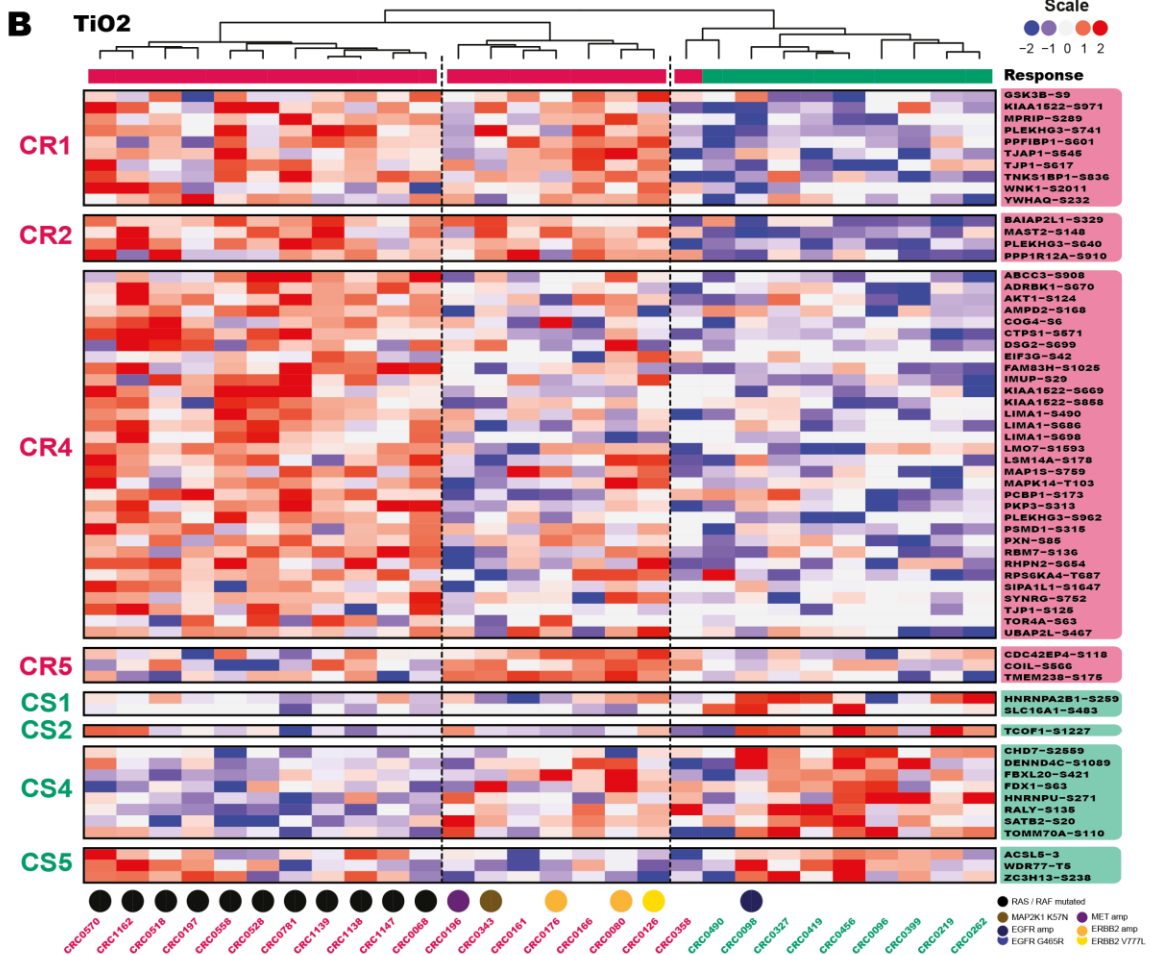
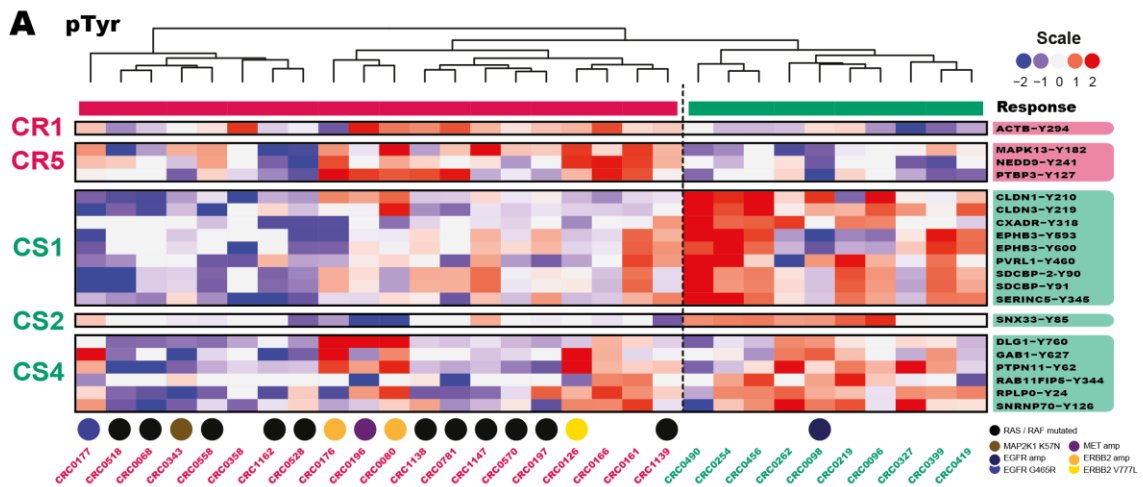
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Suppl. Fig. 4. Overview (phospho) proteomics results. (A), The number of identified proteins and phosphoproteins in the total dataset using mass spectrometry-based expression proteomics, global and tyrosine phosphoproteomics.



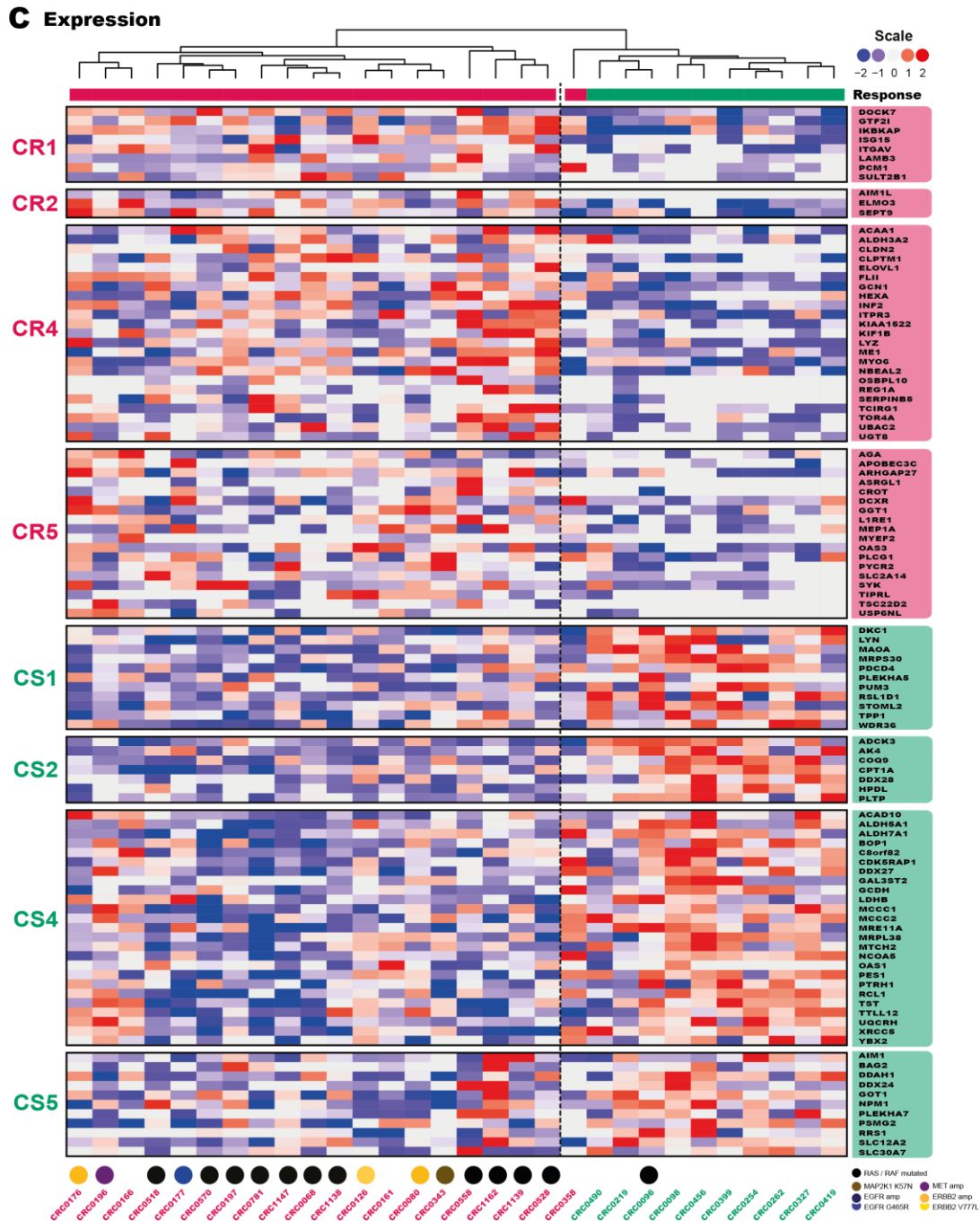
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Suppl. Fig 5. Unsupervised clustering of datasets. Unsupervised clustering of tyrosine (pTyr) and global (TiO2) phosphoproteomics and protein expression dataset. Clusters were annotated with cetuximab response, genomic aberrations, tumor percentage and number of peptides. Clusters do not show sub-clustering of CS and CR tumors.



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Suppl. Fig 6.



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 1156 **Suppl. Fig 6. Comparative analysis of PDX models sensitive and resistant to cetuximab**
 1157 **identifies differential signature.** Clustering of the combined signature of the top differential
 1158 phospho-sites (rows) from the comparisons in the pTyr (A), TiO2 (B) and Expression (C)
 1159 dataset. Response is indicated for sensitive (green) and resistant (red) models. Genomic
 1160 aberrations of models are indicated with colored circles below. Clustering shows separation
 1161 between sensitive and resistant models in pTyr and near-complete separation in TiO2 and
 1162 Expression with separate clusters for RES WT and RES MUT in TiO2.

A

Overview number of models considered per comparison for protein and RNA expression data

	Comparison	Protein	RNA
1.	CS vs. CR-ALL	10 vs. 20	10 vs. 10
2.	CS vs. CR-WT	9 vs. 9	9 vs. 8
3.	CS vs. CR-MUT	9 vs. 11	(9 vs. 2)

No RNA-sequencing data was available for 9 CR-MUT models. In addition, model CRC0358 (CR-WT) was not considered for RNA analyses due to outlier behavior. Due to the unbalanced and underpowered CS vs. CR-MUT comparison on RNA level, this comparison was excluded from all further analyses.

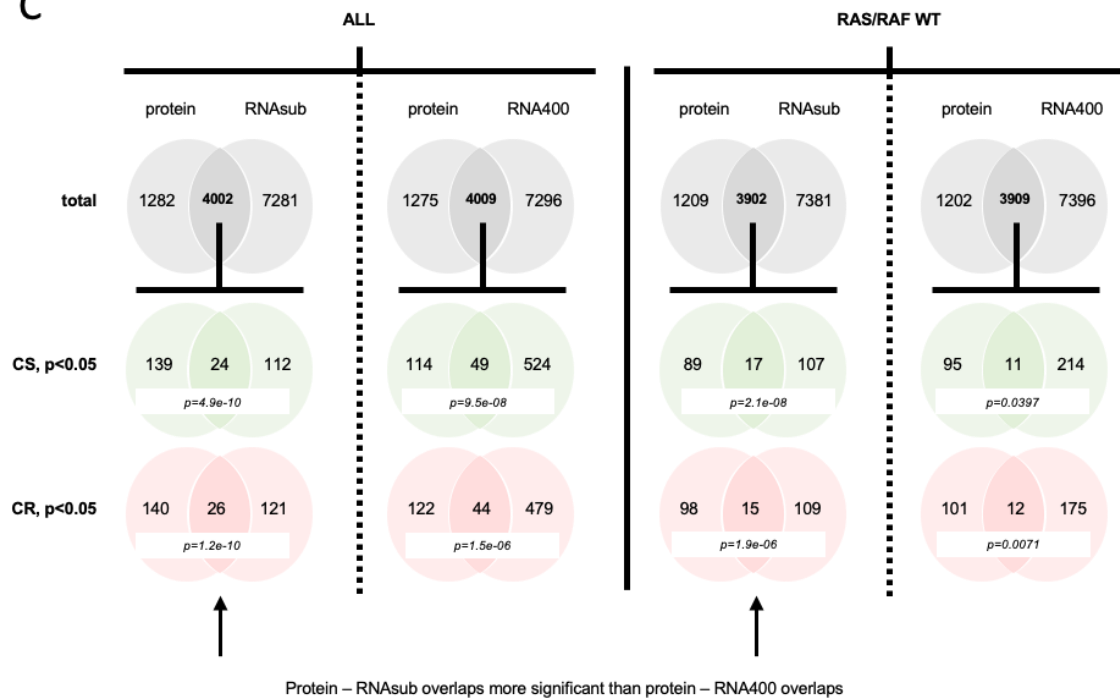
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Overview number of models considered per comparison for protein phosphorylation data

	Comparison	pTyr	TiOx
1.	CS vs. CR-ALL	10 vs. 20	9 vs. 19
2.	CS vs. CR-WT	9 vs. 9	8 vs. 8
3.	CS vs. CR-MUT	9 vs. 11	9 vs. 11

No TiOx data was available for models CRC0254 (CS-WT) and CRC0177 (CR-WT) due to sample loss during TiOx enrichment

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Suppl. Fig. 7. Overlap protein and RNAsub / RNA400 expression data. (A), Overview number of models considered per comparison for protein and RNA expression data. (B), Overview number of models considered per comparison for protein phosphorylation data. (C), Overlap in total identifications on protein and RNA level, and in significantly ($p < 0.05$) differentially expressed genes between CS and CR PDX models. RNAsub refers to a comparison among the same subset of PDX models also used for proteomics profiling, RNA400 refers to a comparison among a large collection of ~400 PDX models (Isella et al., 2017). Hypergeometric test was performed to test for significance of overlap.

Type 1 Interferon Signaling

GO-ID	Description	p-val	corr p-val	cluster freq
45071	negative regulation of viral genome replication	1.5880E-7	2.4969E-5	3/4 75.0%
71357	cellular response to type I interferon	2.0110E-7	2.4969E-5	3/4 75.0%
50337	type I interferon signaling pathway	2.0110E-7	2.4969E-5	3/4 75.0%
34340	response to type I interferon	2.5031E-7	2.4969E-5	3/4 75.0%
1903901	negative regulation of viral life cycle	4.1431E-7	3.3062E-5	3/4 75.0%

Cell-cell Junction Organization

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
45216	cell-cell junction organization	3.7658E-10	1.5892E-7	5/6 83.3%	165/17866 0...
98742	cell-cell adhesion via plasma-membrane adhesion molecules	3.4457E-9	7.2704E-7	5/6 83.3%	256/17866 1...
16338	calcium-independent cell-cell adhesion via plasma membrane cell-adhesion molecules	2.7928E-8	3.9224E-6	3/6 50.0%	21/17866 0....
2000810	regulation of bicellular tight junction assembly	3.7179E-8	3.9224E-6	3/6 50.0%	23/17866 0....

Ribonucleoprotein complex biogenesis

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
22613	ribonucleoprotein complex biogenesis	1.2718E-17	7.0332E-15	12/15 80.0%	427/17866 2...
42254	ribosome biogenesis	2.9761E-17	8.2290E-15	11/15 73.3%	298/17866 1...
42273	ribosomal large subunit biogenesis	1.3075E-13	2.4102E-11	7/15 46.6%	77/17866 0....
6364	rRNA processing	2.2259E-12	3.0772E-10	8/15 53.3%	214/17866 1...
16072	rRNA metabolic process	3.4539E-12	3.8200E-10	8/15 53.3%	226/17866 1...

Enzyme linked receptor protein signaling

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
7167	enzyme linked receptor protein signaling pathway	2.6502E-12	4.4311E-9	10/13 76.9%	718/17866 4.0%
7169	transmembrane receptor protein tyrosine kinase signaling pathway	8.5684E-12	7.1632E-9	9/13 69.2%	517/17866 2.8%
16477	cell migration	1.9664E-9	1.0959E-6	9/13 69.2%	952/17866 5.3%
51674	localization of cell	5.5580E-9	1.8586E-6	9/13 69.2%	1071/17866 5.9%
48870	cell motility	5.5580E-9	1.8586E-6	9/13 69.2%	1071/17866 5.9%

Carboxylic acid metabolic process

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
19752	carboxylic acid metabolic process	4.8227E-9	1.2865E-6	7/8 87.5%	869/17866 4.8%
43436	oxoacid metabolic process	9.6609E-9	1.2865E-6	7/8 87.5%	960/17866 5.3%
6082	organic acid metabolic process	1.1155E-8	1.2865E-6	7/8 87.5%	980/17866 5.4%
44281	small molecule metabolic process	5.1445E-7	4.4500E-5	7/8 87.5%	1701/17866 9.5%
32787	monocarboxylic acid metabolic process	1.0372E-6	7.1771E-5	5/8 62.5%	517/17866 2.8%

Viral Process

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
16032	viral process	3.4604E-6	1.0030E-3	4/4 100.0%	772/17866 4...
44403	symbiotic process	5.2101E-6	1.0030E-3	4/4 100.0%	855/17866 4...
377	RNA splicing, via transesterification reactions with bulged adenosine as nucleophile	1.8151E-5	1.4402E-3	3/4 75.0%	298/17866 1...
398	mRNA splicing, via spliceosome	1.8151E-5	1.4402E-3	3/4 75.0%	298/17866 1...
375	RNA splicing, via transesterification reactions	1.8704E-5	1.4402E-3	3/4 75.0%	301/17866 1...

Organelle Localization

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
51656	establishment of organelle localization	2.1016E-7	1.2862E-4	4/4 100.0%	384/17866 2...
51640	organelle localization	1.2347E-6	3.7782E-4	4/4 100.0%	597/17866 3...
30705	cytoskeleton-dependent intracellular transport	4.8493E-6	9.8926E-4	3/4 75.0%	192/17866 1...
47497	mitochondrion transport along microtubule	8.6719E-6	1.0614E-3	2/4 50.0%	22/17866 0....
34643	establishment of mitochondrion localization, microtubule-mediated	8.6719E-6	1.0614E-3	2/4 50.0%	22/17866 0....

Peptidyl-serine Phosphorylation

GO-ID	Description	p-val	corr p-val	cluster freq	total freq
18105	peptidyl-serine phosphorylation	8.9507E-6	3.7657E-3	3/5 60.0%	174/17866 0.9%
18209	peptidyl-serine modification	1.3190E-5	3.7657E-3	3/5 60.0%	198/17866 1.1%
72740	cellular response to anisomycin	2.7986E-4	3.3249E-2	1/5 20.0%	1/17866 0.0%
51403	stress-activated MAPK cascade	3.3180E-4	3.3249E-2	2/5 40.0%	104/17866 0.5%
72739	response to anisomycin	5.5966E-4	3.3249E-2	1/5 20.0%	2/17866 0.0%

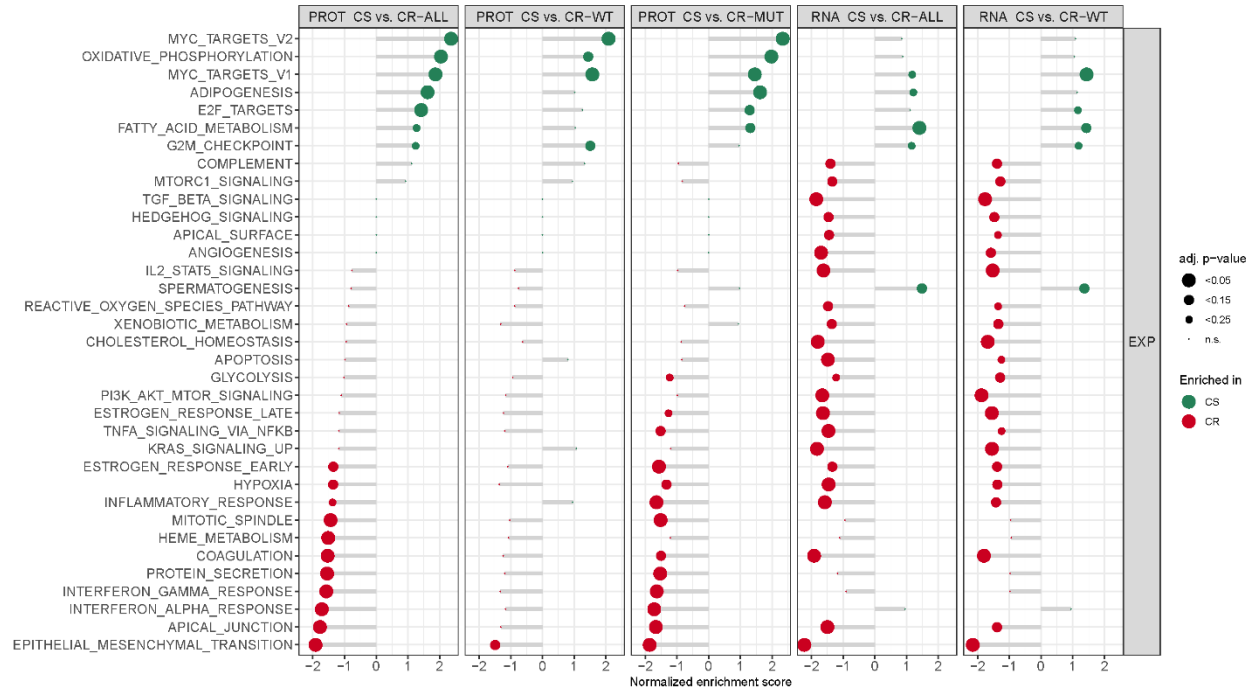
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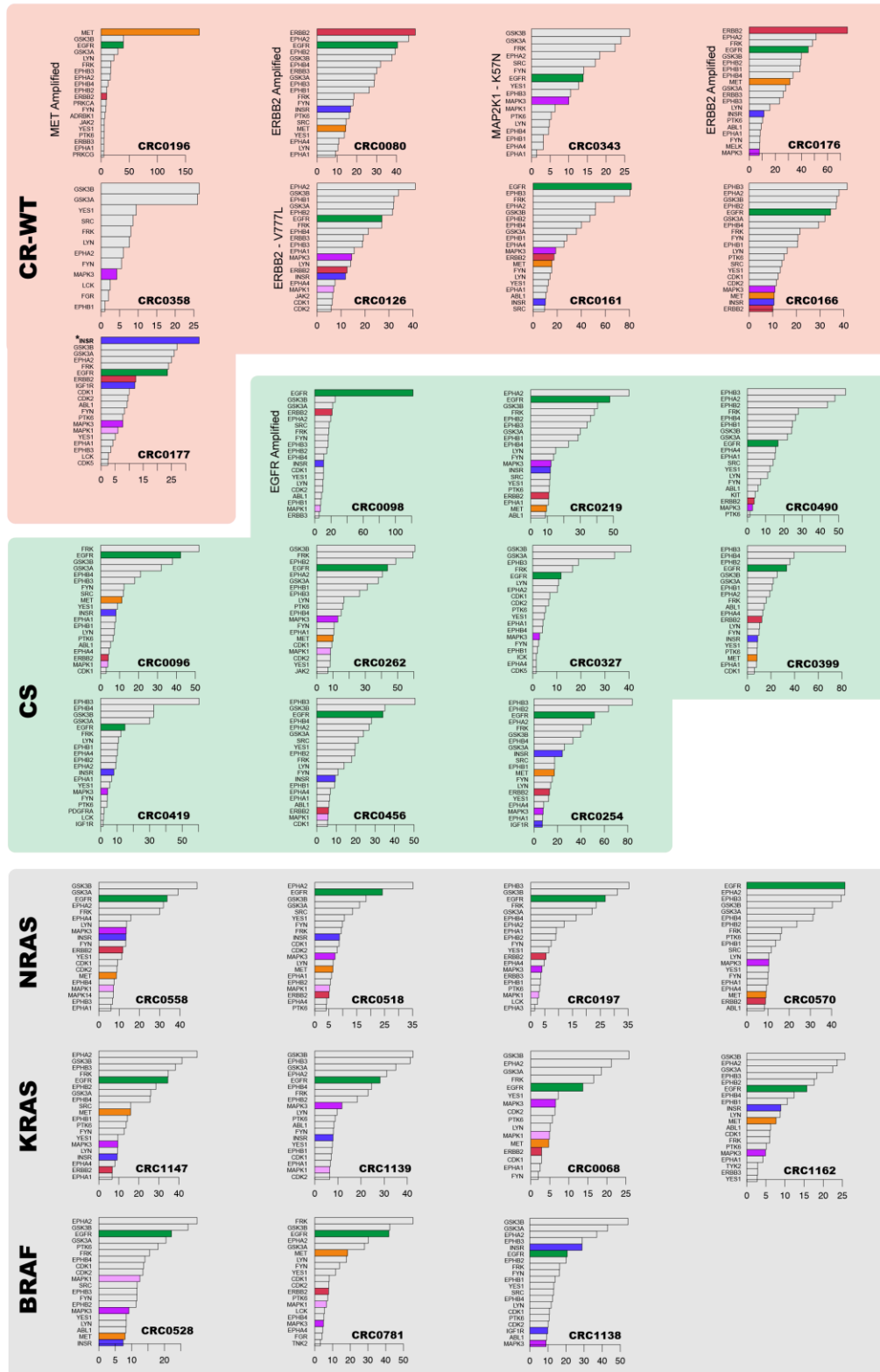
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Suppl. Fig. 8. BinGO clusters. Markov clustering combined with BinGO gene ontology analysis revealed 8 biologically relevant protein clusters.



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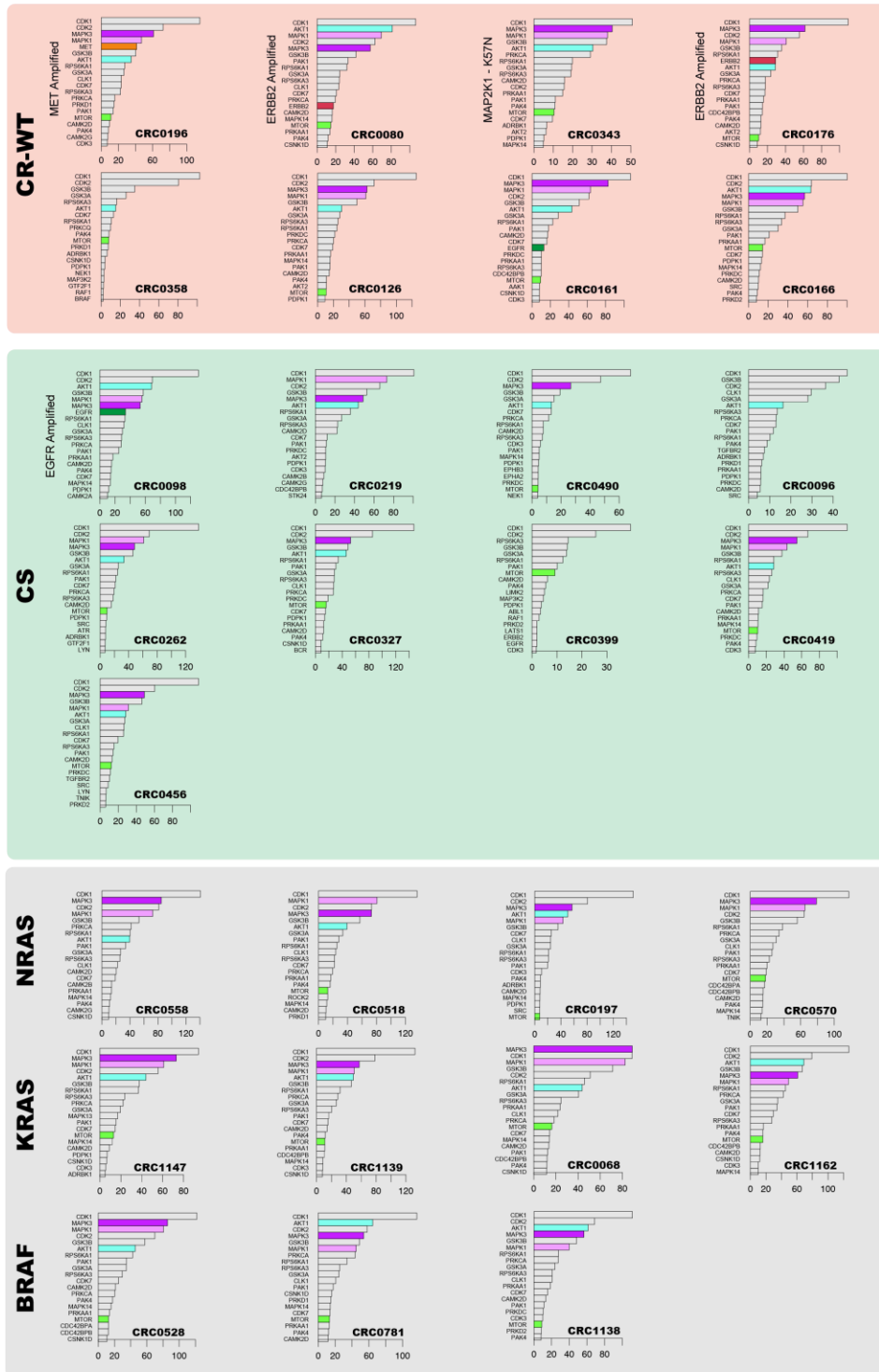
Suppl. Fig 9. Protein and RNA expression-driven enrichment of CS- and CR-specific biology. Lollipop plots showing the normalized enrichment scores of differentially regulated expression-driven HALLMARK signatures between CS and CR PDX models as determined by gene set enrichment analysis (GSEA). The size of the circles correspond to significance of enrichment. Shown are processes that are at least sub-significant (adj. p-value <0.15) in at least one of the comparisons.



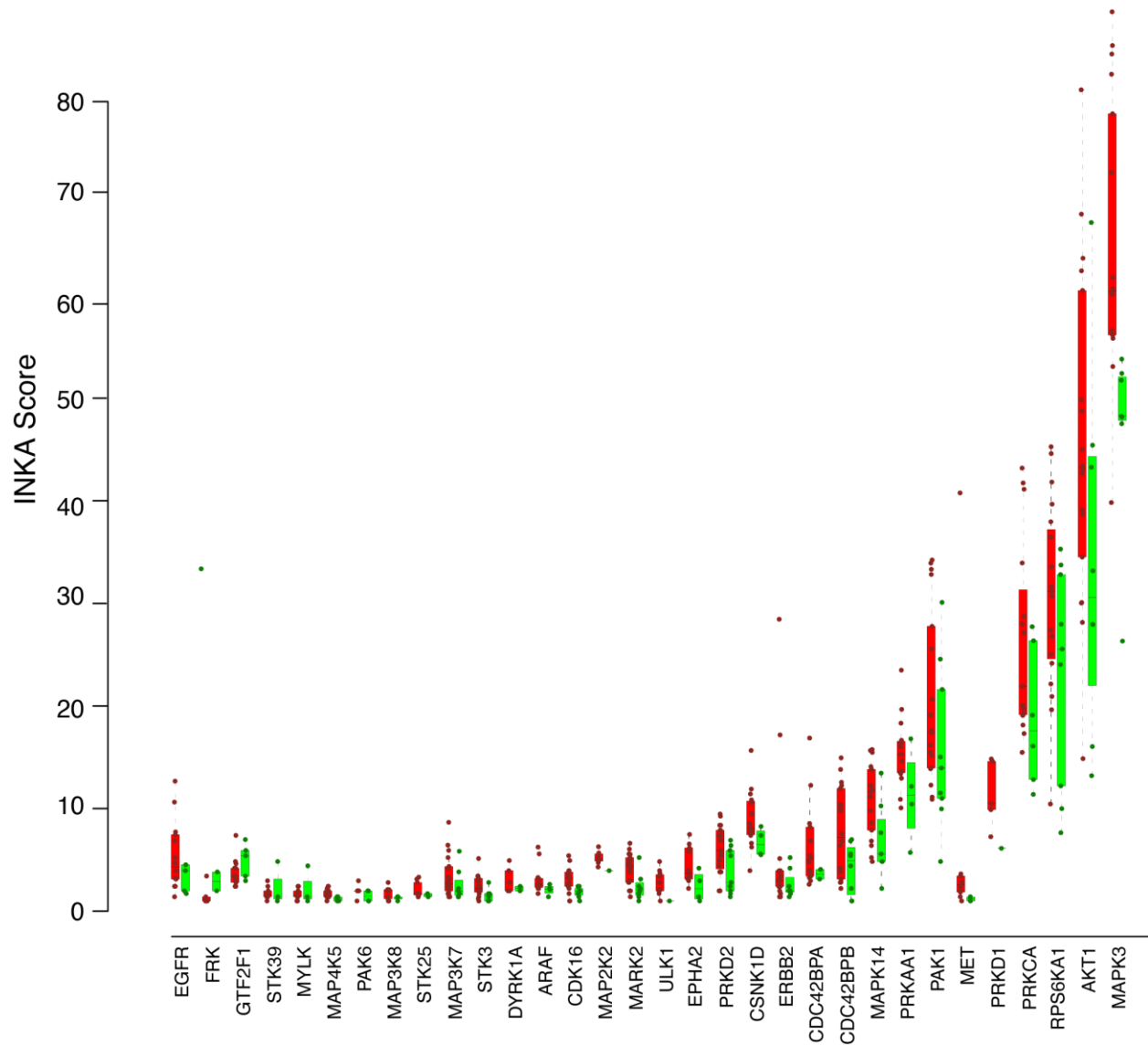
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Suppl. Fig. 10. pTyr INKA Bargraph of all PDX-Models. Bargraph show INKA score of all models. EGFR (green), ERBB2 (red), MET (orange), MAPK1 (pink), MAPK3 (purple), and

1188 INSR/IGF1R (blue) are highlighted. (*) In CRC0177 INSR/IGF1R was confirmed as co-target
 1189 (Beekhof et al., Mol.Sys Bio 2019).
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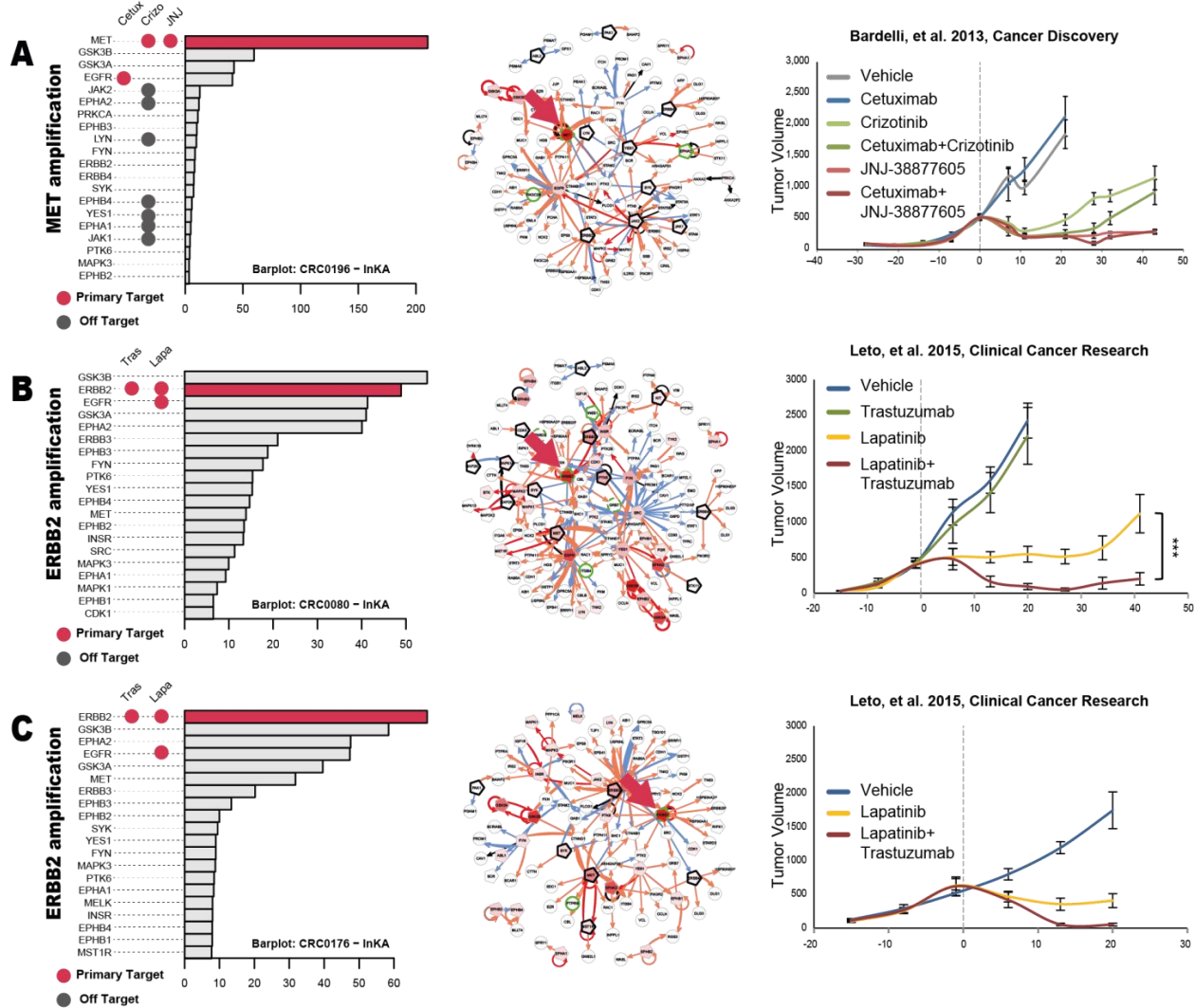


1191 **Suppl. Fig.11. TiO2 INKA Bargraph of all PDX-Models.** Bargraph show INKA score of all
 1192 models. EGFR (green), ERBB2 (red), MET (orange), MAPK1 (pink), MAPK3 (purple), MTOR
 1193 (light green) and AKT1 (light blue) are highlighted.
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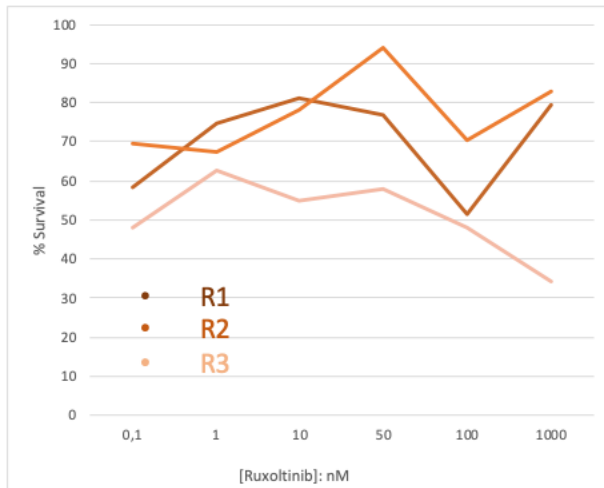
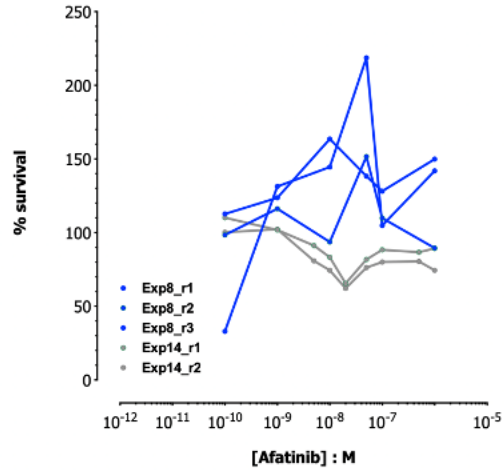
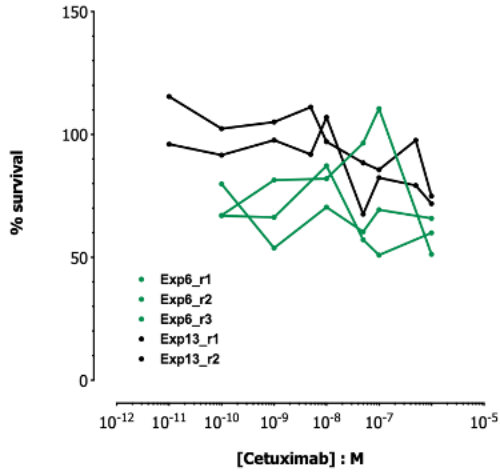
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Suppl. Fig. 12. TiO2 mean INKA Bargraph. Boxplot depicting mean INKA score of CS (green) to CR models (red), showing only kinases with more than 25% difference between CS and CR. Kinases must have been measured in more than 5 models. Dots indicate individual PDX models.



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Suppl. Fig. 13. INKA analysis of tumors with known cetuximab resistance through gene amplification. (A), CRC0196; Left, ranking of top Kinases with their INKA score. Middle, kinase interaction network (red arrow indicates amplified Kinase MET). Right, response to MET inhibitors as described in Bardelli et al., 2013. (B), CRC0080; Left, ranking of top Kinases with their INKA score. Middle, kinase interaction network (red arrow indicates amplified Kinase ERBB2). Right, response to ERBB2 inhibitors as described in Bertotti et al., 2015 and Leto et al., 2015. (C), CRC0176; Left, ranking of top Kinases with their INKA score. Middle, kinase interaction network (red arrow indicates amplified Kinase ERBB2). Right, response to ERBB2 inhibitors as described in Bertotti et al., 2015 and Leto et al., 2015.



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Suppl. Fig. 15. Viability of CRC-0161. Treatment in CRC0161 with cetuximab, afinitinib or the inhibition of JAK with ruxolitinib, a “negative control” that did not show high INKA scoring in CRC0161, did not result in reduction of organoid viability.