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**Systems analysis of protein signatures predicting
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wild-type patient-derived xenografts models of metastatic
colorectal cancer**

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Systems analysis of protein signatures predicting Cetuximab responses in *KRAS*, *NRAS*, *BRAF* and *PIK3CA* wild-type patient-derived xenografts models of metastatic colorectal cancer

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Running title: Systems analysis of cetuximab responses

Novelty and Impact: A large fraction of patients with metastatic colorectal cancer do not respond to anti-EGFR therapy despite *KRAS* wild type tumours. Statistical analysis of RPPA data of colorectal cancer *KRAS*, *BRAF*, *NRAS* and *PI3KCA* wild type PDX models revealed a 14 - 20 (phospho)protein signature that was predicting responses to cetuximab. Our findings furthermore emphasise GSK-3 β to be potentially targetable for a co-treatment with cetuximab.

Keywords: anti-EGFR, metastatic colorectal cancer, molecular subtyping, reverse-phase protein array, deterministic modelling, apoptosis, proliferation

Abbreviations: 5-FU, fluorouracil; ANOVA, analysis of variance; CMS, consensus molecular subtypes; CRC, colorectal cancer; CRIS, CRC intrinsic subtype; EGF, epidermal growth factor; EGFR, EGF receptor; LASSO, least absolute shrinkage and selection operator; NMF, non-negative matrix factorization; P, p-value; PAM, Prediction Analysis for Microarrays; PDX, patient-derived mouse xenograft; RPPA, reverse phase protein array; SC, substrate cleavage.

Abstract

Antibodies targeting the human epidermal growth factor receptor (*EGFR*) are used for the treatment of *RAS* wild-type metastatic colorectal cancer. A significant proportion of patients remains unresponsive to this therapy. Here, we performed a reverse phase protein array-based (phospho)protein analysis of 63 'quadruple-negative' (*KRAS*, *NRAS*, *BRAF* and *PIK3CA* wild-type) metastatic CRC tumours. Responses of tumours to anti-EGFR therapy with cetuximab were recorded in patient-derived xenograft (PDX) models. Unsupervised hierarchical clustering of pre-treatment tumour tissue identified three clusters, of which cluster C3 was exclusively composed of responders. Clusters C1 and C2 showed mixed responses. None of the three protein clusters showed a significant correlation with transcriptome-based subtypes. Analysis of protein signatures across all PDXs identified 14 markers that discriminated cetuximab-sensitive and -resistant tumours: PDK1 (S241), Caspase-8, Shc (Y317), Stat3 (Y705), p27, GSK-3 β (S9), HER3, PKC- α (S657), EGFR (Y1068), Akt (S473), S6 Ribosomal Protein (S240/244), HER3 (Y1289), NF- κ B-p65 (S536) and Gab-1 (Y627). Least absolute shrinkage and selection operator and binomial logistic regression analysis delivered refined protein signatures for predicting response to cetuximab. (Phospho-)protein analysis of matched pre- and post-treated models furthermore showed significant reduction of Gab-1 (Y627) and GSK-3 β (S9) exclusively in responding models, suggesting novel targets for treatment.

Background

Colorectal cancer (CRC) is the third and second most commonly diagnosed cancer in males and females, and the second most common cause of cancer-related deaths in the developed world. In the advanced setting, CRC is routinely treated with fluorouracil (5-FU)-based chemotherapy. 30% of CRC patients present in the metastatic setting¹ where response rates to palliative 5-FU/oxaliplatin- or 5-FU/irinotecan-based chemotherapy range between 40-50%. Median overall survival remains poor at around 16-19 months². Identifying the importance of epidermal growth factor (*EGF*) signalling for the survival of CRC cells resulted in the development of targeted therapies that neutralize the oncogenic activity of EGF receptors (*EGFR*). Anti-EGFR therapies have significantly improved survival in metastatic CRC patients³. Guidelines recommend to test for *KRAS*, *NRAS* and *BRAF* mutations as well as microsatellite instability status in CRC patients being considered for anti-EGFR therapy^{4, 5} on the bases of the ineffectiveness of anti-EGFR therapy ~~is not effective~~ in patients with activating *KRAS*, *BRAF*, and *NRAS* mutations⁶, and favourable responses to immune check point inhibitors in microsatellite instability-high patients⁴. While *PI3KCA* mutational analysis is not recommended yet⁴, *PIK3CA* exon 20 mutations were linked with a worse outcome compared with wild-type status in patients with metastatic colorectal cancer ⁷. Nevertheless, between 50–60% of patients will not benefit from anti-EGFR treatment even when these are *KRAS*, *BRAF*,

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2
3 *NRAS* and *PI3KCA* wild type (~~quadruple negative~~) have a 'quadruple
4
5 negative' status⁷.
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8 Mutations and copy number alterations in genes encoding for other
9
10 survival signaling proteins have been shown to contribute to anti-EGFR
11
12 resistance. For example, *HER2*-amplification, *IGF2* overexpression or
13
14 increased *MET* activity resulted in reduced responses to anti-EGFR
15
16 therapy, as demonstrated in patient-derived xenograft (PDX) models of
17
18 metastatic CRC and in patients^{8, 9}. Analysis of the genomic and
19
20 transcriptomic landscape of anti-EGFR resistance in PDX models and
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22 patients furthermore identified mutations in *EGFR*, *FGFR1*, *PDGFRA*, and
23
24 *MAP2K1* or loss of *NF1* to contribute to anti-EGF resistance^{9, 10}.
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28 While identification of patient-specific genome alterations provides a
29
30 personalised diagnosis that provides insights into anti-EGFR therapy
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32 responses and may open opportunities for personalised therapies,
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34 interpretation of often multiple genomic alterations found in most patients
35
36 is not always straightforward. Other efforts to identify responders and non-
37
38 responders to anti-EGFR therapy have therefore focussed on the power of
39
40 unsupervised molecular subtyping of tumours. An international meta-
41
42 analysis and bioinformatics effort led to the identification of four distinct
43
44 subtypes in CRC, termed 'Consensus Molecular Subtypes' (CMS1-
45
46 CMS4)¹¹. A recent study demonstrated that CMS2 patients benefitted
47
48 more from anti-EGFR therapy than patients treated with anti-angiogenic
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50 therapy, while the opposite was the case in CMS1 patients¹². However
51
52 predictions of anti-EGFR therapy responses in CMS3 and CMS4 patients
53
54 were not possible, and significant variability in overall and progression free
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3 survival are still seen across all four CMS subtypes. Because stroma-
4 derived mRNAs in whole tumour transcriptomes may obscure
5 transcriptional features displayed by cancer cells, other efforts leveraged
6 the power of patient-derived mouse xenograft (PDX) models in which
7 human stroma is replaced by mouse stroma to obtain five CRC 'intrinsic'
8 (CRIS) molecular subtypes, termed CRIS-A to E¹³. CRIS-C was identified
9 as a subtype associated with EGFR signalling and increased sensitivity to
10 anti-EGFR therapy. However responses to anti-EGFR therapy strongly
11 varied among the other four CRIS subtypes¹³.

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24 EGFR activation results in the activation of several downstream signalling
25 pathways, including the PI3K/AKT and MAPK pathways¹⁴. The activation
26 status of these key signalling pathways influences a variety of biological
27 processes such as proliferation, apoptosis, cell migration, bioenergetics,
28 immune responses, and angiogenesis. A different approach to investigate
29 responses to anti-EGFR therapy is to determine the activation status of
30 key signalling branches activated by EGFR receptors and their
31 downstream effectors, supported by statistical or deterministic modelling¹⁵.
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Because processes such as proliferation and apoptosis are controlled by
complex networks that show significant signalling redundancies,
deterministic systems models have been developed to estimate more
precisely proliferative capacity or apoptosis sensitivity of tumours. One
such tool developed by our group is the systems model, DR_MOMP,
which calculates the apoptosis sensitivity of tumours based on a
quantitative analysis of BCL-2 family proteins and their interactions^{16, 17}.
To identify novel prognostic markers of anti-EGFR therapy, we here

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2
3 comprehensively profiled 83 signalling proteins and (phospho)proteins
4 related to EGFR and key cancer signalling pathways in a cohort of 63
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8 ~~'quadruple negative' (KRAS, BRAF, NRAS and PI3KCA wild type)~~ PDX
9
10 models isolated from liver biopsies that were derived from metastatic CRC
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12 patients^{9, 18}. We performed both statistical and systems modelling
13
14 analyses to identify novel protein signatures of anti-EGFR responsiveness.
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18 **Methods**

21 **CRC PDX in vivo model**

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25 108 PDX models derived from colorectal cancer liver metastasis originally
26
27 at the Institute for Cancer Research and Treatment, and Mauriziano
28
29 Umberto I (Torino, Italy)¹⁸ were used in this study. 63 of 108 were *KRAS*,
30
31 *BRAF*, *NRAS* and *PI3KCA* wild type ~~quadruple negative (with wild-type~~
32
33 ~~*KRas*, *NRas*, *PI3KCA*, and *B-Raf*)~~ based on matched next-generation
34
35 sequencing analysis data from Bertotti *et al.*⁹ and used for statistical
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37 analysis. Tumour tissues were implanted subcutaneously and passaged in
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39 *NOD/SCID* mice. Response data is available for each tumour to cetuximab
40
41 treatment after 3 and 6 weeks¹⁹.
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47 **Reverse phase protein array**

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49 Protein was extracted from PDX tumour tissue and cell line standards and
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51 RPPA was performed as described previously²⁰. Protein lysates
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53 normalized to 1µg/µL concentration as assessed by bicinchoninic acid
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55 assay (BCA, Biorad). Reverse phase protein array (RPPA) with a panel of
56
57 antibodies targeting various key cancer related proteins was used for
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3 measuring protein levels in untreated tumours. The response is from
4 matching samples of same tumour in different mice. The DAKO
5 (Carpinteria, CA) catalyzed signal amplification system was used for
6 antibody blotting.
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13 **PDX Protein clustering**

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16 RPPA data for 93 PDX samples have been clustered using consensus
17 Non-negative Matrix Factorization (R package 'NMF'²¹, version 0.21.0) on
18 centred RPPA data^{22, 23}. NMF was performed 1 000 times with the number
19 of clusters k varying from 2 to 8. $k = 3$ was selected based on visual
20 inspection of co-clustering matrices and heatmap of clustered RPPA data.
21
22 To represent graphically the correspondence between CRIS subtypes
23 classifiers and the RPPA clusters or cetuximab response, Factorial
24 Correspondence Analysis (FCA) was used. For each comparison, χ^2
25 independence test was carried out. In order to have large enough
26 numbers in the contingency table so that the χ^2 approximation is correct,
27 we combined together the closest CRIS subtypes.
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43 **DR MOMP, APOPTO-CELL and proliferation signature**

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45 The normalised gene expression of *BIRC5*, *CCNB1*, *CDC20*, *CDCA1*,
46 *CEP55*, *NDC80*, *MKI67*, *PTTG1*, *RRM2*, *TYMS* and *UBE2C* was averaged
47 and used as proliferation signature^{24, 25} of each PDX. The gene expression
48 data for respective PDX models was downloaded from GSE76402¹³.
49
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54 To calculate the sensitivity of patients' cancer cells to undergo apoptosis,
55 the mathematical models APOPTO-CELL²⁶ and DR_MOMP¹⁶ were
56 applied, using PRO-CASPASE-3, PRO-CASPASE-9, SMAC, and XIAP
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3 protein for APOPTO-CELL, and BAK, BAX, BCL2 and BCL(X)L for
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5 DR_MOMP as input for the models. MCL1 protein levels were assumed to
6
7 be 0 nM for DR_MOMP. SMAC concentrations were assumed to be 122.7
8
9 nM for APOPTO-CELL²⁶. Protein levels were normalized to HeLa cells that
10
11 were placed on the RPPA together with the cancer tissue^{16, 26}.
12
13

14 15 16 **Statistical analysis**

17
18 Statistical analysis of RPPA data was done using 'SAMR'²⁷ (Significance
19
20 Analysis of Microarrays, version 3.0) and 'PAMR'²⁸ (Prediction Analysis for
21
22 Microarrays; version 1.56.1) R Packages (R version 3.6.2). LASSO was
23
24 performed using the 'glmnet' R package (version 2.0-18). The packages
25
26 'ComplexHeatmap'²⁹ (version 2.1.0) and 'Circlize'³⁰ (version 0.4.7) were
27
28 used to create Figure 1. Week 3 response was used for all the statistical
29
30 analysis as not all the mice were followed through after 3 weeks. Student's
31
32 t-test and ANOVA was used for measuring statistical significance. ANOVA
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34 was followed by Tukey's HSD (honest significant difference) test for
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36 multiple pair comparison. Fisher's exact test was used for count data.
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43 44 45 **Results**

46 47 **Characterisation of *KRAS*, *BRAF*, *NRAS* and *PI3KCA* wild** 48 49 **type metastatic CRC (phospho)protein signatures**

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51 To investigate cetuximab responses in patients with metastatic CRC, we
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53 analyzed a large collection of genomically annotated PDX models, for
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55 which information on response to cetuximab in mice was available¹⁸. Of
56
57 the 108 patient-derived xenografts (PDX) '*KRAS* wild-type' models
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2
3 originally collected (determined by Sanger sequencing), 63 samples were
4 identified to bear no somatic sequence alteration of the *KRAS*, *NRAS*,
5 *BRAF* and *PIK3CA* genes as identified by exome sequencing with an
6 average coverage within the target regions of nearly 150-fold for each
7 sample⁹. Protein levels were quantitatively profiled by Reverse Phase
8 Protein Array (RPPA) analysis of fresh-frozen pre-treatment tumour
9 samples derived from each PDX model (Figure 1A; Supplementary Table
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To explore whether cetuximab responses were related to differences in cell signalling pathways as evaluated by RPPA (phospho)protein analysis, we first performed unsupervised clustering using Nonnegative Matrix Factorizations (NMF) of the 63 quadruple negative samples (Supplementary Table 2). Clustering identified three distinct protein clusters termed C1, C2 and C3 (Figure 1A). We also performed clustering in all $n = 93$ *KRAS* wild type samples and found 88.9% consistency of the clusters (Supplementary Table 1).

Protein cluster C1 contained 35 PDX models of which 13 were regressing, 14 showed no change in volume, and 8 were progressing at week 3 (Figure 1B). Samples in C1 had predominantly high levels of phosphorylated Chk-1 (S345), c-RAF (S338), S6 Ribosomal Protein (S235/236 and S240/244), Gab-1 (Y627) and GSK-3 β (S9; Figure 1A and Supplementary Figure 1). In contrast, C1 samples had low levels of phosphorylated p38 MAPK (T180/Y182), AMPK (T172), FAK (Y925), Src (Y527), and Src (Y416). Furthermore, samples had low levels of SMAC, BCL(X) and STAT3 proteins.

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3 Cluster C2 contained 18 PDXs of which 4 were regressing, 10 showed no
4
5 change in volume, and 4 were progressing after cetuximab treatment
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7 (Figure 1B). C2 tissues were characterised by high levels of
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9 phosphorylated EGFR (Y1068), BCL2 (S70 and T56), Src (Y527), and
10
11 STAT3 (Y705) (Figure 1A and Supplementary Figure 1). Furthermore, the
12
13 cluster had low p27 and PTEN levels. This cluster was also characterised
14
15 by low levels of phosphorylated GSK-3 β (S9), MAPK (T202/Y204) and
16
17 MEK1/2 (S217/221).
18
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21 Interestingly, cluster C3 contained no progressing tumour models, 6 with
22
23 no change in volume and 4 regressing PDX models (Figure 1B). C3
24
25 tissues had high levels of phosphorylated p38 MAPK (T180/Y182), AKT
26
27 (S473), MEK1/2 (S217/221), MAPK (T202/Y204) and PDK1 (S241),
28
29 together with high levels of p70 S6 Kinase and p27 protein levels (Figure
30
31 1A and Supplementary Figure 1). Compared to clusters C1 and C2, C3
32
33 showed low IGF1-R β , PARP, cIAP-1, APAF-1 and EGFR protein levels,
34
35 together with low levels of cleaved caspase 9 (D330).
36
37

38
39 There was no difference in genetic alterations between the clusters (not
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41 shown). Overall, *TP53* mutations were found in 90% (n = 57; from 89% in
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43 C1 to 94% in C2), *APC* mutations in 89% (n = 56; from 89% in C1 to 90%
44
45 in C3) and *TTN* mutations in 48% (n = 30; from 40% in C1 to 70% in C3)
46
47 of PDX models (genetic data from Bertotti *et al.*⁹). Further, we did not find
48
49 protein clusters to be significantly associated with a specific CRIS
50
51 molecular subtype (Figure 1C). C1 consisted of 4 CRIS-A, 7 CRIS-B, 16
52
53 CRIS- C, 5 CRIS-D and 3 CRIS- E. C2 consisted of 2 CRIS-A, 3 CRIS-B,
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55 16 CRIS-C, 3 CRIS-D and 4 CRIS-E. C3 consisted of zero CRIS-A, 1
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3 CRIS-B, 7 CRIS-C, 1 CRIS-D and 1 CRIS-E. Likely due to the small size
4
5 of the tested collection, we did not find significant differences in response
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7 relative to the CRIS subtypes (Fisher's exact $p = 0.49$; Figure 1D).
8
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10 11 **Identification of a (phospho)protein signature predicting** 12 13 **responses to cetuximab** 14 15

16
17 In a subsequent analysis we used a statistical method for class prediction
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19 from gene expression data using nearest shrunken centroids (prediction
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21 analysis for microarrays; PAM)²⁸ to determine to what extent proteins were
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23 either up- or down-regulated in all PDX models when grouped according to
24
25 their response to cetuximab at week 3 (Figure 2; Supplementary Table 3).
26
27 Overall, proteins levels were found to be inverted when comparing
28
29 regressing models with progressing models. Progressing tumour models
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31 had high levels of phosphorylated EGFR (Y1173 and Y1068), AKT (S373),
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33 S6 ribosomal protein (S235/236 and S240/244), HER3 (Y1289), cRAF
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35 (S338), Gab-1 (Y627) and BCL2 (T56), together with high protein levels of
36
37 cIAP-1, IGF1-R β , PARP, BAK, BAX, EGFR and APAF-1 compared to
38
39 regressing models. In contrast, levels of phosphorylated PDK1 (S241),
40
41 Shc (Y317), STAT3 (Y705), FAK (Y925), phosphorylated GSK-3 β (S9),
42
43 Src (Y416), MAPK (T202/Y204), NF- κ B-p65 (S536), Caspase-8, p27, Src,
44
45 Xiap and SMAC were low in progressing compared to regressing models.
46
47 When comparing responses at week 6, we observed high levels of AKT
48
49 (S473), HIAP-2 and PARP, and low p27 levels in progressing compared to
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51 regressing models (Supplementary Figure 2).
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Refinement of a (phospho)protein response score

As a next step, we aimed to further reduce the number of proteins required for a predictive (phospho)protein signature. For this purpose we employed least absolute shrinkage and selection operator (LASSO; L1 regularization) and binominal logistic regression (progression *versus* regression) to identify the variables strongest associated with treatment response from the markers identified above. The advantage of LASSO is that the method exploits sparsity by shrinking less important features' coefficients to zero. Using only progressing ($n = 12$) or regressing ($n = 22$) PDX models, LASSO reduced the required proteins to 22 markers (Figure 3AB): PDK1 (S241; $\beta = 2.4687$), Caspase-8 ($\beta = 2.3486$), Shc (Y317; $\beta = 0.2415$), Stat3 (Y705; $\beta = 1.4916$), p27 ($\beta = 1.5234$), XIAP ($\beta = 0.2372$), GSK-3 β (S9; $\beta = 1.3425$), PI3-Kinase p110 α ($\beta = 0.4648$), HER3 ($\beta = 0.2071$), cleaved Caspase-9 (D330; $\beta = 0.0043$), MAPK - ERK 1/2 ($\beta = 0.2350$) and PKC-alpha (S657; $\beta = 0.9340$) were found with a positive coefficient (Figure 3B). BAK ($\beta = -1.6263$), EGFR (Y1068; $\beta = -0.1290$), Akt (S473; $\beta = -2.5973$), S6 Ribosomal Protein (S240/244; $\beta = -1.6658$), HER3 (Y1289; $\beta = -1.9349$), mTOR ($\beta = -1.600$), NF- κ B-p65 (S536; $\beta = -1.9424$), Gab-1 (Y627; $\beta = -1.5928$) and Bcl-2 (T56; $\beta = -0.5066$) were found with a negative coefficient (Figure 3B). The interception was 2.2000. To gain a deeper understanding of the role of these markers, we used the Spearman correlation coefficients (Figure 3A) to construct a co-expression network (Figure 3B). While proteins such as EGFR (Y1068) and NF- κ B-p65 (S536) had the same coefficient in the LASSO model and were co-expressed, Shc (Y317), GSK-3 β (S9), HER3, Caspase-8, PDK1 (S241),

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2
3 BAK and mTOR had disagreeing signs. Assuming that co-expressed
4 proteins fell in the same, active or respectively inactive, signalling pathway
5 and hence conducted a similar signal, the disagreement in the coefficients'
6 sign suggested a critical difference of the proteins' role in responses to
7 cetuximab.
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9

10
11 We then applied the regression model to the PDXs that showed no or only
12 minor changes in tumour volume (n = 30), in order to test whether the
13 model is able to define models with any increase in tumour volume as
14 "progressing" (n = 16) or "regressing" (n = 14). Although this is a
15 challenging task, the model identified 12 models as true "progressing"
16 (true positive), 9 as true "regressing" (true negative), 5 "regressing" as
17 "progressing" and 4 "progressing" as "regressing" models. Hence the
18 majority of marginally progressing or regressing PDXs were correctly
19 identified by the regression model.
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36 **Comparison of pre- and post-treatment protein profiles**

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38 In further exploratory analysis, we also investigated whether cetuximab
39 treatment altered protein levels during treatment. We randomly selected
40 15 PDX models, one from protein cluster C1, seven from cluster C2 and
41 seven from cluster C3. Protein quantification using RPPA were repeated
42 for pre- and post-treatment tumour tissues on a separate RPPA run. The
43 pre-treated PDX tissues had a mean correlation coefficient of 0.79 (25th -
44 75th percentile = 0.74 – 0.85) compared with the post-treated tissues
45 (Supplementary Figure 3). Pairwise comparison of pre- and post-treatment
46 samples showed that 6 out of 69 (phospho)proteins were significantly
47 altered by more (or less) than factor 2 (or ½) in response to cetuximab.
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3 Levels of phosphorylated Gab-1 (Y627; $p < 0.001$), MEK1/2 (S217/221; p
4 < 0.001), p70 S6 kinase (T389; $p < 0.001$) and GSK-3 β (S9; $p < 0.01$),
5
6 together with levels of MEK1 ($p < 0.001$), cleaved Caspase-7 (D198; $p <$
7
8 0.1) proteins, were significantly lower in post-treatment compared to pre-
9
10 treatment tissues (Figure 3C). The full list of changes in protein levels can
11
12 be seen in Supplementary Table 4.
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17 Levels of only 2 of the 6 proteins that were differential expressed were
18
19 prognostic for the response to cetuximab when measured prior to
20
21 treatment. Models not responding to cetuximab were more likely to lack
22
23 Gab-1 (Y627) and GSK-3 β (S9; Figure 2). Abundance of MEK1/2
24
25 (S217/221) was characteristic for models of the protein cluster without
26
27 progressing tumours (C3, Supplementary Figure 1). Levels of p70 S6
28
29 kinase (T389; $p < 0.001$), MEK1 ($p < 0.001$) and cleaved Caspase-7
30
31 (D198; $p < 0.1$) were neither associated with a specific response to
32
33 cetuximab nor a protein cluster.
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39 **Proliferation rather than apoptosis systems score predicts** 40 **responses to cetuximab** 41 42 43

44
45 To determine whether apoptosis competence was a prognostic marker for
46
47 anti-EGFR therapy responses, we used protein levels of BCL-2, BCL-XL,
48
49 MCL-1, BAX, BAK, APAF1, SMAC, XIAP, PROCASPASE-3 and -9 in the
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51 63 PDX models as model inputs for two deterministic models of apoptosis
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53 competence, one describing the process of mitochondrial
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55 permeabilization, DR_MOMP¹⁶, and one the process of caspase activation
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57 downstream of mitochondrial permeabilization, APOPTO-CELL²⁶ (Figure
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3 4A). Both models were developed and validated by our group and
4
5 previously shown to be prognostic for survival of stage 2 and 3 CRC
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7 patients^{16, 17, 31}. DR_MOMP calculates the 'stress dose' of tumour cells
8
9 required to undergo mitochondrial permeabilisation, with low values
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11 indicating a high apoptosis competence¹⁶. For quantitative evaluation of
12
13 protein levels, cell lysates of the PDX models were normalized to lysates
14
15 of HeLa cells in which absolute protein levels were previously determined
16
17 by quantitative Western blotting using purified proteins^{16, 26}. The mean
18
19 levels of the proteins required as model inputs are shown in [Figure 4BC](#).
20
21 Employing DR_MOMP using the generated quantitative protein profiles,
22
23 we determined a mean 'stress dose' of 171.4 nM (SD 56.4 nM) across all
24
25 PDXs. PDXs with a 'stress dose' greater than the mean also had
26
27 significantly less cleaved caspase 9 (D330) compared to models with
28
29 'stress dose' less than the mean (t-test $p < 0.01$), confirming impaired
30
31 apoptosis in models with high DR_MOMP 'stress dose' values. However,
32
33 the DR_MOMP score did not correlate with cetuximab responses (ANOVA
34
35 $p = 0.6$; [Figure 4E](#)). The DR_MOMP apoptosis score was lowest in PDX
36
37 models in cluster C1 (mean = 152.9 nM) and, greatest in C3 (mean =
38
39 246.0 nM; ANOVA $p < 0.0001$, Tukey post-hoc $p \leq 0.02$; [Figure 4F](#)). There
40
41 were no significant differences in DR_MOMP apoptosis scores when
42
43 PDXs were grouped based on the CRIS subtypes (ANOVA $p = 0.6$; [Figure](#)
44
45 [4G](#)).
46
47 APOPTO-CELL predicts apoptosis susceptibility of cells by modelling
48
49 activation of executioner caspases and cleavage of their downstream
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51 substrates²⁶. Exceeding a threshold of 25% substrate cleavage within 300
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3 minutes served in previous studies as a surrogate for the competence of
4 cells to undergo executioner (caspase 3) activation, in line with previous
5 single-cell imaging findings^{26, 31}. APOPTO-CELL identified 24 PDX
6 samples with less than 25% predicted substrate cleavage and 36 models
7 with more than 25 % predicted substrate cleavage. However the predicted
8 substrate cleavage did not correlate with responses of the PDX models to
9 cetuximab (Fisher's exact $p = 0.89$; [Figure 4E](#)). Further, there was no
10 significant difference in the number of PDXs with substrate cleavage less
11 or greater than 25% between protein clusters C1-C3 (Fisher's exact $p =$
12 0.09) or CRIS subtypes (Fisher's exact $p = 0.85$; [Figures 4FG](#)).

13
14 We also questioned whether apoptosis signalling contributed to cetuximab
15 responses only in specific protein clusters/molecular subtypes. There was
16 no significant differences between DR_MOMP 'stress dose' scores and
17 treatment responses when PDX models broken down into the three protein
18 clusters C1, C2 and C3 (ANOVA interaction $p = 0.9$) or into the CRIS
19 subtypes (ANOVA interaction $p = 0.9$). Similarly, there was no significant
20 differences between the APOPTO-CELL class and treatment responses
21 after stratifying for the protein cluster or CRIS (not-adjusted Fisher's exact
22 $p > 0.12$). Collectively, these data suggest that BCL2-dependent
23 mitochondrial apoptosis and caspase-3 activation does not play a major
24 role in cetuximab responses.

25
26 Next, we calculated the individual proliferative capacity of each PDX using
27 an 11 gene signature index^{24, 25} using existing gene expression profiles¹³.
28 Numerically, proliferation indices were lowest in protein cluster C3, and
29 highest in C2. Statistical analysis revealed no significantly differences
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3 between protein clusters (ANOVA $p = 0.1$; [Figure 4H](#)). CRIS-D had
4 significant higher indices compared to the CRIS-B molecular subtype
5 (Tukey post-hoc $p = 0.02$) and C (Tukey post-hoc $p < 0.001$; ANOVA $p =$
6 0.001). Across all PDXs, the proliferation index gradually increased from
7 PDXs with regressing toward progressing responses to cetuximab
8 (ANOVA p -value of 0.01 , [Figure 4](#)
9
10 [J](#)). Progressing PDX models had higher proliferation indices compared to
11 stable (Tukey post-hoc $p = 0.01$ and 0.03) or regressing PDX models
12 (Tukey post-hoc $p = 0.001$ and 0.02) if adjusted for either CRIS (ANOVA p
13 $= 0.01$) or protein clusters (ANOVA $p = 0.02$). Collectively, these data
14 suggested that proliferation rather than apoptosis score is a key
15 determinant of cetuximab responses in 'quadruple negative' metastatic
16 CRC PDX models.

17
18
19 ~~We also condensed the cell death scores of DR_MOMP and APOPTO-~~
20 ~~CELL and the proliferation score to an overall growth score by classifying~~
21 ~~models with impaired apoptosis and high proliferation as high growth (n =~~
22 ~~19), models with impeccable apoptosis competency and low proliferation~~
23 ~~as low growth (n = 6), and all other models as intermediate growth (n = 35;~~
24 ~~Figure 3K). Growths score did not reflect response to cetuximab with the~~
25 ~~PDX models being equally likely to show progression or regression in~~
26 ~~response to cetuximab (Fisher's Exact $p = 0.18$; Figure 3L).~~

Development of an improved (phospho)protein response score

Because our previous protein analysis identified cell death markers (Figure 2 and 3B; BAK, BCL2, cleaved Caspase-9, XIAP, etc.) that indicated responses to cetuximab, we finally decided to repeat the LASSO analysis with the 22 proteins, but replaced the apoptosis-related markers (BAK, BCL-2 (T56), cleaved Caspase-9 (D330) and XIAP) with the normalised DR_MOMP score. In addition, we removed the protein markers for AKT, mTOR, MAPK-ERK1/2 and PI3-Kinase p110 α based on the assumption that these markers will likely not indicate the activation status of their respective signalling pathway. This enabled us to reduce the overall number of proteins analysed. The LASSO analysis set only the coefficient of DR_MOMP to zero: PDK1 (S241; β = 6.3505), Caspase-8 (β = 5.2772), Shc (Y317; β = 4.2598), Stat3 (Y705; β = 2.6455), p27 (β = 0.6169), GSK-3 β (S9; β = 6.0001), HER3 (β = 3.5702) and PKC-alpha (S657; β = 0.8191) were found with a positive coefficient. EGFR (Y1068; β = -1.065), Akt (S473; β = -5.5777), S6 Ribosomal Protein (S240/244; β = -4.3452), HER3 (Y1289; β = -5.4732), NF-kB-p65 (S536; β = -6.3106) and Gab-1 (Y627; β = -4.6551) were found with a negative coefficients. The interception was 4.9424. The coefficients were in line with the first LASSO model (Spearman's rank correlation ρ = 0.88, p < 0.0001). Testing the updated regression model (14 markers) on PDX models showing no or only minor changes in tumour volume (n = 30), showed a significant improvement compared with the initial score, with 13 PDX models identified as true "progressing" (true positive), 10 as true "regressing" (true

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3 negative), 4 “regressing” as “progressing” and 3 “progressing” as
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5 “regressing” models.
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8 9 **Discussion**

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12 The discovery of new prognostic biomarkers for cetuximab response is of
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14 crucial importance for improving efficiency, and efficacy, of the treatment
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16 of metastatic CRC. The genetic heterogeneity of metastatic CRC cancer
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18 makes it unlikely that one single protein will serve as a biomarker in all
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20 instances, and high throughput techniques such as RPPA may therefore
21
22 be helpful in identifying predictive biomarker sets. Statistical analysis of
23
24 our RPPA data showed significant correlation between levels of 20
25
26 (phospho)proteins with changes in tumour volume, as detected in PDX
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28 models. We identified markers indicating active signalling of the EGFR
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30 pathway such as EGFR (Y1068) itself and Akt (S473), Gab-1 (Y627), Shc
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32 (Y317), Stat3 (Y705) and PDK1 (S241) to significantly predict responses
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34 to cetuximab. Overall we found a high cross correlation between levels of
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36 these proteins markers across all samples, emphasising their potential to
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38 act as predictive biomarkers for cetuximab responses.
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45 Interestingly, we found that high levels of phosphorylated EGFR at
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47 Tyr1068 and Akt at Ser473 indicated tumour progression, whereas
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49 regressing tumours showed a lack of phosphorylated Shc at Tyr317 and
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51 Stat3 at Tyr705. Phosphorylation of EGFR on Tyr1068 (and Tyr1086)
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53 leads to activation of the MAPK cascade and AKT activation³². *Signal*
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55 *transducer and activator of transcription 3* (STAT3) and its phosphorylation
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57 are associated with cell growth and transformation³³. The scaffolding
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3 protein *Src homology and collagen domain protein* (Shc) directs the EGF
4 stimuli to pro-mitogenic, pro-survival and invasion signalling pathways in a
5 time-dependent manner³⁴. *Phosphoinositide Dependent Protein Kinase 1*
6 (PDK1) is a crucial enzyme in transducing signals to multiple effector
7 pathways including *phosphoinositide 3-kinase* (PI3K/AKT), *Ras/mitogen-*
8 *activated protein kinase* (MAPK), *serum/glucocorticoid regulated kinase*
9 (SGK), *p70 ribosomal protein S6 kinase* (p70 S6 K) and members of
10 *protein kinase C* (PKC) family. Phosphorylation of PDK1 on Ser241 is
11 necessary for its activation³⁵. Some of its substrates require a prior
12 conformational switch to allow subsequent phosphorylation by PDK1³⁵
13 rendering it as gatekeeper for those signalling pathways. We also found
14 that models expressing the *human epidermal growth factor receptor 3*
15 (HER3, also EGFR3) were more likely to respond with tumour regression
16 in response to cetuximab. In contrast, phosphorylation of HER3 on
17 Tyr1289 was indicative for tumour progression. HER3 cannot be activated
18 by ligand alone but its heterodimer with EGFR and HER2 is highly
19 mitogenic³⁶. Existing literature on the expression and relevance of *HER3* is
20 inconsistent, reporting association with either increased or decreased
21 survival of CRC patients³⁶. In advanced non-small cell lung cancer,
22 abundant *HER3* expression identifies gefitinib (EGFR inhibitor) sensitive
23 cell lines³⁷. In addition, Bosch-Vilaró *et al.*³⁸ described a cetuximab-
24 induced feedback *HER3* activation that reduces the response to
25 cetuximab, and in pancreatic cancer, dimerization of EGFR and *HER3* was
26 reported to be necessary for downstream signalling³⁹.
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3 Further LASSO and binominal logistic regression analysis of these protein
4 biomarkers delivered a refined protein signatures for predicting responses
5 to cetuximab. Given that many of the identified markers in our signature
6 are predicted to regulate cell proliferation, we also investigated a
7 previously published, transcriptome-based proliferation score as to its
8 predictive power^{24, 25}. Using this score, we also found a significant
9 correlation between cetuximab responses and the transcriptome-based
10 proliferation score across all 63 PDX models investigated. Although the
11 focus of our study was the delivery of a (phospho)protein signature,
12 combining our protein score with the transcriptome-based proliferation
13 score did not further increase the predictive power of the protein
14 signature, suggesting that the signature was sufficient to describe the
15 proliferation status of the PDX models in relation to cetuximab responses.
16
17 We also found that responses to cetuximab were dependent on protein
18 clusters identified through unsupervised cluster analysis. One of the
19 clusters, protein cluster 3 (C3), represented a cluster without progressing
20 PDX models. C3 was characterised by PDK1-dependend active AKT
21 signalling and inhibition of the cell cycle. The largest protein cluster (C1) in
22 contrast showed mixed responses, and was characterised by genotoxic
23 stress, inflammation and cell survival signalling. Cluster C2 was also
24 composed of mixed responders and characterised by active EGFR
25 signalling and inhibition of apoptosis. Compared to PDX models in C1 and
26 C2, PDX models in C3 had lower levels of phosphorylated MEK1/2
27 (S217/221). This suggests that cetuximab-resistant models in C1 and C2
28 may potentially benefit from MEK inhibitors. We also explored the
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3 relationship between protein clusters and transcriptome-based molecular
4 subtypes. CRIS molecular subtypes capture very well differences in
5 intrinsic tumour cell gene expression¹³. CRIS-C was previously associated
6 with sensitivity to cetuximab¹³, potentially a consequence of the lower
7 representation of *KRAS* and *NRAS* mutations in this subtype¹³. We did not
8 find that any of the three protein clusters showed a significant association
9 with CRIS molecular subtypes. We also found that, when focusing on
10 *KRAS*, *NRAS*, *BRAF* and *PIK3CA* quadruple wild type models, CRIS-C
11 was not enriched in cetuximab responders (Figure 1D). Overall, this
12 suggests that sensitivity to anti-EGFR therapy is predicted well by an
13 analysis of (phospho)protein clusters.
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17 While we observed that increased proliferative capacity was associated
18 with disease progression during cetuximab treatment (Figure 4J),
19 competence to undergo mitochondrial apoptosis was not a major
20 determinant of cetuximab responses. Both the DR_MOMP and APOPTO-
21 CELL apoptosis models have been shown to be prognostic for stage II and
22 III CRC patients, but have not yet not been tested in the setting of
23 metastatic CRC^{17, 31}.
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27 Our data suggest that resistance to mitochondrial apoptosis is not critical
28 for responses of metastatic CRC to cetuximab. While cetuximab was
29 shown to induce apoptosis to a minor extent in colorectal cancer cells in
30 previous studies⁴⁰, combination therapy for example with regorafenib has
31 been shown to be required for significant apoptosis induction by
32 cetuximab⁴¹. In the setting of colorectal cancer, we have previously also
33 shown that activation of Caspase-3 may be associated with a
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3 compensatory stimulation of cancer cell proliferation and adverse effects
4 on clinical outcome⁴². Here, we also observed that PDX models with
5 progressing tumours tended to have higher levels cleaved Caspase-3
6 compared to models with stable or regressing tumours (Figure 2). It might
7 be possible that activating apoptosis may have both beneficial and
8 detrimental effects in the setting of metastatic CRC.
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17 By comparing matched pre- and post-treatment samples, we also found
18 that levels of GSK-3 β (S9) were reduced in tissue after cetuximab
19 treatment. The *Glycogen synthase kinase 3 β* (GSK-3 β) is a key player in
20 the β -catenin/Wnt signalling pathway but also phosphorylates various
21 transcription factors and structural, metabolic and signalling proteins^{43, 44}.
22 Inhibition of GSK-3 β activity by phosphorylation at Ser9⁴⁵ is a critical factor
23 to allow many coupled signalling pathways to proceed^{43, 44}. 96% of CRCs
24 harbour increased oncogenic Wnt pathway alteration⁴⁶ and dysregulation
25 of GSK-3 β signalling is associated with cancer and metabolic and
26 degenerative disorders⁴⁷. Inhibition of GSK-3 β was reported to induce
27 apoptosis and attenuated proliferation in colon cancer cells *in vitro*⁴⁸ and in
28 colon cancer xenografts⁴⁹. It is possible that inhibition of GSK-3 β would be
29 desirable co-treatment with cetuximab. Lithium, which also acts as an
30 inhibitor of GSK-3 β ⁵⁰, was reported to suppress cell proliferation in prostate
31 cancer xenografts⁵¹ and may inhibit colon cancer metastasis by blocking
32 *transforming growth factor- β -induced* protein (TGFB1p) expression⁵²
33 downstream of GSK-3⁵³. Combining cetuximab with lithium or other GSK-
34 3 β inhibitors may improve response to cetuximab.
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3 In conclusion, we present here a 14 (phospho)protein marker signature
4 that was predicting responses to cetuximab in mCRC tissue. Likewise, our
5 findings emphasises GSK-3 β to be potentially targetable for a co-
6 treatment with cetuximab.
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13 Further Disclosures

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16 **Ethics approval and consent to participate:** Informed consent for
17 research use was obtained from all patients and the study was conducted
18 under the approval of the RCSI Research Ethics Committee and *Comitato*
19 *Etico Istituto di Candiolo-FPO IRCCS*. All animal procedures were
20 approved by the Ethical Commission of the Candiolo Cancer Institute and
21 by the Italian Ministry of Health (806/2016-PR).
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30 **Data Accessibility:** Data is provided as supplementary materials.
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32 Extended data and scripts will be made available upon reasonable
33 request.
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37 **Conflict of interest:** The authors declare no conflict of interest.

38
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3 **Authors' contributions:** ABa, AUL, SC, NM, MS and JHMP wrote the
4 manuscript. ABa, AUL, SC, NM and MS performed data analysis and
5 prepared figures. ABe and ERZ performed acquisition of sample data.
6 BTH, ERZ, ROB, SC and MC collected samples and conducted the protein
7 quantification using RPPA. ABe, BTH, LT and JHMP supervised the
8 project. All authors read, reviewed and approved the final manuscript for
9 publication.
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For Peer Review

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Figure Legends

Figure 1

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21 (A) Heatmap of protein levels determined by RPPA. PDX models were annotated
22 with the, CRIS, the consensus protein cluster subtype, and response to cetuximab
23 (top). Clustering was performed using Nonnegative Matrix Factorization (NMF)
24 consensus clustering algorithm. The right annotations indicates proteins'
25 association to the protein clusters (Supplementary Figure 1). Chord diagrams show
26 overlap between RPPA clusters and (B) response to cetuximab and (C) CRIS, and
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(D) overlap between CRIS and response to cetuximab.

Figure 2

Protein scores indicating proteins' association to the PDX models' response to
cetuximab. Proteins' scores for response to cetuximab after 3 week was calculated
using PAM²⁷.

Figure 3

(A) Heatmap of Spearman's rank correlation coefficients for proteins associated
with differences in response to cetuximab from Figure 2. (B) Undirected graph of
proteins found to be relevant in LASSO analysis. Intensity and colour of the edges
indicate the correlation coefficient of (A). Grouping based on the signs of the

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3 correlation coefficients and signs of the coefficients found by LASSO are indicated
4 black & white nodes and plus & minus icons, respectively. (C) Protein found to be
5 differential expressed in PDX models after treatment with cetuximab, based on
6 pairwise comparison and Benjamin & Hochberg adjusted p-value. Dashed red lines
7 indicate 0.05 significance threshold for p-value, and 2-fold or 1/2-fold protein level.
8 The protein marker names and n-fold differences (treated to un-treated) in brackets
9 were added for proteins passing all thresholds.
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20 Figure 4

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23 (A) Simplified illustration of the apoptotic signalling modelled in DR_MOMP and
24 APOPTO-CELL. Absolute protein levels normalised to HeLa cells were measured
25 using RPPA and used as input for (B) DR_MOMP and (C) APOPTO-CELL.
26 Calculated DR MOMP values against (D) APOPTO-CELLs' calculated substrate
27 cleavage class with (E) differences in response to cetuximab, (F) RPPA protein
28 cluster C3 and (G) CRIS. Calculated proliferation against (H) protein clusters, (I)
29 CRIS and (J) response to cetuximab. (K) ~~The proliferation score was combined with~~
30 ~~both models to a tumour growth score. (L) n-numbers of tumour growth score~~
31 ~~classes against response to cetuximab, RPPA protein cluster and CRIS.~~
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Systems analysis of protein signatures predicting Cetuximab responses in *KRAS*, *NRAS*, *BRAF* and *PIK3CA* wild-type patient-derived xenografts models of metastatic colorectal cancer

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36 **Running title:** Systems analysis of cetuximab responses

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38 **Novelty and Impact:** A large fraction of patients with metastatic colorectal cancer do
39 not respond to anti-EGFR therapy despite *KRAS* wild type tumours. Statistical
40 analysis of RPPA data of colorectal cancer *KRAS*, *BRAF*, *NRAS* and *PI3KCA* wild
41 type PDX models revealed a 14 - 20 (phospho)protein signature that was predicting
42 responses to cetuximab. Our findings furthermore emphasise GSK-3 β to be
43 potentially targetable for a co-treatment with cetuximab.
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47 **Keywords:** anti-EGFR, metastatic colorectal cancer, molecular subtyping, reverse-
48 phase protein array, deterministic modelling, apoptosis, proliferation
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51 **Abbreviations:** 5-FU, fluorouracil; ANOVA, analysis of variance; CMS, consensus
52 molecular subtypes; CRC, colorectal cancer; CRIS, CRC intrinsic subtype; EGF,
53 epidermal growth factor; EGFR, EGF receptor; LASSO, least absolute shrinkage and
54 selection operator; NMF, non-negative matrix factorization; P, p-value; PAM,
55 Prediction Analysis for Microarrays; PDX, patient-derived mouse xenograft; RPPA,
56 reverse phase protein array; SC, substrate cleavage.
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Abstract

Antibodies targeting the human epidermal growth factor receptor (*EGFR*) are used for the treatment of *RAS* wild-type metastatic colorectal cancer. A significant proportion of patients remains unresponsive to this therapy. Here, we performed a reverse phase protein array-based (phospho)protein analysis of 63 *KRAS*, *NRAS*, *BRAF* and *PIK3CA* wild-type metastatic CRC tumours. Responses of tumours to anti-EGFR therapy with cetuximab were recorded in patient-derived xenograft (PDX) models. Unsupervised hierarchical clustering of pre-treatment tumour tissue identified three clusters, of which cluster C3 was exclusively composed of responders. Clusters C1 and C2 showed mixed responses. None of the three protein clusters showed a significant correlation with transcriptome-based subtypes. Analysis of protein signatures across all PDXs identified 14 markers that discriminated cetuximab-sensitive and -resistant tumours: PDK1 (S241), Caspase-8, Shc (Y317), Stat3 (Y705), p27, GSK-3 β (S9), HER3, PKC- α (S657), EGFR (Y1068), Akt (S473), S6 Ribosomal Protein (S240/244), HER3 (Y1289), NF- κ B-p65 (S536) and Gab-1 (Y627). Least absolute shrinkage and selection operator and binomial logistic regression analysis delivered refined protein signatures for predicting response to cetuximab. (Phospho-)protein analysis of matched pre- and post-treated models furthermore showed significant reduction of Gab-1 (Y627) and GSK-3 β (S9) exclusively in responding models, suggesting novel targets for treatment.

Background

Colorectal cancer (CRC) is the third and second most commonly diagnosed cancer in males and females, and the second most common cause of cancer-related deaths in the developed world. In the advanced setting, CRC is routinely treated with fluorouracil (5-FU)-based chemotherapy. 30% of CRC patients present in the metastatic setting¹ where response rates to palliative 5-FU/oxaliplatin- or 5-FU/irinotecan-based chemotherapy range between 40-50%. Median overall survival remains poor at around 16-19 months². Identifying the importance of epidermal growth factor (*EGF*) signalling for the survival of CRC cells resulted in the development of targeted therapies that neutralize the oncogenic activity of EGF receptors (*EGFR*). Anti-EGFR therapies have significantly improved survival in metastatic CRC patients³. Guidelines recommend to test for *KRAS*, *NRAS* and *BRAF* mutations as well as microsatellite instability status in CRC patients being considered for anti-EGFR therapy^{4, 5} on the bases of the ineffectiveness of anti-EGFR therapy in patients with activating *KRAS*, *BRAF*, and *NRAS* mutations⁶, and favourable responses to immune check point inhibitors in microsatellite instability-high patients⁴. While *PI3KCA* mutational analysis is not recommended yet⁴, *PIK3CA* exon 20 mutations were linked with a worse outcome compared with wild-type status in patients with metastatic colorectal cancer⁷. Nevertheless, between 50–60% of patients will not benefit from anti-EGFR treatment even when these are *KRAS*, *BRAF*, *NRAS* and *PI3KCA* wild type⁷.

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3 Mutations and copy number alterations in genes encoding for other
4 survival signaling proteins have been shown to contribute to anti-EGFR
5 resistance. For example, *HER2*-amplification, *IGF2* overexpression or
6 increased MET activity resulted in reduced responses to anti-EGFR
7 therapy, as demonstrated in patient-derived xenograft (PDX) models of
8 metastatic CRC and in patients^{8, 9}. Analysis of the genomic and
9 transcriptomic landscape of anti-EGFR resistance in PDX models and
10 patients furthermore identified mutations in *EGFR*, *FGFR1*, *PDGFRA*, and
11 *MAP2K1* or loss of *NF1* to contribute to anti-EGF resistance^{9, 10}.

12 While identification of patient-specific genome alterations provides a
13 personalised diagnosis that provides insights into anti-EGFR therapy
14 responses and may open opportunities for personalised therapies,
15 interpretation of often multiple genomic alterations found in most patients
16 is not always straightforward. Other efforts to identify responders and non-
17 responders to anti-EGFR therapy have therefore focussed on the power of
18 unsupervised molecular subtyping of tumours. An international meta-
19 analysis and bioinformatics effort led to the identification of four distinct
20 subtypes in CRC, termed 'Consensus Molecular Subtypes' (CMS1-
21 CMS4)¹¹. A recent study demonstrated that CMS2 patients benefitted
22 more from anti-EGFR therapy than patients treated with anti-angiogenic
23 therapy, while the opposite was the case in CMS1 patients¹². However
24 predictions of anti-EGFR therapy responses in CMS3 and CMS4 patients
25 were not possible, and significant variability in overall and progression free
26 survival are still seen across all four CMS subtypes. Because stroma-
27 derived mRNAs in whole tumour transcriptomes may obscure
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3 transcriptional features displayed by cancer cells, other efforts leveraged
4 the power of patient-derived mouse xenograft (PDX) models in which
5 human stroma is replaced by mouse stroma to obtain five CRC 'intrinsic'
6 (CRIS) molecular subtypes, termed CRIS-A to E¹³. CRIS-C was identified
7 as a subtype associated with EGFR signalling and increased sensitivity to
8 anti-EGFR therapy. However responses to anti-EGFR therapy strongly
9 varied among the other four CRIS subtypes¹³.

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EGFR activation results in the activation of several downstream signalling
pathways, including the PI3K/AKT and MAPK pathways¹⁴. The activation
status of these key signalling pathways influences a variety of biological
processes such as proliferation, apoptosis, cell migration, bioenergetics,
immune responses, and angiogenesis. A different approach to investigate
responses to anti-EGFR therapy is to determine the activation status of
key signalling branches activated by EGFR receptors and their
downstream effectors, supported by statistical or deterministic modelling¹⁵.
Because processes such as proliferation and apoptosis are controlled by
complex networks that show significant signalling redundancies,
deterministic systems models have been developed to estimate more
precisely proliferative capacity or apoptosis sensitivity of tumours. One
such tool developed by our group is the systems model, DR_MOMP,
which calculates the apoptosis sensitivity of tumours based on a
quantitative analysis of BCL-2 family proteins and their interactions^{16, 17}.
To identify novel prognostic markers of anti-EGFR therapy, we here
comprehensively profiled 83 signalling proteins and (phospho)proteins
related to EGFR and key cancer signalling pathways in a cohort of 63

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3 *KRAS*, *BRAF*, *NRAS* and *PI3KCA* wild type PDX models isolated from
4 liver biopsies that were derived from metastatic CRC patients^{9, 18}. We
5 performed both statistical and systems modelling analyses to identify novel
6 protein signatures of anti-EGFR responsiveness.
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13 **Methods**

14 **CRC PDX in vivo model**

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17 108 PDX models derived from colorectal cancer liver metastasis originally
18 at the Institute for Cancer Research and Treatment, and Mauriziano
19 Umberto I (Torino, Italy)¹⁸ were used in this study. 63 of 108 were *KRAS*,
20 *BRAF*, *NRAS* and *PI3KCA* wild type based on matched next-generation
21 sequencing analysis data from Bertotti *et al.*⁹ and used for statistical
22 analysis. Tumour tissues were implanted subcutaneously and passaged in
23 *NOD/SCID* mice. Response data is available for each tumour to cetuximab
24 treatment after 3 and 6 weeks¹⁹.
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40 **Reverse phase protein array**

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42 Protein was extracted from PDX tumour tissue and cell line standards and
43 RPPA was performed as described previously²⁰. Protein lysates
44 normalized to 1µg/µL concentration as assessed by bicinchoninic acid
45 assay (BCA, Biorad). Reverse phase protein array (RPPA) with a panel of
46 antibodies targeting various key cancer related proteins was used for
47 measuring protein levels in untreated tumours. The response is from
48 matching samples of same tumour in different mice. The DAKO
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(Carpinteria, CA) catalyzed signal amplification system was used for antibody blotting.

PDX Protein clustering

RPPA data for 93 PDX samples have been clustered using consensus Non-negative Matrix Factorization (R package 'NMF'²¹, version 0.21.0) on centred RPPA data^{22, 23}. NMF was performed 1 000 times with the number of clusters k varying from 2 to 8. $k = 3$ was selected based on visual inspection of co-clustering matrices and heatmap of clustered RPPA data.

To represent graphically the correspondence between CRIS subtypes classifiers and the RPPA clusters or cetuximab response, Factorial Correspondence Analysis (FCA) was used. For each comparison, χ^2 independence test was carried out. In order to have large enough numbers in the contingency table so that the χ^2 approximation is correct, we combined together the closest CRIS subtypes.

DR MOMP, APOPTO-CELL and proliferation signature

The normalised gene expression of *BIRC5*, *CCNB1*, *CDC20*, *CDCA1*, *CEP55*, *NDC80*, *MKI67*, *PTTG1*, *RRM2*, *TYMS* and *UBE2C* was averaged and used as proliferation signature^{24, 25} of each PDX. The gene expression data for respective PDX models was downloaded from GSE76402¹³.

To calculate the sensitivity of patients' cancer cells to undergo apoptosis, the mathematical models APOPTO-CELL²⁶ and DR_MOMP¹⁶ were applied, using PRO-CASPASE-3, PRO-CASPASE-9, SMAC, and XIAP protein for APOPTO-CELL, and BAK, BAX, BCL2 and BCL(X)L for DR_MOMP as input for the models. MCL1 protein levels were assumed to

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3 be 0 nM for DR_MOMP. SMAC concentrations were assumed to be 122.7
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5 nM for APOPTO-CELL²⁶. Protein levels were normalized to HeLa cells that
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7 were placed on the RPPA together with the cancer tissue^{16, 26}.
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10 **Statistical analysis**

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14 Statistical analysis of RPPA data was done using 'SAMR'²⁷ (Significance
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16 Analysis of Microarrays, version 3.0) and 'PAMR'²⁸ (Prediction Analysis for
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18 Microarrays; version 1.56.1) R Packages (R version 3.6.2). LASSO was
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20 performed using the 'glmnet' R package (version 2.0-18). The packages
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22 'ComplexHeatmap'²⁹ (version 2.1.0) and 'Circlize'³⁰ (version 0.4.7) were
23
24 used to create Figure 1. Week 3 response was used for all the statistical
25
26 analysis as not all the mice were followed through after 3 weeks. Student's
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28 t-test and ANOVA was used for measuring statistical significance. ANOVA
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30 was followed by Tukey's HSD (honest significant difference) test for
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32 multiple pair comparison. Fisher's exact test was used for count data.
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38 **Results**

39 40 41 42 **Characterisation of *KRAS*, *BRAF*, *NRAS* and *PI3KCA* wild** 43 44 **type metastatic CRC (phospho)protein signatures**

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47 To investigate cetuximab responses in patients with metastatic CRC, we
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49 analyzed a large collection of genomically annotated PDX models, for
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51 which information on response to cetuximab in mice was available¹⁸. Of
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53 the 108 patient-derived xenografts (PDX) '*KRAS* wild-type' models
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55 originally collected (determined by Sanger sequencing), 63 samples were
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57 identified to bear no somatic sequence alteration of the *KRAS*, *NRAS*,
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3 *BRAF* and *PIK3CA* genes as identified by exome sequencing with an
4 average coverage within the target regions of nearly 150-fold for each
5 sample ⁹. Protein levels were quantitatively profiled by Reverse Phase
6 Protein Array (RPPA) analysis of fresh-frozen pre-treatment tumour
7 samples derived from each PDX model (Figure 1A; Supplementary Table
8 1).

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17 To explore whether cetuximab responses were related to differences in
18 cell signalling pathways as evaluated by RPPA (phospho)protein analysis,
19 we first performed unsupervised clustering using Nonnegative Matrix
20 Factorizations (NMF) of the 63 quadruple negative samples
21 (Supplementary Table 2). Clustering identified three distinct protein
22 clusters termed C1, C2 and C3 (Figure 1A). We also performed clustering
23 in all n = 93 *KRAS* wild type samples and found 88.9% consistency of the
24 clusters (Supplementary Table 1).

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35 Protein cluster C1 contained 35 PDX models of which 13 were regressing,
36 14 showed no change in volume, and 8 were progressing at week 3
37 (Figure 1B). Samples in C1 had predominantly high levels of
38 phosphorylated Chk-1 (S345), c-RAF (S338), S6 Ribosomal Protein
39 (S235/236 and S240/244), Gab-1 (Y627) and GSK-3 β (S9; Figure 1A and
40 Supplementary Figure 1). In contrast, C1 samples had low levels of
41 phosphorylated p38 MAPK (T180/Y182), AMPK (T172), FAK (Y925), Src
42 (Y527), and Src (Y416). Furthermore, samples had low levels of SMAC,
43 BCL(X) and STAT3 proteins.

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56 Cluster C2 contained 18 PDXs of which 4 were regressing, 10 showed no
57 change in volume, and 4 were progressing after cetuximab treatment
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3 (Figure 1B). C2 tissues were characterised by high levels of
4 phosphorylated EGFR (Y1068), BCL2 (S70 and T56), Src (Y527), and
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6 STAT3 (Y705) (Figure 1A and Supplementary Figure 1). Furthermore, the
7
8 cluster had low p27 and PTEN levels. This cluster was also characterised
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10 by low levels of phosphorylated GSK-3 β (S9), MAPK (T202/Y204) and
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12 MEK1/2 (S217/221).
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17 Interestingly, cluster C3 contained no progressing tumour models, 6 with
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19 no change in volume and 4 regressing PDX models (Figure 1B). C3
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21 tissues had high levels of phosphorylated p38 MAPK (T180/Y182), AKT
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23 (S473), MEK1/2 (S217/221), MAPK (T202/Y204) and PDK1 (S241),
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25 together with high levels of p70 S6 Kinase and p27 protein levels (Figure
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27 1A and Supplementary Figure 1). Compared to clusters C1 and C2, C3
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29 showed low IGF1-R β , PARP, cIAP-1, APAF-1 and EGFR protein levels,
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31 together with low levels of cleaved caspase 9 (D330).
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36 There was no difference in genetic alterations between the clusters (not
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38 shown). Overall, *TP53* mutations were found in 90% (n = 57; from 89% in
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40 C1 to 94% in C2), *APC* mutations in 89% (n = 56; from 89% in C1 to 90%
41
42 in C3) and *TTN* mutations in 48% (n = 30; from 40% in C1 to 70% in C3)
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44 of PDX models (genetic data from Bertotti *et al.*⁹). Further, we did not find
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46 protein clusters to be significantly associated with a specific CRIS
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48 molecular subtype (Figure 1C). C1 consisted of 4 CRIS-A, 7 CRIS-B, 16
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50 CRIS- C, 5 CRIS-D and 3 CRIS- E. C2 consisted of 2 CRIS-A, 3 CRIS-B,
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52 16 CRIS-C, 3 CRIS-D and 4 CRIS-E. C3 consisted of zero CRIS-A, 1
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54 CRIS-B, 7 CRIS-C, 1 CRIS-D and 1 CRIS-E. Likely due to the small size
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3 of the tested collection, we did not find significant differences in response
4 relative to the CRIS subtypes (Fisher's exact $p = 0.49$; Figure 1D).
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8 **Identification of a (phospho)protein signature predicting** 9 **responses to cetuximab** 10 11 12

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14 In a subsequent analysis we used a statistical method for class prediction
15 from gene expression data using nearest shrunken centroids (prediction
16 analysis for microarrays; PAM)²⁸ to determine to what extent proteins were
17 either up- or down-regulated in all PDX models when grouped according to
18 their response to cetuximab at week 3 (Figure 2; Supplementary Table 3).
19 Overall, proteins levels were found to be inverted when comparing
20 regressing models with progressing models. Progressing tumour models
21 had high levels of phosphorylated EGFR (Y1173 and Y1068), AKT (S373),
22 S6 ribosomal protein (S235/236 and S240/244), HER3 (Y1289), cRAF
23 (S338), Gab-1 (Y627) and BCL2 (T56), together with high protein levels of
24 cIAP-1, IGF1-R β , PARP, BAK, BAX, EGFR and APAF-1 compared to
25 regressing models. In contrast, levels of phosphorylated PDK1 (S241),
26 Shc (Y317), STAT3 (Y705), FAK (Y925), phosphorylated GSK-3 β (S9),
27 Src (Y416), MAPK (T202/Y204), NF- κ B-p65 (S536), Caspase-8, p27, Src,
28 Xiap and SMAC were low in progressing compared to regressing models.
29 When comparing responses at week 6, we observed high levels of AKT
30 (S473), HIAP-2 and PARP, and low p27 levels in progressing compared to
31 regressing models (Supplementary Figure 2).
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Refinement of a (phospho)protein response score

As a next step, we aimed to further reduce the number of proteins required for a predictive (phospho)protein signature. For this purpose we employed least absolute shrinkage and selection operator (LASSO; L1 regularization) and binominal logistic regression (progression *versus* regression) to identify the variables strongest associated with treatment response from the markers identified above. The advantage of LASSO is that the method exploits sparsity by shrinking less important features' coefficients to zero. Using only progressing (n = 12) or regressing (n = 22) PDX models, LASSO reduced the required proteins to 22 markers (Figure 3AB): PDK1 (S241; $\beta = 2.4687$), Caspase-8 ($\beta = 2.3486$), Shc (Y317; $\beta = 0.2415$), Stat3 (Y705; $\beta = 1.4916$), p27 ($\beta = 1.5234$), XIAP ($\beta = 0.2372$), GSK-3 β (S9; $\beta = 1.3425$), PI3-Kinase p110 α ($\beta = 0.4648$), HER3 ($\beta = 0.2071$), cleaved Caspase-9 (D330; $\beta = 0.0043$), MAPK - ERK 1/2 ($\beta = 0.2350$) and PKC-alpha (S657; $\beta = 0.9340$) were found with a positive coefficient (Figure 3B). BAK ($\beta = -1.6263$), EGFR (Y1068; $\beta = -0.1290$), Akt (S473; $\beta = -2.5973$), S6 Ribosomal Protein (S240/244; $\beta = -1.6658$), HER3 (Y1289; $\beta = -1.9349$), mTOR ($\beta = -1.600$), NF- κ B-p65 (S536; $\beta = -1.9424$), Gab-1 (Y627; $\beta = -1.5928$) and Bcl-2 (T56; $\beta = -0.5066$) were found with a negative coefficient (Figure 3B). The interception was 2.2000. To gain a deeper understanding of the role of these markers, we used the Spearman correlation coefficients (Figure 3A) to construct a co-expression network (Figure 3B). While proteins such as EGFR (Y1068) and NF- κ B-p65 (S536) had the same coefficient in the LASSO model and were co-expressed, Shc (Y317), GSK-3 β (S9), HER3, Caspase-8, PDK1 (S241),

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3 BAK and mTOR had disagreeing signs. Assuming that co-expressed
4 proteins fell in the same, active or respectively inactive, signalling pathway
5 and hence conducted a similar signal, the disagreement in the coefficients'
6 sign suggested a critical difference of the proteins' role in responses to
7 cetuximab.
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10 We then applied the regression model to the PDXs that showed no or only
11 minor changes in tumour volume (n = 30), in order to test whether the
12 model is able to define models with any increase in tumour volume as
13 "progressing" (n = 16) or "regressing" (n = 14). Although this is a
14 challenging task, the model identified 12 models as true "progressing"
15 (true positive), 9 as true "regressing" (true negative), 5 "regressing" as
16 "progressing" and 4 "progressing" as "regressing" models. Hence the
17 majority of marginally progressing or regressing PDXs were correctly
18 identified by the regression model.
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36 **Comparison of pre- and post-treatment protein profiles**

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39 In further exploratory analysis, we also investigated whether cetuximab
40 treatment altered protein levels during treatment. We randomly selected
41 15 PDX models, one from protein cluster C1, seven from cluster C2 and
42 seven from cluster C3. Protein quantification using RPPA were repeated
43 for pre- and post-treatment tumour tissues on a separate RPPA run. The
44 pre-treated PDX tissues had a mean correlation coefficient of 0.79 (25th -
45 75th percentile = 0.74 – 0.85) compared with the post-treated tissues
46 (Supplementary Figure 3). Pairwise comparison of pre- and post-treatment
47 samples showed that 6 out of 69 (phospho)proteins were significantly
48 altered by more (or less) than factor 2 (or ½) in response to cetuximab.
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3 Levels of phosphorylated Gab-1 (Y627; $p < 0.001$), MEK1/2 (S217/221; p
4 < 0.001), p70 S6 kinase (T389; $p < 0.001$) and GSK-3 β (S9; $p < 0.01$),
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6 together with levels of MEK1 ($p < 0.001$), cleaved Caspase-7 (D198; $p <$
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8 0.1) proteins, were significantly lower in post-treatment compared to pre-
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10 treatment tissues (Figure 3C). The full list of changes in protein levels can
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12 be seen in Supplementary Table 4.
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17 Levels of only 2 of the 6 proteins that were differential expressed were
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19 prognostic for the response to cetuximab when measured prior to
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21 treatment. Models not responding to cetuximab were more likely to lack
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23 Gab-1 (Y627) and GSK-3 β (S9; Figure 2). Abundance of MEK1/2
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25 (S217/221) was characteristic for models of the protein cluster without
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27 progressing tumours (C3, Supplementary Figure 1). Levels of p70 S6
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29 kinase (T389; $p < 0.001$), MEK1 ($p < 0.001$) and cleaved Caspase-7
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31 (D198; $p < 0.1$) were neither associated with a specific response to
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33 cetuximab nor a protein cluster.
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39 **Proliferation rather than apoptosis systems score predicts** 40 **responses to cetuximab** 41 42 43

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45 To determine whether apoptosis competence was a prognostic marker for
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47 anti-EGFR therapy responses, we used protein levels of BCL-2, BCL-XL,
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49 MCL-1, BAX, BAK, APAF1, SMAC, XIAP, PROCASPASE-3 and -9 in the
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51 63 PDX models as model inputs for two deterministic models of apoptosis
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53 competence, one describing the process of mitochondrial
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55 permeabilization, DR_MOMP¹⁶, and one the process of caspase activation
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57 downstream of mitochondrial permeabilization, APOPTO-CELL²⁶ (Figure
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3 4A). Both models were developed and validated by our group and
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5 previously shown to be prognostic for survival of stage 2 and 3 CRC
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7 patients^{16, 17, 31}. DR_MOMP calculates the 'stress dose' of tumour cells
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9 required to undergo mitochondrial permeabilisation, with low values
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11 indicating a high apoptosis competence¹⁶. For quantitative evaluation of
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13 protein levels, cell lysates of the PDX models were normalized to lysates
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15 of HeLa cells in which absolute protein levels were previously determined
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17 by quantitative Western blotting using purified proteins^{16, 26}. The mean
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19 levels of the proteins required as model inputs are shown in Figure 4BC.
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21 Employing DR_MOMP using the generated quantitative protein profiles,
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23 we determined a mean 'stress dose' of 171.4 nM (SD 56.4 nM) across all
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25 PDXs. PDXs with a 'stress dose' greater than the mean also had
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27 significantly less cleaved caspase 9 (D330) compared to models with
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29 'stress dose' less than the mean (t-test $p < 0.01$), confirming impaired
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31 apoptosis in models with high DR_MOMP 'stress dose' values. However,
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33 the DR_MOMP score did not correlate with cetuximab responses (ANOVA
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35 $p = 0.6$; Figure 4E). The DR_MOMP apoptosis score was lowest in PDX
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37 models in cluster C1 (mean = 152.9 nM) and, greatest in C3 (mean =
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39 246.0 nM; ANOVA $p < 0.0001$, Tukey post-hoc $p \leq 0.02$; Figure 4F). There
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41 were no significant differences in DR_MOMP apoptosis scores when
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43 PDXs were grouped based on the CRIS subtypes (ANOVA $p = 0.6$; Figure
44
45 4G).

53 APOPTO-CELL predicts apoptosis susceptibility of cells by modelling
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55 activation of executioner caspases and cleavage of their downstream
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57 substrates²⁶. Exceeding a threshold of 25% substrate cleavage within 300
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3 minutes served in previous studies as a surrogate for the competence of
4 cells to undergo executioner (caspase 3) activation, in line with previous
5 single-cell imaging findings^{26, 31}. APOPTO-CELL identified 24 PDX
6 samples with less than 25% predicted substrate cleavage and 36 models
7 with more than 25 % predicted substrate cleavage. However the predicted
8 substrate cleavage did not correlate with responses of the PDX models to
9 cetuximab (Fisher's exact $p = 0.89$; Figure 4E). Further, there was no
10 significant difference in the number of PDXs with substrate cleavage less
11 or greater than 25% between protein clusters C1-C3 (Fisher's exact $p =$
12 0.09) or CRIS subtypes (Fisher's exact $p = 0.85$; Figures 4FG).

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14 We also questioned whether apoptosis signalling contributed to cetuximab
15 responses only in specific protein clusters/molecular subtypes. There was
16 no significant differences between DR_MOMP 'stress dose' scores and
17 treatment responses when PDX models broken down into the three protein
18 clusters C1, C2 and C3 (ANOVA interaction $p = 0.9$) or into the CRIS
19 subtypes (ANOVA interaction $p = 0.9$). Similarly, there was no significant
20 differences between the APOPTO-CELL class and treatment responses
21 after stratifying for the protein cluster or CRIS (not-adjusted Fisher's exact
22 $p > 0.12$). Collectively, these data suggest that BCL2-dependent
23 mitochondrial apoptosis and caspase-3 activation does not play a major
24 role in cetuximab responses.

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26 Next, we calculated the individual proliferative capacity of each PDX using
27 an 11 gene signature index^{24, 25} using existing gene expression profiles¹³.
28 Numerically, proliferation indices were lowest in protein cluster C3, and
29 highest in C2. Statistical analysis revealed no significantly differences
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3 between protein clusters (ANOVA $p = 0.1$; Figure 4H). CRIS-D had
4 significant higher indices compared to the CRIS-B molecular subtype
5 (Tukey post-hoc $p = 0.02$) and C (Tukey post-hoc $p < 0.001$; ANOVA $p =$
6 0.001). Across all PDXs, the proliferation index gradually increased from
7 PDXs with regressing toward progressing responses to cetuximab
8 (ANOVA p -value of 0.01 , Figure 4
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17 J). Progressing PDX models had higher proliferation indices compared to
18 stable (Tukey post-hoc $p = 0.01$ and 0.03) or regressing PDX models
19 (Tukey post-hoc $p = 0.001$ and 0.02) if adjusted for either CRIS (ANOVA p
20 $= 0.01$) or protein clusters (ANOVA $p = 0.02$). Collectively, these data
21 suggested that proliferation rather than apoptosis score is a key
22 determinant of cetuximab responses in 'quadruple negative' metastatic
23 CRC PDX models.
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33 34 **Development of an improved (phospho)protein response** 35 36 37 **score**

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40 Because our previous protein analysis identified cell death markers (Figure
41 2 and 3B; BAK, BCL2, cleaved Caspase-9, XIAP, etc.) that indicated
42 responses to cetuximab, we finally decided to repeat the LASSO analysis
43 with the 22 proteins, but replaced the apoptosis-related markers (BAK,
44 BCL-2 (T56), cleaved Caspase-9 (D330) and XIAP) with the normalised
45 DR_MOMP score. In addition, we removed the protein markers for AKT,
46 mTOR, MAPK-ERK1/2 and PI3-Kinase p110 α based on the assumption
47 that these markers will likely not indicate the activation status of their
48 respective signalling pathway. This enabled us to reduce the overall
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number of proteins analysed. The LASSO analysis set only the coefficient of DR_MOMP to zero: PDK1 (S241; $\beta = 6.3505$), Caspase-8 ($\beta = 5.2772$), Shc (Y317; $\beta = 4.2598$), Stat3 (Y705; $\beta = 2.6455$), p27 ($\beta = 0.6169$), GSK-3 β (S9; $\beta = 6.0001$), HER3 ($\beta = 3.5702$) and PKC-alpha (S657; $\beta = 0.8191$) were found with a positive coefficient. EGFR (Y1068; $\beta = -1.065$), Akt (S473; $\beta = -5.5777$), S6 Ribosomal Protein (S240/244; $\beta = -4.3452$), HER3 (Y1289; $\beta = -5.4732$), NF-kB-p65 (S536; $\beta = -6.3106$) and Gab-1 (Y627; $\beta = -4.6551$) were found with a negative coefficients. The interception was 4.9424. The coefficients were in line with the first LASSO model (Spearman's rank correlation $\rho = 0.88$, $p < 0.0001$). Testing the updated regression model (14 markers) on PDX models showing no or only minor changes in tumour volume ($n = 30$), showed a significant improvement compared with the initial score, with 13 PDX models identified as true "progressing" (true positive), 10 as true "regressing" (true negative), 4 "regressing" as "progressing" and 3 "progressing" as "regressing" models.

Discussion

The discovery of new prognostic biomarkers for cetuximab response is of crucial importance for improving efficiency, and efficacy, of the treatment of metastatic CRC. The genetic heterogeneity of metastatic CRC cancer makes it unlikely that one single protein will serve as a biomarker in all instances, and high throughput techniques such as RPPA may therefore be helpful in identifying predictive biomarker sets. Statistical analysis of our RPPA data showed significant correlation between levels of 20

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3 (phospho)proteins with changes in tumour volume, as detected in PDX
4 models. We identified markers indicating active signalling of the EGFR
5 pathway such as EGFR (Y1068) itself and Akt (S473), Gab-1 (Y627), Shc
6 (Y317), Stat3 (Y705) and PDK1 (S241) to significantly predict responses
7 to cetuximab. Overall we found a high cross correlation between levels of
8 these proteins markers across all samples, emphasising their potential to
9 act as predictive biomarkers for cetuximab responses.

10
11 Interestingly, we found that high levels of phosphorylated EGFR at
12 Tyr1068 and Akt at Ser473 indicated tumour progression, whereas
13 regressing tumours showed a lack of phosphorylated Shc at Tyr317 and
14 Stat3 at Tyr705. Phosphorylation of EGFR on Tyr1068 (and Tyr1086)
15 leads to activation of the MAPK cascade and AKT activation³². *Signal*
16 *transducer and activator of transcription 3* (STAT3) and its phosphorylation
17 are associated with cell growth and transformation³³. The scaffolding
18 protein *Src homology and collagen domain protein* (Shc) directs the EGF
19 stimuli to pro-mitogenic, pro-survival and invasion signalling pathways in a
20 time-dependent manner³⁴. *Phosphoinositide Dependent Protein Kinase 1*
21 (PDK1) is a crucial enzyme in transducing signals to multiple effector
22 pathways including *phosphoinositide 3-kinase* (PI3K/AKT), *Ras/mitogen-*
23 *activated protein kinase* (MAPK), *serum/glucocorticoid regulated kinase*
24 (SGK), *p70 ribosomal protein S6 kinase* (p70 S6 K) and members of
25 *protein kinase C* (PKC) family. Phosphorylation of PDK1 on Ser241 is
26 necessary for its activation³⁵. Some of its substrates require a prior
27 conformational switch to allow subsequent phosphorylation by PDK1³⁵
28 rendering it as gatekeeper for those signalling pathways. We also found
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3 that models expressing the *human epidermal growth factor receptor 3*
4 (HER3, also EGFR3) were more likely to respond with tumour regression
5 in response to cetuximab. In contrast, phosphorylation of HER3 on
6 Tyr1289 was indicative for tumour progression. HER3 cannot be activated
7 by ligand alone but its heterodimer with EGFR and HER2 is highly
8 mitogenic³⁶. Existing literature on the expression and relevance of *HER3* is
9 inconsistent, reporting association with either increased or decreased
10 survival of CRC patients³⁶. In advanced non-small cell lung cancer,
11 abundant *HER3* expression identifies gefitinib (EGFR inhibitor) sensitive
12 cell lines³⁷. In addition, Bosch-Vilaró *et al.*³⁸ described a cetuximab-
13 induced feedback HER3 activation that reduces the response to
14 cetuximab, and in pancreatic cancer, dimerization of EGFR and HER3 was
15 reported to be necessary for downstream signalling³⁹.

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33 Further LASSO and binominal logistic regression analysis of these protein
34 biomarkers delivered a refined protein signatures for predicting responses
35 to cetuximab. Given that many of the identified markers in our signature
36 are predicted to regulate cell proliferation, we also investigated a
37 previously published, transcriptome-based proliferation score as to its
38 predictive power^{24, 25}. Using this score, we also found a significant
39 correlation between cetuximab responses and the transcriptome-based
40 proliferation score across all 63 PDX models investigated. Although the
41 focus of our study was the delivery of a (phospho)protein signature,
42 combining our protein score with the transcriptome-based proliferation
43 score did not further increase the predictive power of the protein
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signature, suggesting that the signature was sufficient to describe the proliferation status of the PDX models in relation to cetuximab responses. We also found that responses to cetuximab were dependent on protein clusters identified through unsupervised cluster analysis. One of the clusters, protein cluster 3 (C3), represented a cluster without progressing PDX models. C3 was characterised by PDK1-dependent active AKT signalling and inhibition of the cell cycle. The largest protein cluster (C1) in contrast showed mixed responses, and was characterised by genotoxic stress, inflammation and cell survival signalling. Cluster C2 was also composed of mixed responders and characterised by active EGFR signalling and inhibition of apoptosis. Compared to PDX models in C1 and C2, PDX models in C3 had lower levels of phosphorylated MEK1/2 (S217/221). This suggests that cetuximab-resistant models in C1 and C2 may potentially benefit from MEK inhibitors. We also explored the relationship between protein clusters and transcriptome-based molecular subtypes. CRIS molecular subtypes capture very well differences in intrinsic tumour cell gene expression¹³. CRIS-C was previously associated with sensitivity to cetuximab¹³, potentially a consequence of the lower representation of *KRAS* and *NRAS* mutations in this subtype¹³. We did not find that any of the three protein clusters showed a significant association with CRIS molecular subtypes. We also found that, when focusing on *KRAS*, *NRAS*, *BRAF* and *PIK3CA* wild type models, CRIS-C was not enriched in cetuximab responders (Figure 1D). Overall, this suggests that sensitivity to anti-EGFR therapy is predicted well by an analysis of (phospho)protein clusters.

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3 While we observed that increased proliferative capacity was associated
4 with disease progression during cetuximab treatment (Figure 4J),
5 competence to undergo mitochondrial apoptosis was not a major
6 determinant of cetuximab responses. Both the DR_MOMP and APOPTO-
7 CELL apoptosis models have been shown to be prognostic for stage II and
8 III CRC patients, but have not yet not been tested in the setting of
9 metastatic CRC^{17, 31}.

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11 Our data suggest that resistance to mitochondrial apoptosis is not critical
12 for responses of metastatic CRC to cetuximab. While cetuximab was
13 shown to induce apoptosis to a minor extent in colorectal cancer cells in
14 previous studies⁴⁰, combination therapy for example with regorafenib has
15 been shown to be required for significant apoptosis induction by
16 cetuximab⁴¹. In the setting of colorectal cancer, we have previously also
17 shown that activation of Caspase-3 may be associated with a
18 compensatory stimulation of cancer cell proliferation and adverse effects
19 on clinical outcome⁴². Here, we also observed that PDX models with
20 progressing tumours tended to have higher levels cleaved Caspase-3
21 compared to models with stable or regressing tumours (Figure 2). It might
22 be possible that activating apoptosis may have both beneficial and
23 detrimental effects in the setting of metastatic CRC.

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25 By comparing matched pre- and post-treatment samples, we also found
26 that levels of GSK-3 β (S9) were reduced in tissue after cetuximab
27 treatment. The *Glycogen synthase kinase 3 β* (GSK-3 β) is a key player in
28 the β -catenin/Wnt signalling pathway but also phosphorylates various
29 transcription factors and structural, metabolic and signalling proteins^{43, 44}.

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3 Inhibition of GSK-3 β activity by phosphorylation at Ser9⁴⁵ is a critical factor
4 to allow many coupled signalling pathways to proceed^{43, 44}. 96% of CRCs
5 harbour increased oncogenic Wnt pathway alteration⁴⁶ and dysregulation
6 of GSK-3 β signalling is associated with cancer and metabolic and
7 degenerative disorders⁴⁷. Inhibition of GSK-3 β was reported to induce
8 apoptosis and attenuated proliferation in colon cancer cells *in vitro*⁴⁸ and in
9 colon cancer xenografts⁴⁹. It is possible that inhibition of GSK-3 β would be
10 desirable co-treatment with cetuximab. Lithium, which also acts as an
11 inhibitor of GSK-3 β ⁵⁰, was reported to suppress cell proliferation in prostate
12 cancer xenografts⁵¹ and may inhibit colon cancer metastasis by blocking
13 *transforming growth factor- β -induced* protein (TGFB1p) expression⁵²
14 downstream of GSK-3⁵³. Combining cetuximab with lithium or other GSK-
15 3 β inhibitors may improve response to cetuximab.
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46 In conclusion, we present here a 14 (phospho)protein marker signature
47 that was predicting responses to cetuximab in mCRC tissue. Likewise, our
48 findings emphasises GSK-3 β to be potentially targetable for a co-
49 treatment with cetuximab.
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Further Disclosures

Ethics approval and consent to participate: Informed consent for research use was obtained from all patients and the study was conducted under the approval of the RCSI Research Ethics Committee and *Comitato Etico Istituto di Candiolo-FPO IRCCS*. All animal procedures were

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3 approved by the Ethical Commission of the Candiolo Cancer Institute and
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5 by the Italian Ministry of Health (806/2016-PR).
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8 **Data Accessibility:** Data is provided as supplementary materials.
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10 Extended data and scripts will be made available upon reasonable
11
12 request.
13

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15 **Conflict of interest:** The authors declare no conflict of interest.
16

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18
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20
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28
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31 LT is a member of the EuroPDX Consortium.
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35 **Authors' contributions:** ABa, AUL, SC, NM, MS and JHMP wrote the
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37 manuscript. ABa, AUL, SC, NM and MS performed data analysis and
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39 prepared figures. ABe and ERZ performed acquisition of sample data.
40
41 BTH, ERZ, ROB, SC and MC collected samples and conducted the protein
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43 quantification using RPPA. ABe, BTH, LT and JHMP supervised the
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45 project. All authors read, reviewed and approved the final manuscript for
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47 publication.
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14 671 **Figure Legends**

17 672 **Figure 1**

18 673 **(A)** Heatmap of protein levels determined by RPPA. PDX models were annotated
19 674 with the, CRIS, the consensus protein cluster subtype, and response to cetuximab
20 675 (top). Clustering was performed using Nonnegative Matrix Factorization (NMF)
21 676 consensus clustering algorithm. The right annotations indicates proteins'
22 677 association to the protein clusters (Supplementary Figure 1). Chord diagrams show
23 678 overlap between RPPA clusters and **(B)** response to cetuximab and **(C)** CRIS, and
24 679 **(D)** overlap between CRIS and response to cetuximab.
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38 680 **Figure 2**

39 681 Protein scores indicating proteins' association to the PDX models' response to
40 682 cetuximab. Proteins' scores for response to cetuximab after 3 week was calculated
41 683 using PAM²⁷.
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49 684 **Figure 3**

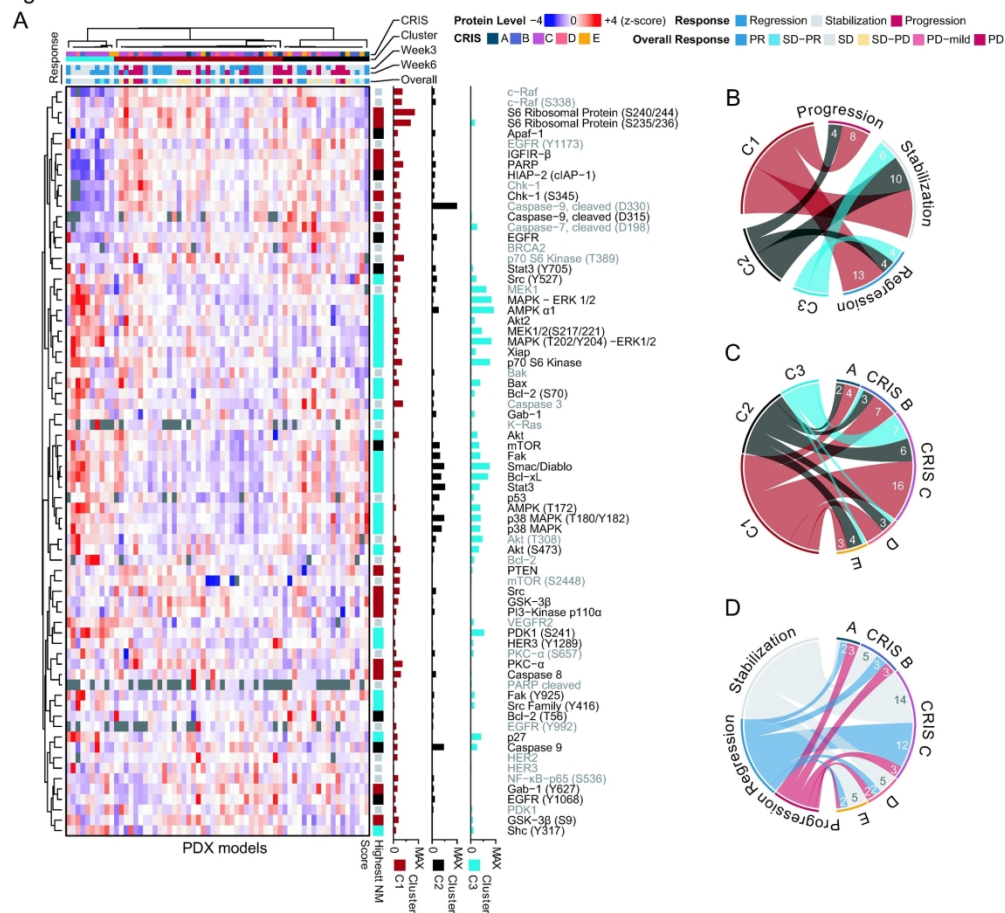
50 685 **(A)** Heatmap of Spearman's rank correlation coefficients for proteins associated
51 686 with differences in response to cetuximab from Figure 2. **(B)** Undirected graph of
52 687 proteins found to be relevant in LASSO analysis. Intensity and colour of the edges
53 688 indicate the correlation coefficient of **(A)**. Grouping based on the signs of the
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3 689 correlation coefficients and signs of the coefficients found by LASSO are indicated
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5 690 black & white nodes and plus & minus icons, respectively. **(C)** Protein found to be
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7 691 differential expressed in PDX models after treatment with cetuximab, based on
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10 692 pairwise comparison and Benjamin & Hochberg adjusted p-value. Dashed red lines
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12 693 indicate 0.05 significance threshold for p-value, and 2-fold or 1/2-fold protein level.
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14 694 The protein marker names and n-fold differences (treated to un-treated) in brackets
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16 695 were added for proteins passing all thresholds.

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20 696 **Figure 4**

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23 697 **(A)** Simplified illustration of the apoptotic signalling modelled in DR_MOMP and
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25 698 APOPTO-CELL. Absolute protein levels normalised to HeLa cells were measured
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27 699 using RPPA and used as input for **(B)** DR_MOMP and **(C)** APOPTO-CELL.
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29 700 Calculated DR MOMP values against **(D)** APOPTO-CELLs' calculated substrate
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31 701 cleavage class with **(E)** differences in response to cetuximab, **(F)** RPPA protein
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33 702 cluster C3 and **(G)** CRIS. Calculated proliferation against **(H)** protein clusters, **(I)**
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35 703 CRIS and **(J)** response to cetuximab.
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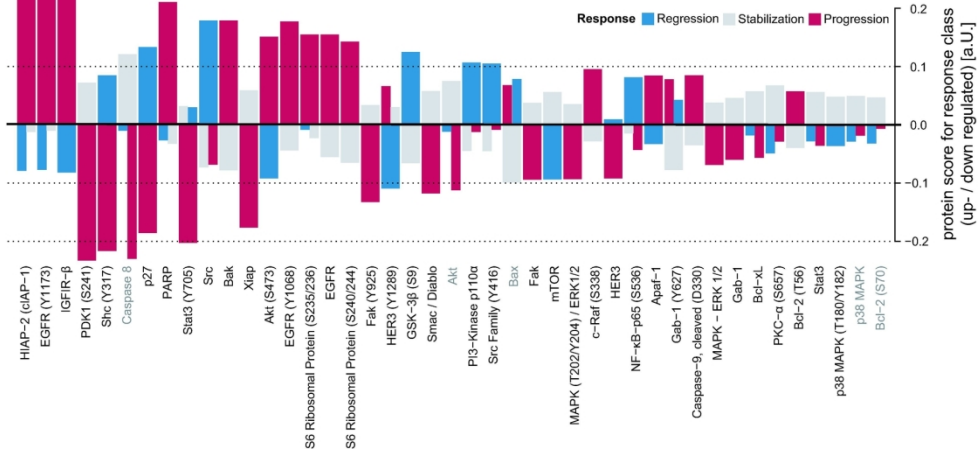
Figure 1



(A) Heatmap of protein levels determined by RPPA. PDX models were annotated with the, CRIS, the consensus protein cluster subtype, and response to cetuximab (top). Clustering was performed using Nonnegative Matrix Factorization (NMF) consensus clustering algorithm. The right annotations indicates proteins' association to the protein clusters (Supplementary Figure 1). Chord diagrams show overlap between RPPA clusters and (B) response to cetuximab and (C) CRIS, and (D) overlap between CRIS and response to cetuximab.

184x172mm (300 x 300 DPI)

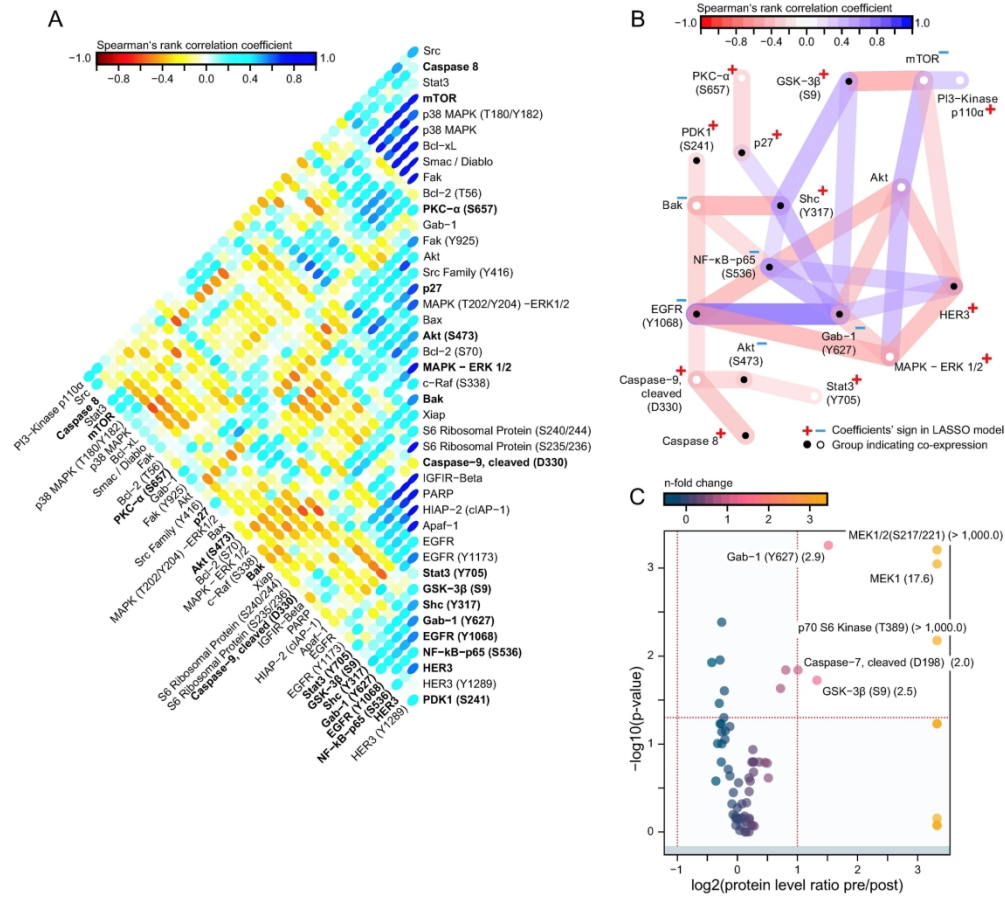
Figure 2



Protein scores indicating proteins' association to the PDX models' response to cetuximab. Proteins' scores for response to cetuximab after 3 week was calculated using PAM[27].

171x90mm (300 x 300 DPI)

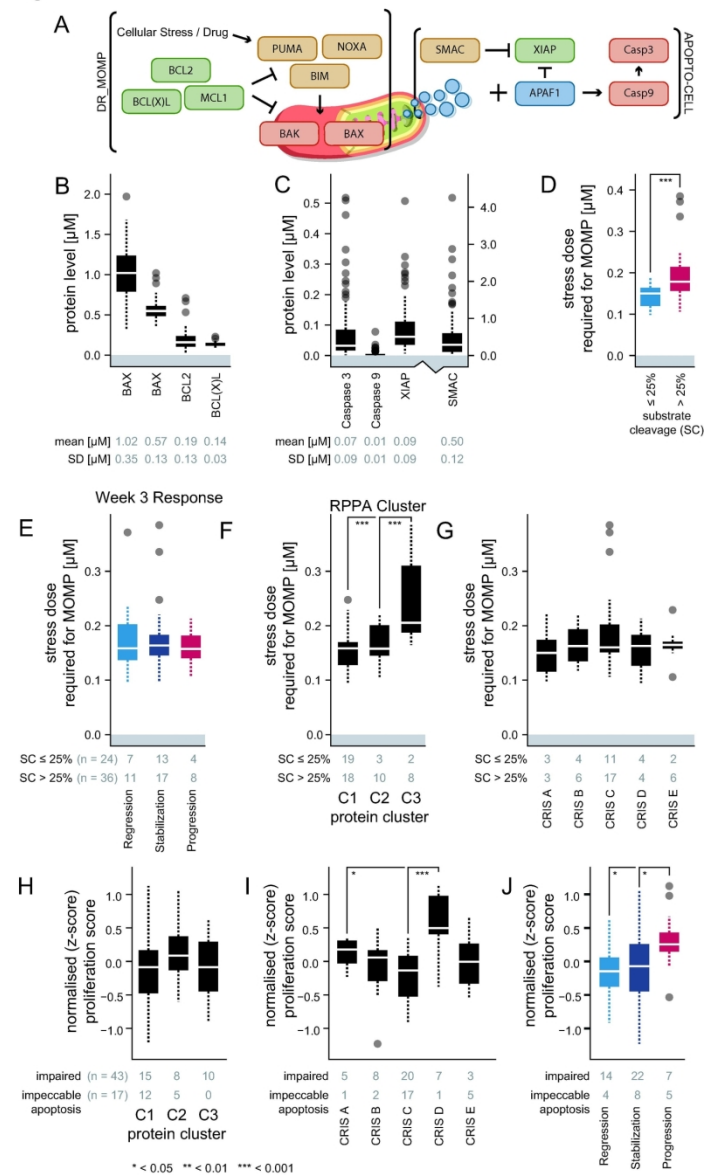
Figure 3



(A) Heatmap of Spearman's rank correlation coefficients for proteins associated with differences in response to cetuximab from Figure 2. (B) Undirected graph of proteins found to be relevant in LASSO analysis. Intensity and colour of the edges indicate the correlation coefficient of (A). Grouping based on the signs of the correlation coefficients and signs of the coefficients found by LASSO are indicated black & white nodes and plus & minus icons, respectively. (C) Protein found to be differential expressed in PDX models after treatment with cetuximab, based on pairwise comparison and Benjamin & Hochberg adjusted p-value. Dashed red lines indicate 0.05 significance threshold for p-value, and 2-fold or 1/2-fold protein level. The protein marker names and n-fold differences (treated to un-treated) in brackets were added for proteins passing all thresholds.

187x174mm (300 x 300 DPI)

Figure 4



(A) Simplified illustration of the apoptotic signalling modelled in DR_MOMP and APOPTO-CELL. Absolute protein levels normalised to HeLa cells were measured using RPPA and used as input for (B) DR_MOMP and (C) APOPTO-CELL. Calculated DR MOMP values against (D) APOPTO-CELLS' calculated substrate cleavage class with (E) differences in response to cetuximab, (F) RPPA protein cluster C3 and (G) CRIS. Calculated proliferation against (H) protein clusters, (I) CRIS and (J) response to cetuximab.

124x215mm (300 x 300 DPI)