Interferon/STAT1 and *neuregulin* signaling pathways are exploratory biomarkers of cetuximab (Erbitux®) efficacy in *KRAS* wild-type squamous carcinomas: A pathway-based analysis of whole human-genome microarray data from cetuximab-adapted tumor cell-line models

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Abstract. *KRAS* mutation status is being used as the sole biomarker to predict therapeutic efficacy of cetuximab in metastatic colorectal cancer (mCRC). A significant number of mCRC patients with *KRAS* wild-type (WT) tumors, however, do not benefit from cetuximab. We are also lacking efficacy predictors in head and neck squamous cell carcinomas with an intact *KRAS* signaling and in non-small cell lung cancer in which *KRAS* mutations do not predict cetuximab efficacy. We recently established pre-clinical models of *EGFR* gene-amplified *KRAS* WT A431 squamous carcinoma cells chronically adapted to grow in the presence of cetuximab. We employed the ingenuity pathway analysis software to functionally interpret data from Agilent's whole human genome arrays in the context of biological processes, networks, and pathways. Cetuximab-induced activation of the interferon (IFN)/STAT1 appeared to switch from 'growth inhibitory' in acutely-treated cells to 'pro-survival' in chronically-adapted cells. Cetuximab treatment appeared to negatively select initially dominant IFN-sensitive clones and promoted selection of IFN- and cetuximab-refractory tumor clones constitutively bearing an up-regulated *IFN/STAT1* signaling. High-levels of mRNAs coding for the EGFR ligands *amphiregulin (AREG), epiregulin (EREG),* and *neuregulin-1/heregulin (NRG1)* predicted for acute cetuximab's functioning. Chronic cetuximab, however, appeared to negatively select initially dominant *AREG/EREG/NRG1*-positive clones to promote selection of cetuximab-refractory clones exhibiting a knocked-down *neuregulin* signaling. Our current evolutionary mapping of the transcriptomic changes that occur during cetuximab-induced chronic blockade of *EGFR/KRAS* WT signaling strongly suggests that mRNAs coding for *IFN/STAT1*- and *EGFR ligands*-related genes can be evaluated as novel predictors of efficacy in *KRAS* WT squamous cancer patients being treated with cetuximab.

Introduction

'The presence of a wild-type KRAS does not predict response to cetuximab, but a mutant KRAS is associated with the high likelihood of nonresponse' (1). This sentence is, at the present time, the sole *Holy Grail* for prediction of sensitive cetuximab response that clinicians can utilize before treating metastatic colorectal cancer (mCRC) with cetuximab-based regimens (2). The presence of *KRAS* mutations has been consistently associated with a lack of response to cetuximab in a series of small observational studies [i.e., mCRC patients with *KRAS* mutations had significantly shorter time to tumor progression compared with *KRAS* wild-type (WT) mCRC patients whereas tumors not harboring *KRAS* mutations were more likely to decrease in size upon treatment]. Importantly, the association between *KRAS* status and outcome appears to be maintained across lines of treatment and in both monotherapy and combination settings (with the exception of first-line dual biological combination of

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anti-VEGF and cetuximab) (3). Retrospective analyses of larger randomized clinical trials have further cemented a pivotal role of *KRAS* mutations as predictors of lack of benefit with cetuximab treatment (4,5). In this scenario, *KRAS* gene mutation status as selection parameter poses a tremendous logistic challenge in the actual testing of tumors in mCRC because there is currently no US Food and Drug Administration-approved standardized *KRAS* mutation detection test (6). Moreover, having an intact *KRAS* gene is necessary but not sufficient to derive benefit from cetuximab in EGFR-driven mCRC (6,7).

With *KRAS* mutations accounting for approximately 30-40% patients who are not response to EGFR-targeted therapies including cetuximab and panitumumab, it is obvious that we need to develop novel treatment alternatives for mCRC patients bearing *KRAS* mutations. Given that a significant number of patients with *KRAS* WT do not benefit from cetuximab therapy (i.e., less than half of *KRAS* WT mCRC patients will benefit from cetuximab treatment) (8), we should consider further selection based not only on *KRAS* mutational status but also on additional potential predictors of benefit from cetuximab. Elucidating mechanisms by which mCRC exhibit *de novo* refractoriness (i.e., failure of cetuximab to elicit any detectable response to initial treatment) and acquired auto-resistance (i.e., progression of a mCRC that had previously responded to treatment, despite continued administration of cetuximab) may be critical to improving the successful clinical management of mCRC patients treated with cetuximab. We should therefore increase also our scrutiny for molecular mechanisms of resistance and predictive biomarkers in *KRAS* WT mCRC patients. In this regard, we are beginning to accumulate evidence that mRNA levels of the EGFR-ligands amphiregulin (*AREG*) and epiregulin (*EREG*) can be employed as potential predictors of response to cetuximab among *KRAS* WT mCRC patients. Using a pharmacogenomic approach in pre-treatment biopsies from metastatic sites, Khambata-Ford *et al* pioneeringly reported that high expression levels of *AREG* and *EREG* were highly predictive of clinical outcome from cetuximab monotherapy in mCRC (9). In their hands, constitutive expression of *AREG* and *EREG* in EGFR-positive L2987 lung carcinoma cells was stimulated by EGF treatment and decreased upon exposure to cetuximab. Since cetuximab-induced inhibition of EGFR-dependent L2987 cell growth was accompanied by coordinated down-regulation of *AREG* and *EREG*, the authors suggested that these EGFR ligands should be considered beacons of an activated EGFR pathway and perhaps a necessary positive feedback loop for cetuximab efficacy. From a clinical perspective, a significant correlation between expression of *AREG* and *EREG* and outcome of mCRC patients treated with cetuximab-based regimens has been confirmed in other studies (10,11). Further expanding the highly significant ability of *AREG* and *EREG* to predict response and survival benefit after treatment with cetuximab, Jacobs *et al* revealed that high levels of *AREG* and *EREG* mRNAs in the primary tumor positively associate with increased responsiveness to cetuximab (12). When assessing the predictive effect of a) high versus low *EREG* among *KRAS* WT and b) high *EREG*/*KRAS* WT status ('combimarker') versus all other patients on overall survival and progression-free survival, Jonker *et al* recently reported that mCRC patients with both high *EREG* gene expression and *KRAS* WT status did benefit from significantly larger cetuximab treatment effects (13).

In addition to high expression levels of *AREG* and *EREG*, mRNA expression levels of the MAPK phosphatases *DUSP4* and *DUSP6* have been also suggested as predictor candidates of outcome after cetuximab treatment in *KRAS* WT and mutant mCRC (14). *DUSPs* are key regulators of the balance between MAP kinase pathway activation and inactivation and have previously been reported to be up-regulated in an adaptive response, thus creating a negative feedback loop following MAPK pathway activation (15,16). Indeed, *DUSP4* and *DUSP6* were originally identified as top resistance markers to cetuximab in unselected patients and the use of a four-gene expression model, including *AREG*, *EREG* and *DUSP6* (as well as *SLC26A3* - solute carrier family 26 member 3) has been shown to improve the identification of responders among pre-selected *KRAS* WT mCRC (9,11). Recently, Montagut *et al* have revealed that DUSP4 protein can be considered a promising negative marker of response to cetuximab-based treatment in mCRC with WT *KRAS* (17). When they assessed DUSP4 expression by immunohistochemistry in a small series of mCRC patients, overexpression of DUSP4 protein significantly associated with lower response rates and shorter median time to progression among *KRAS* WT patients (17).

The development of pre-clinical models exhibiting long-term adaptation to cetuximab can be highly desired to preliminary elucidate if changes in the expression status of EGFR ligands relate to the occurrence of cetuximab refractoriness. We recently envisioned that cetuximab-imposed chronic prevention of binding of the EGFR ligands to the EGFR receptor should provoke a long-term deactivation of the EGFR/RAS/MAPK signaling cascade that might generate cetuximab-adapted tumor cells bearing molecular features equivalent to those expected in the important sub-group of *KRAS* WT mCRC patients that do not benefit from cetuximab *ab initio*. We established pre-clinical models of EGFR-positive A431 vulvar squamous carcinoma cells chronically adapted to grow in the continuous presence of cetuximab. A431 epidermoid carcinoma cells were chosen based on three primary criteria: a) these EGFR-overexpressing cells are exquisitely sensitive to cetuximab; b) A431 cells has not either EGFR tyrosine kinase domain or *KRAS* mutations, and c) apoptotic cell death is usually weak or sometimes not observed after cetuximab treatment (i.e., cetuximab-induced EGFR down-regulation effectively abrogates mitogenic signals from autocrine or paracrine growth factors, thus resulting in arrest of cell cycle progression but nor active cell killing) (18-20). In this scenario, we aimed to minimize the detection of gene expression changes related to general alterations in the apoptotic cell machinery among the transcriptional events induced by chronic cetuximab-induced EGFR inhibition. Our results confirm a crucial role for EGFR ligands in dictating the outcome in response to cetuximab treatment and reveal for the first time that tumor clones that constitutively exhibit high transcriptional levels of interferon (IFN)/STAT pathway expression, and are thus resistant to the cytotoxic effects of this pathway, are preferentially selected by chronic exposure to molecularly targeted agents such as cetuximab. We propose for the first time that loss of the main mechanism for EGFR activation (i.e., autocrine/paracrine loop by EGFR ligands) together with overexpression of the INF/STAT1 pathway may actively contribute to cetuximab refractoriness and cancer relapsing during cetuximab-based therapy.

Materials and methods

Materials. RayBio™ Human Cytokine Array 3 (Cat no. AAH-CYT-3) was purchased from RayBiotech, Inc. (Norcross, GA, USA). Cetuximab (Erbitux®) was kindly provided by Hospital Universitari de Girona Dr Josep Trueta Pharmacy (Girona, Spain). Cetuximab was solubilized with 10 mmol/l NaCl of potassium phosphate buffer (PBS) pH 7.2 in bacteriostatic water for injection purposes (stock solution at 2 mg/ml), stored at 4˚C and used within 1 month.

Culture conditions. Parental A431 vulvar squamous carcinoma cells (originally obtained from the American Type Culture Collection, Manassas, VA, USA) were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Gibco® Cell Culture Systems, Invitrogen S.A., Barcelona, Spain) containing 10% heat-inactivated fetal bovine serum (FBS, Bio-Whittaker, Inc., Walkersville, MD, USA), 1% L-glutamine, 1% sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin. Cells were maintained at 37˚C in a humidified atmosphere of 95% air/5% CO_2. Cells were screened periodically for *Mycoplasma* contamination.

Establishment of long-term (LT)-cetuximab adapted KRAS WT tumor cell populations. Commencing with the IC_{50} of cetuximab (~25 μg/ml) against A431 parental cells, the exposure dose of cetuximab was progressively increased 2-3 weeks until four dose doubling had been successfully achieved. Controlled parental cells were cultured strictly in parallel and exposed to the phosphate-buffered saline (PBS) vehicle. This approach resulted in the establishment of two LT-cetuximab adapted A431 POOLs that were then maintained in continuous culture with the maximal achieved dose of cetuximab. When challenged to cetuximab doses as high as 200 μg/ml, LT-cetuximab A431 POOL1/2 retained a >90% active metabolic status (as assessed by MTT-based cell viability assays) compared to cetuximab-naïve A431 parental cells (which decreased their ability to metabolize MTT by ~50% following treatment with 200 μg/ml cetuximab).

Agilent GeneChip analyses. Total RNA isolated from A431, LT-cetuximab A431 POOL1, LT-cetuximab A431 POOL2 cells grown in the absence of cetuximab was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA quantity and quality were determined using the RNA 6000 Nano Assay kit on an Agilent 2100 BioAnalyzer (Agilent Technologies, Palo Alto, CA, USA), as recommended. Agilent human whole genome microarrays (G4112F), containing 45,220 probes, were then hybridized. Briefly, 500 ng of total RNA from each sample were amplified by Oligo-dT-T7 reverse transcription and labeled by *in vitro* transcription with T7 RNA polymerase in the presence of Cy5-CTP or Cy3-CTP using the Quick Amp Labeling Kit (Agilent) and purified using RNAeasy columns (Qiagen). After fragmentation, 825 ng of labeled cRNA from each of the two samples were co-hybridized in *in situ* hybridization buffer (Agilent) for 17 h at 65˚C and washed at room temperature (RT) 1 min in Gene Expression Wash Buffer 1 (Agilent) and 1 min at 37˚C in Gene Expression Wash Buffer 2 (Agilent).

Statistical analysis of microarray data. The images were generated on a confocal microarray scanner (G2565BA, Agilent) at 5 μm resolution and quantified using GenePix 6.0 (Molecular Dynamics). Spots with signal intensities twice above the local background, not saturated and not flagged by GenePix were considered reliable. Extracted intensities were background-corrected and the log2 ratios were normalized in an intensity-dependent fashion by the global LOWESS method (intra-chip normalization). Normalized log2 ratios were scaled between arrays to make all data comparable. Raw data were processed using MMARGE, a web implementation of LIMMA - a microarray analysis library developed within the Bioconductor project in the R statistical environment. To determine genes that were differentially-expressed, the multi-class SAM (significance analysis of microarrays) procedure was applied. Probes with q-value (FDR) below 5% and additionally a fold change exceeding 2.0 in absolute value were initially selected as the relevant ones. Microarray probes were collapsed to genes by taking the median log2 ratio of the respective probes per gene.

Ingenuity analysis. Gene networks were constructed using ingenuity pathway analysis (Ingenuity® Systems, Redwood City, CA, USA). Data sets containing identifiers of genes with >2.0-fold up- and down-regulatory changes were uploaded into the application. These 'focus genes' were overlaid onto a global molecular network developed from information contained in the ingenuity pathway knowledge base. Networks of these 'focus genes' (nodes) are algorithmically generated based on the principle that highly connected gene networks are most biologically meaningful. All edges are supported by, at least, one reference from the literature stored in the ingenuity pathway knowledge base (the IPA interaction database is manually curated by scientists and updated quarterly). Briefly, the user-input or 'focus genes' gene list is compared to the 'global molecular network' (GMN) database consisting of thousands of genes and interactions. The focus genes are sorted based on highest to lowest connectivity within the GMN, and then networks of approximately 35 genes are grown starting with the most connected focus gene. IPA assigns a p-value for a network of size *n* and an input focus gene list of size *f* by calculating the probability of finding *f* or more focus genes in a randomly selected set of *n* genes from the GMN. The intensity of the node color indicates the degree of expression (green scale for down-regulated nodes; red scale for up-regulated nodes). Nodes are displayed using various shapes each representing functional class of the gene product. The score indicates the likelihood of the genes in a network being found together because of random chance. Using a 99% confidence interval, scores of ≥3 are significant.

Conditioned medium. To prepare conditioned medium, cells were plated in 100-mm tissue culture dishes and cultured in DMEM with 10% FBS until they reached 75-80% confluence. Cell cultures were then washed twice with serum-free DMEM, and incubated overnight in serum-free DMEM. Cells were finally cultured for 48 h in low-serum (0.1% v/v FBS) DMEM. The supernatants were collected, centrifuged at 1,000 x g, aliquoted, and stored at -80˚C until testing.

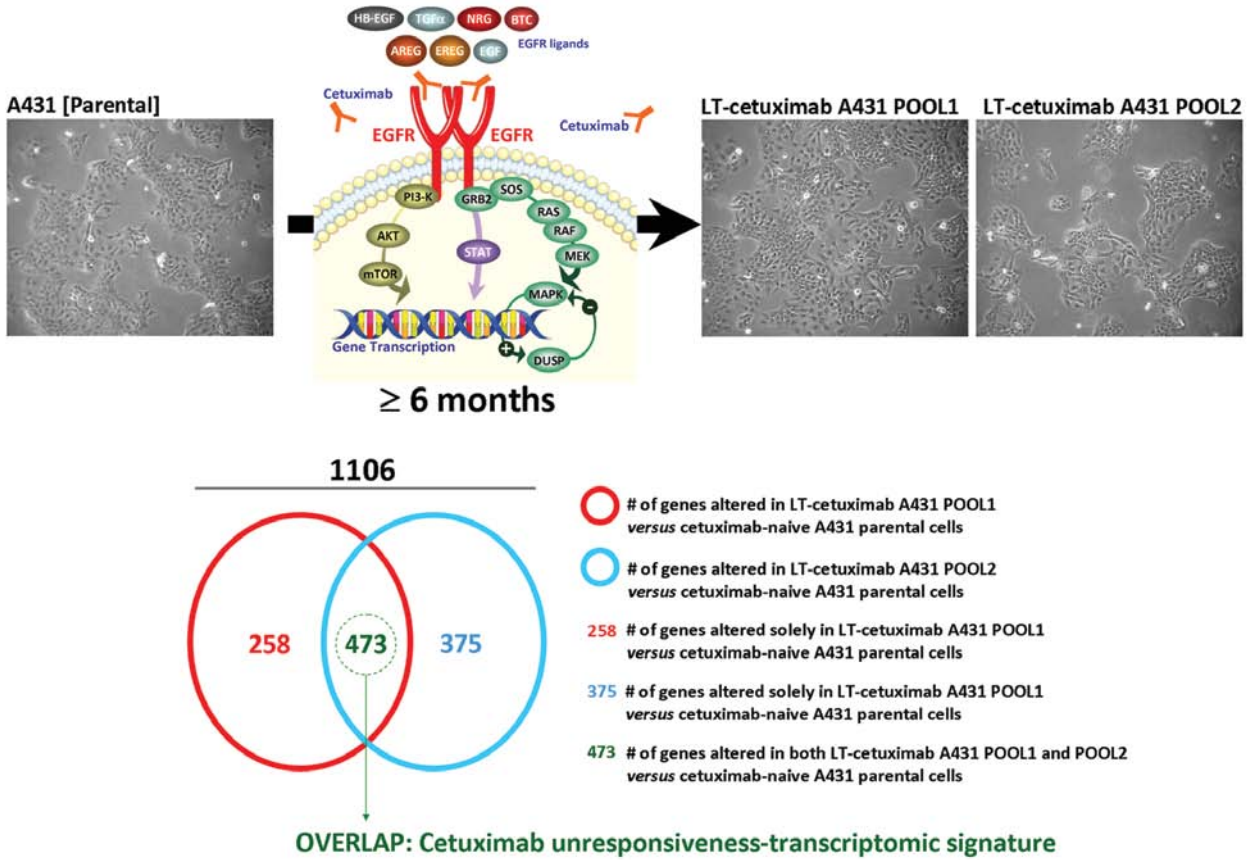

Figure 1. Top: A schematic depicting the experimental approach designed to establish long-term (LT) cetuximab-adapted populations (pools) of *KRAS* WT tumor cell populations. LT-cetuximab adapted A431 POOLs may contain cells with various degrees of cetuximab resistance. Also, the POOLs developed during the study may harbour resistance acquired over time or may represent a selected subpopulation of cells with inherent resistance. Despite these drawbacks to our approach, POOLs also represent the alterations that are represented in the majority of heterogeneous tumor cell populations, whereas clones - another approach that would have been used to answer our research question - represent just one isolated alteration. Bottom: Ven diagram showing overlap in genes whose expression is significantly altered following acquisition of resistance to cetuximab-induced cell growth inhibition. To investigate how A431 cells adapted to grow in the continuous presence of cetuximab, we performed gene-expression profiling experiments. RNA was extracted from POOL1, POOL2 and parental A431 cells. The RNA was then hybridized to G4112F Agilent human whole genome microarray chips, and gene expression was analyzed as described in Materials and methods. Figure summarizes the differences observed in the LT-cetuximab adapted A431 POOLs versus parental A431 cells expressed as fold-change of individual probes for a given gene in the microarray (for complete gene data see Tables I and II).

Cytokine antibody arrays. Assays for cytokine antibody arrays was carried out as per the manufacturer's instructions with minor modifications. Briefly, cytokine array membranes were blocked with 5% BSA/TBS (0.01 mol/l Tris-HCl pH 7.6/0.15 mol/l NaCl) for 1 h. Membranes were then incubated with about 2 ml of conditioned media prepared from different cell lines at 4˚C for overnight. After extensive washing with TBS/0.1% v/v Tween-20 (3 times, 5 min each), the membranes were incubated with a cocktail of biotin-labeled antibodies against different, individual cytokines. The membranes were washed and incubated with HRP-conjugated streptavidin (2.5 pg/ml) for 1 h at room temperature. Unbound HRP-streptavidin was washed out with TBS/0.1% v/v Tween-20 and TBS. The signals were finally detected by ECL system. Densitometric values of spots were quantified using Scion Imaging Software (Scion Corp., Frederick, MD, USA).

Results

First, we performed genome-wide analyses by comparing global transcriptomic profiles of cetuximab-naïve A431 parental cells and A431-derived cetuximab-adapted A431 POOLs. After RNA hybridization to Agilent 44K (double density) whole human genome oligo microarray (containing 45,220 features - probes - representing 41,000 unique human genes and transcripts), normalized and filtered data from cetuximab-responsive A431 parental cells and cetuximab-unresponsive A431 POOLs were both analyzed simultaneously using the significance analysis of microarray (SAM) algorithm. We set the significance cut-off at a median false discovery rate (FDR) of <5.0%. Using a 2.0-fold change cut-off relative to the transcriptome of cetuximab-naïve A431 parental cells, and when identification of genes that showed significant expression changes was made only considering well-annotated transcripts (not partial *cds* for hypothetical proteins, hypothetical insert cDNA clones, etc.) and genes that could not be identified were rule out, the analysis software package ingenuity pathway analysis (IPA, Ingenuity System Inc., USA) identified 731 and 848 genes differentially-expressed in LT-cetuximab adapted A431 POOL1 and POOL2, respectively (Fig. 1). We decided to investigate the overlap of 473 genes (43%) between the gene signatures obtained from both LT-cetuximab adapted A431 POOLs (1106 genes in all were differentially-expressed in both the cetuximab-adapted POOL1 and the cetuximab-adapted

Table I. Gene transcripts differentially up-regulated in LT-cetuximab adapted A431 POOLs (POOL1 and POOL2) versus cetuximab-naïve *EGFR* gene-amplified *KRAS* WT A431 tumor cells.

ID	Entrez gene name	Δ
C3orf23	Chromosome 3 open reading frame 23	2,025
SAA4	Serum amyloid A4, constitutive	2,042
VWF	von Willebrand factor	2,065
ANXA8	Annexin A8	2,080
TUG1	Taurine up-regulated 1 (non-protein coding)	2,089
MLLT4	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	2,089
ING3	Inhibitor of growth family, member 3	2,092
SLC1A3	Solute carrier family 1 (glial high affinity glutamate transporter), member 3	2,104
ZNF278	POZ (BTB) and AT hook containing zinc finger 1	2,110
UBE2L3	Ubiquitin-conjugating enzyme E2L 3	2,119
TEF	Thyrotrophic embryonic factor	2,122
AGPAT3	1-acylglycerol-3-phosphate O-acyltransferase 3	2,123
STAT1	Signal transducer and activator of transcription 1, 91 kDa	2,135
PRKCA	Protein kinase C, alpha	2,136
ITGB2	Integrin, beta 2 (complement component 3 receptor 3 and 4 subunit)	2,144
ABR	Active BCR-related gene	2,145
ERBB3	v-erb-B2 erythroblastic leukemia viral oncogene homolog 3 (avian)	2,149
RBM5	RNA binding motif protein 5	2,150
TPM1	Tropomyosin 1 (alpha)	2,150
SAA1	Serum amyloid A1	2,170
IL17RA	Interleukin 17 receptor A	2,179
PLEKHM1	Pleckstrin homology domain containing, family M (with RUN domain) member 1	2,183
GUSBL2	Glucuronidase, beta-like 2	2,192
TNFRSF9	Tumor necrosis factor receptor superfamily, member 9	2,204
CGN	Cingulin	2,212
SCCPDH	Saccharopine dehydrogenase (putative)	2,220
GBP3	Guanylate binding protein 3	2,226
ITGAV	Integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	2,230
HIST2H2AA3	Histone cluster 2, H2aa3	2,240
NUP50	Nucleoporin 50 kDa	2,250
TIAM2	T-cell lymphoma invasion and metastasis 2	2,258
ECGF1	Thymidine phosphorylase	2,259
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1	2,262
CARD11	Caspase recruitment domain family, member 11	2,273
OAS3	2'-5'-oligoadenylate synthetase 3, 100 kDa	2,274
TAGLN	Transgelin	2,280
UBE1L	Ubiquitin-like modifier activating enzyme 7	2,282
TNFRSF21	Tumor necrosis factor receptor superfamily, member 21	2,285
BSPRY	B-box and SPRY domain containing	2,288
N48043	Thrombospondin 1	2,293
TRIM16	Tripartite motif-containing 16	2,303
TJP2	Tight junction protein 2 (zona occludens 2)	2,305
IFNGR1	Interferon gamma receptor 1	2,307
ETV7	Ets variant 7	2,309
DEPDC5	DEP domain containing 5	2,311
MTAC2D1	Tandem C2 domains, nuclear	2,316
DGCR2	DiGeorge syndrome critical region gene 2	2,317
C12orf26	Chromosome 12 open reading frame 26	2,319

Table I. Continued.

ID	Entrez gene name	Δ
BC045174	Family with sequence similarity 126, member B	2,320
TNFRSF14	Tumor necrosis factor receptor superfamily, member 14 (herpesvirus entry mediator)	2,328
S100A10	S100 calcium binding protein A10	2,333
PODXL	Podocalyxin-like	2,340
TYMS	Thymidylate synthetase	2,346
POMT1	Protein-O-mannosyltransferase 1	2,356
EPC2	Enhancer of polycomb homolog 2 (Drosophila)	2,357
BC022417	TBC1 domain family, member 22A	2,363
HLA-F	Major histocompatibility complex, class I, F	2,372
CYP4V2	Cytochrome P450, family 4, subfamily V, polypeptide 2	2,380
SAA2	Serum amyloid A2	2,380
PVRL4	Poliovirus receptor-related 4	2,391
SORT1	Sortilin 1	2,393
CLDN1	Claudin 1	2,395
LRRC61	Leucine rich repeat containing 61	2,401
PAFAH1B1	Platelet-activating factor acetylhydrolase 1b, regulatory subunit 1 (45 kDa)	2,406
OPTN	Optineurin	2,409
CARD6	Caspase recruitment domain family, member 6	2,430
CIITA	Class II, major histocompatibility complex, transactivator	2,432
KRTAP4-10	Keratin associated protein 4-1	2,433
HD	Huntingtin	2,442
MYD88	Myeloid differentiation primary response gene (88)	2,450
EFNA1	Ephrin-A1	2,453
BDNF	Brain-derived neurotrophic factor	2,455
ZNF268	Zinc finger protein 268	2,461
TACSTD2	Tumor-associated calcium signal transducer 2	2,467
IFI27	Interferon, alpha-inducible protein 27	2,469
IFITM1	Interferon induced transmembrane protein 1 (9-27)	2,479
IL23A	Interleukin 23, alpha subunit p19	2,484
SLC44A3	Solute carrier family 44, member 3	2,490
EPSTI1	Epithelial stromal interaction 1 (breast)	2,495
TRAF1	TNF receptor-associated factor 1	2,500
MDK	Midkine (neurite growth-promoting factor 2)	2,502
C10orf10	Chromosome 10 open reading frame 10	2,503
INPP5D	Inositol polyphosphate-5-phosphatase, 145 kDa	2,517
ANKRD37	Ankyrin repeat domain 37	2,523
FGFR2	Fibroblast growth factor receptor 2	2,524
NR2F2	Nuclear receptor subfamily 2, group F, member 2	2,530
CCL5	Chemokine (C-C motif) ligand 5	2,535
RAB20	RAB20, member RAS oncogene family	2,540
APPBP2	Amyloid beta precursor protein (cytoplasmic tail) binding protein 2	2,541
TRIM59	Tripartite motif-containing 59	2,553
BC063641	Proline rich Gla (G-carboxyglutamic acid) 4 (transmembrane)	2,560
SAMD9	Sterile alpha motif domain containing 9	2,567
SERTAD4	SERTA domain containing 4	2,585
REC8L1	REC8 homolog (yeast)	2,596
TSPAN2	Tetraspanin 2	2,615
BATF2	Basic leucine zipper transcription factor, ATF-like 2	2,618
C1orf38	Chromosome 1 open reading frame 38	2,620

Table I. Continued.

ID	Entrez gene name	Δ
PARP14	Poly (ADP-ribose) polymerase family, member 14	2,623
TRIM14	Tripartite motif-containing 14	2,654
EPN3	Epsin 3	2,664
GGT6	Gamma-glutamyltransferase 6	2,671
PDZK1IP1	PDZK1 interacting protein 1	2,691
THBS1	Thrombospondin 1	2,695
GTSE1	G-2 and S-phase expressed 1	2,697
AK125038	Calmin (calponin-like, transmembrane)	2,712
BE710618	Insulin-like growth factor binding protein 3	2,728
ISGF3G	Interferon regulatory factor 9	2,739
PIM3	Pim-3 oncogene	2,745
PLEKHA6	Pleckstrin homology domain containing, family A member 6	2,758
ITGB8	Integrin, beta 8	2,758
HERC6	Hect domain and RLD 6	2,763
SLC26A11	Solute carrier family 26, member 11	2,792
TXNIP	Thioredoxin interacting protein	2,794
OAS2	2'-5'-oligoadenylate synthetase 2, 69/71 kDa	2,815
TLR3	Toll-like receptor 3	2,819
C6orf61	Minichromosome maintenance complex component 9	2,825
C1R	Complement component 1, r subcomponent	2,837
BID	BH3 interacting domain death agonist	2,844
PPAP2B	Phosphatidic acid phosphatase type 2B	2,847
EMILIN2	Elastin microfibril interfacer 2	2,850
PEG10	Paternally expressed 10	2,863
MAP3K8	Mitogen-activated protein kinase kinase kinase 8	2,884
OAS1	2',5'-oligoadenylate synthetase 1, 40/46 kDa	2,887
C9orf3	Chromosome 9 open reading frame 3	2,905
FAM111A	Family with sequence similarity 111, member A	2,930
LIPH	Lipase, member H	2,932
UBD	Ubiquitin D	2,949
SIGIRR	Single immunoglobulin and toll-interleukin 1 receptor (TIR) domain	2,966
GJB7	Gap junction protein, beta 7, 25 kDa	2,967
RHBDL2	Rhomboid, veinlet-like 2 (Drosophila)	2,985
POLR3H	Polymerase (RNA) III (DNA directed) polypeptide H (22.9 kDa)	3,004
CFB	Complement factor B	3,013
TWIST2	Twist homolog 2 (Drosophila)	3,017
ATP2B4	ATPase, Ca^{++} transporting, plasma membrane 4	3,023
AL359055	Metastasis associated in colon cancer 1	3,046
ID3	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	3,058
CSF1	Colony stimulating factor 1 (macrophage)	3,060
PLAT	Plasminogen activator, tissue	3,065
C9orf19	GLI pathogenesis-related 2	3,072
GPR30	G protein-coupled estrogen receptor 1	3,073
TM4SF1	Transmembrane 4 L six family member 1	3,074
FA2H	Fatty acid 2-hydroxylase	3,088
ID1	Inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	3,100
APOL3	Apolipoprotein L, 3	3,101
CABLES1	Cdk5 and Abl enzyme substrate 1	3,108
TRIM22	Tripartite motif-containing 22	3,125

Table I. Continued.

ID	Entrez gene name	Δ
APOLD1	Apolipoprotein L domain containing 1	3,169
KIAA1618	Ring finger protein 213	3,170
MAL2	Mal, T-cell differentiation protein 2	3,195
PLEKHK1	Rhotekin 2	3,205
GPRC5A	G protein-coupled receptor, family C, group 5, member A	3,240
RSAD2	Radical S-adenosyl methionine domain containing 2	3,261
CD82	CD82 molecule	3,267
CD24	CD24 molecule	3,278
CLDN4	Claudin 4	3,297
AQP3	Aquaporin 3 (Gill blood group)	3,300
OCLN	Occludin	3,335
APOL6	Apolipoprotein L, 6	3,343
LMO2	LIM domain only 2 (rhombotin-like 1)	3,345
EPPK1	Epiplakin 1	3,357
WNT4	Wingless-type MMTV integration site family, member 4	3,380
PCSK6	Proprotein convertase subtilisin/kexin type 6	3,382
NCOA7	Nuclear receptor coactivator 7	3,405
BCL2L14	BCL2-like 14 (apoptosis facilitator)	3,419
MOBKL2B	MOB1, Mps One Binder kinase activator-like 2B (yeast)	3,430
ZNF143	Zinc finger protein 143	3,463
BG547557	Cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	3,476
APOBEC3D	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3D	3,509
MESP1	Mesoderm posterior 1 homolog (mouse)	3,560
USP18	Ubiquitin specific peptidase 18	3,570
TMEM125	Transmembrane protein 125	3,606
MGAT4A	Mannosyl (alpha-1,3-)-glycoprotein beta-1,4-N-acetylglucosaminyltransferase, isozyme A	3,633
LAMP3	Lysosomal-associated membrane protein 3	3,661
METTL7A	Methyltransferase like 7A	3,750
NFKBIA	Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	3,769
ENC1	Ectodermal-neural cortex 1 (with BTB-like domain)	3,785
KRT33A	Keratin 33A	3,799
SLFN5	Schlafen family member 5	3,826
TNF	Tumor necrosis factor	3,839
SAMD9L	Sterile alpha motif domain containing 9-like	3,877
TIMP2	TIMP metallopeptidase inhibitor 2	3,931
HSH2D	Hematopoietic SH2 domain containing	3,944
APOBEC3B	Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	3,980
TNFSF10	Tumor necrosis factor (ligand) superfamily, member 10	3,985
GDA	Guanine deaminase	3,995
RTP4	Receptor (chemosensory) transporter protein 4	4,035
C3		4,081
IFI6	Interferon, alpha-inducible protein 6	4,104
ADRB2	Adrenergic, beta-2-, receptor, surface	4,110
DUSP10	Dual specificity phosphatase 10	4,185
SLPI	Secretory leukocyte peptidase inhibitor	4,274
MMP7	Matrix metallopeptidase 7 (matrilysin, uterine)	4,283
JPH2	Junctophilin 2	4,285
PPL	Periplakin	4,308
CTSS	Cathepsin S	4,321

Table I. Continued.

ID	Entrez gene name	Δ
C1S	Complement component 1, s subcomponent	4,358
TNS3	Tensin 3	4,388
AW979273	Metastasis associated in colon cancer 1	4,463
MALL	Mal, T-cell differentiation protein-like	4,579
CLIC3	Chloride intracellular channel 3	4,626
IL7	Interleukin 7	4,662
KRT14	Keratin 14	4,667
PLK2	Polo-like kinase 2 (Drosophila)	4,740
ARHGAP23	Rho GTPase activating protein 23	4,759
GBP2	Guanylate binding protein 2, interferon-inducible	4,774
MUC4	Mucin 4, cell surface associated	4,792
JDP2	Jun dimerization protein 2	4,817
PLAC8	Placenta-specific 8	5,040
BIRC3	Baculoviral IAP repeat-containing 3	5,085
LCN2	Lipocalin 2	5,322
SECTM1	Secreted and transmembrane 1	5,366
ELF3	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	5,383
NANOS1	Nanos homolog 1 (Drosophila)	5,457
C19orf21	Chromosome 19 open reading frame 21	5,568
LGALS9	Lectin, galactoside-binding, soluble, 9	5,578
KRT15	Keratin 15	5,597
IL32	Interleukin 32	5,753
BC018597	Ankyrin repeat domain 33B	5,911
KRT19	Keratin 19	5,979
METRNL	Meteorin, glial cell differentiation regulator-like	6,098
AMOT	Angiomotin	6,116
OVOL1	Ovo-like 1(Drosophila)	6,293
MX2	Myxovirus (influenza virus) resistance 2 (mouse)	6,440
MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	6,686
SLC15A3	Solute carrier family 15, member 3	6,805
CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	6,852
ATAD4	Proline rich 15-like	6,998
WFDC2	WAP four-disulfide core domain 2	7,290
MARCO	Macrophage receptor with collagenous structure	7,447
FGD3	FYVE, RhoGEF and PH domain containing 3	8,192
C7orf29	Chromosome 7 open reading frame 29	8,994
GPR110	G protein-coupled receptor 110	9,325
GBP4	Guanylate binding protein 4	9,350
CST6	Cystatin E/M	9,892
VGLL1	Vestigial like 1 (Drosophila)	9,921
KRT13	Keratin 13	10,151
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	10,564
IFI44L	Interferon-induced protein 44-like	10,765
GRHL3	Grainyhead-like 3 (Drosophila)	17,030
IGFBP3	Insulin-like growth factor binding protein 3	18,380
C15orf48	Chromosome 15 open reading frame 48	21,136
LY6D	Lymphocyte antigen 6 complex, locus D	25,090
LTB	Lymphotoxin beta (TNF superfamily, member 3)	26,754
MUC20	Mucin 20, cell surface associated	33,420

Table II. Gene transcripts differentially down-regulated in LT-cetuximab adapted A431 POOLs (POOL1 and POOL2) versus cetuximab-naïve *EGFR* gene-amplified *KRAS* WT A431 tumor cells.

ID	Entrez gene name	Δ
FNDC6	Interleukin 20 receptor beta	-27,918
DUSP6	Dual specificity phosphatase 6	-16,491
TGFBI	Transforming growth factor, beta-induced, 68 kDa	-16,453
VAV3	Vav 3 guanine nucleotide exchange factor	-12,012
TMEPAI	Prostate transmembrane protein, androgen induced 1	-10,610
FHOD3	Formin homology 2 domain containing 3	-10,567
GLDC	Glycine dehydrogenase (decarboxylating)	-10,553
THBD	Thrombomodulin	-10,526
FAM113B	Family with sequence similarity 113, member B	-9,293
ACP5	Acid phosphatase 5, tartrate resistant	-9,084
EGFR	Epidermal growth factor receptor [erythroblastic leukemia viral (v-erb-B) oncogene homolog, avian]	-8,745
SPP1	Secreted phosphoprotein 1	-8,720
AREG	Amphiregulin	-8,382
TNS4	Tensin 4	-7,967
RNF130	Ring finger protein 130	-7,826
TMEM45A	Transmembrane protein 45A	-7,749
CMKOR1	Chemokine (C-X-C motif) receptor 7	-7,512
AK095472	Transmembrane protein 64	-6,525
STEAP1	Six transmembrane epithelial antigen of the prostate 1	-6,500
HOXD10	Homeobox D10	-6,415
FOXA2	Forkhead box A2	-6,250
STC2	Stanniocalcin 2	-6,130
NT5E	5'-nucleotidase, ecto (CD73)	-5,784
HAS3	Hyaluronan synthase 3	-5,761
EREG	Epiregulin	-5,694
PPARGC1A	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha	-5,580
SERPINE1	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	-5,365
EFS	Embryonal Fyn-associated substrate	-5,269
PDGFA	Platelet-derived growth factor alpha polypeptide	-5,253
GJA1	Gap junction protein, alpha 1, 43 kDa	-5,237
SLC20A1	Solute carrier family 20 (phosphate transporter), member 1	-5,095
VSNL1	Visinin-like 1	-5,095
CYP2R1	Cytochrome P450, family 2, subfamily R, polypeptide 1	-5,007
KRT83	Keratin 83	-4,948
GPX2	Glutathione peroxidase 2 (gastrointestinal)	-4,936
AMIGO2	Adhesion molecule with Ig-like domain 2	-4,926
NRG1	Neuregulin 1	-4,750
FABP6	Fatty acid binding protein 6, ileal	-4,745
SOX2	SRY (sex determining region Y)-box 2	-4,730
DUSP23	Dual specificity phosphatase 23	-4,687
C20orf19	Polo-like kinase 1 substrate 1	-4,546
KCTD12	Potassium channel tetramerisation domain containing 12	-4,521
TXNDC13	Thioredoxin-related transmembrane protein 4	-4,260
GPSM1	G-protein signaling modulator 1 (AGS3-like, C. elegans)	-4,228
KCNG1	Potassium voltage-gated channel, subfamily G, member 1	-4,227
APOC1	Apolipoprotein C-I	-4,205
SNAI2	Snail homolog 2 (Drosophila)	-4,153
C6orf141	Chromosome 6 open reading frame 141	-4,093

Table II. Continued.

ID	Entrez gene name	Δ
IER3	Immediate early response 3	-4,089
KRT81	Keratin 81	-4,078
TMEM64	Transmembrane protein 64	-4,070
GNAZ	Guanine nucleotide binding protein (G protein), alpha z polypeptide	-4,045
C20orf100	TOX high mobility group box family member 2	-4,027
SNAI1	Snail homolog 1 (Drosophila)	-3,990
TMEM30B	Transmembrane protein 30B	-3,966
CR610885	Transmembrane protein 64	-3,931
SLC37A2	Solute carrier family 37 (glycerol-3-phosphate transporter), member 2	-3,860
PRKCDBP	Protein kinase C, delta binding protein	-3,850
KIAA1622	Protein phosphatase 4, regulatory subunit 4	-3,825
DBN1	Drebrin 1	-3,807
CYR61	Cysteine-rich, angiogenic inducer, 61	-3,720
TP73L	Tumor protein p63	-3,651
BEX2	Brain expressed X-linked 2	-3,615
ITGB5	Integrin, beta 5	-3,613
SH3PXD2A	SH3 and PX domains 2A	-3,539
EFNB2	Ephrin-B2	-3,538
C10orf58	Chromosome 10 open reading frame 58	-3,509
GPR68	G protein-coupled receptor 68	-3,477
CA2	Carbonic anhydrase II	-3,472
NSBP1	High-mobility group nucleosome binding domain 5	-3,453
BDKRB1	Bradykinin receptor B1	-3,450
MAOA	Monoamine oxidase A	-3,425
PHLDA1	Pleckstrin homology-like domain, family A, member 1	-3,405
EFNB1	Ephrin-B1	-3,396
ITGA5	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	-3,392
COL17A1	Collagen, type XVII, alpha 1	-3,360
ARHGAP25	Rho GTPase activating protein 25	-3,334
ADA	Adenosine deaminase	-3,328
PDE2A	Phosphodiesterase 2A, cGMP-stimulated	-3,279
IL1A	Interleukin 1, alpha	-3,245
LTBP2	Latent transforming growth factor beta binding protein 2	-3,235
IL1RAP	Interleukin 1 receptor accessory protein	-3,217
FAM20C	Family with sequence similarity 20, member C	-3,198
GPNMB	Glycoprotein (transmembrane) nmb	-3,177
PYCR1	Pyrroline-5-carboxylate reductase 1	-3,175
STAR	Steroidogenic acute regulatory protein	-3,170
TUBAL3	Tubulin, alpha-like 3	-3,152
VEGF	Vascular endothelial growth factor A	-3,145
PC	Pyruvate carboxylase	-3,140
NUPR1	Nuclear protein, transcriptional regulator, 1	-3,127
PPARG	Peroxisome proliferator-activated receptor gamma	-3,095
RNF128	Ring finger protein 128	-3,084
FAM83A	Family with sequence similarity 83, member A	-3,065
LANCL2	LanC lantibiotic synthetase component C-like 2 (bacterial)	-3,037
CHST5	Carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 5	-3,034
HEY1	Hairy/enhancer-of-split related with YRPW motif 1	-2,970
PTPRE	Protein tyrosine phosphatase, receptor type, E	-2,960

Table II. Continued.

ID	Entrez gene name	Δ
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)	-2,952
LTBP4	Latent transforming growth factor beta binding protein 4	-2,950
TFCP2L1	Transcription factor CP2-like 1	-2,944
SPINK2	Serine peptidase inhibitor, Kazal type 2 (acrosin-trypsin inhibitor)	-2,925
TINAGL1	Tubulointerstitial nephritis antigen-like 1	-2,920
SPHK1	Sphingosine kinase 1	-2,890
HOXD11	Homeobox D11	-2,884
LTBP1	Latent transforming growth factor beta binding protein 1	-2,845
HOXD9	Homeobox D9	-2,829
CFDP1	Craniofacial development protein 1	-2,823
C1QDC1	Caprin family member 2	-2,805
LAMB3	Laminin, beta 3	-2,783
LAPTM5	Lysosomal protein transmembrane 5	-2,755
MT1G	Metallothionein 1G	-2,745
MLLT11	Myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 11	-2,731
FSCN1	Fascin homolog 1, actin-bundling protein (Strongylocentrotus purpuratus)	-2,730
PORCN	Porcupine homolog (Drosophila)	-2,728
CSTA	Cystatin A (stefin A)	-2,725
MDFI	MyoD family inhibitor	-2,717
MAGED1	Melanoma antigen family D, 1	-2,701
CYP27B1	Cytochrome P450, family 27, subfamily B, polypeptide 1	-2,688
NRG2	Neuregulin 2	-2,676
ARSI	Arylsulfatase family, member I	-2,661
WDR66	WD repeat domain 66	-2,636
HPCAL1	Hippocalcin-like 1	-2,618
HOMER3	Homer homolog 3 (Drosophila)	-2,611
CTH	Cystathionase (cystathionine gamma-lyase)	-2,606
BCHE	Butyrylcholinesterase	-2,603
KAZALD1	Kazal-type serine peptidase inhibitor domain 1	-2,592
PLTP	Phospholipid transfer protein	-2,590
MT1X	Metallothionein 1X	-2,585
CPE	Carboxypeptidase E	-2,569
ANKH	Ankylosis, progressive homolog (mouse)	-2,564
NOMO1	NODAL modulator 1	-2,563
CR601458	Family with sequence similarity 110, member C	-2,543
RUNX2	Runt-related transcription factor 2	-2,536
C6orf1	Chromosome 6 open reading frame 1	-2,535
FUS	Fused in sarcoma	-2,529
GNG4	Guanine nucleotide binding protein (G protein), gamma 4	-2,515
BQ045216	Tumor protein p63	-2,507
GYLTL1B	Glycosyltransferase-like 1B	-2,503
CSRP2	Cysteine and glycine-rich protein 2	-2,500
CXorf57	Chromosome X open reading frame 57	-2,486
HPCA	Hippocalcin	-2,486
EFCAB1	EF-hand calcium binding domain 1	-2,485
KRT86	Keratin 86	-2,482
PREX1	Phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 1	-2,455
MLXIPL	MLX interacting protein-like	-2,448
TCOF1	Treacher Collins-Franceschetti syndrome 1	-2,446

Table II. Continued.

ID	Entrez gene name	Δ
CCDC52	Coiled-coil domain containing 52	-2,441
MT1B	Metallothionein 1B	-2,439
SLC25A27	Solute carrier family 25, member 27	-2,438
DUSP4	Dual specificity phosphatase 4	-2,426
TFEB	Transcription factor EB	-2,425
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	-2,425
ETV4	Ets variant 4	-2,415
BC031342	Homeobox A2	-2,403
C10orf13	Fibroblast growth factor binding protein 3	-2,393
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11	-2,392
AIM2	Absent in melanoma 2	-2,391
ANKRD57	Ankyrin repeat domain 57	-2,388
C16orf35	Nitrogen permease regulator-like 3 (S. cerevisiae)	-2,385
JAK3	Janus kinase 3	-2,376
GSPT1	G1 to S phase transition 1	-2,375
ACPL2	Acid phosphatase-like 2	-2,374
PRR7	Proline rich 7 (synaptic)	-2,366
PFKFB4	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4	-2,350
TSPAN5	Tetraspanin 5	-2,345
HNRPA0	Heterogeneous nuclear ribonucleoprotein A0	-2,320
ERF	Ets2 repressor factor	-2,319
SLC7A8	Solute carrier family 7 (amino acid transporter, L-type), member 8	-2,310
STEAP2	Six transmembrane epithelial antigen of the prostate 2	-2,290
C6orf105	Chromosome 6 open reading frame 105	-2,289
C5orf13	Chromosome 5 open reading frame 13	-2,280
ARID3B	AT rich interactive domain 3B (BRIGHT-like)	-2,277
FLOT1	Flotillin 1	-2,266
MT1H	Metallothionein 1H	-2,265
GCNT1	Glucosaminyl (N-acetyl) transferase 1, core 2	-2,264
ACSS2	Acyl-CoA synthetase short-chain family member 2	-2,257
SMAD7	SMAD family member 7	-2,254
MT1L	Metallothionein 1L (gene/pseudogene)	-2,251
LONRF1	LON peptidase N-terminal domain and ring finger 1	-2,243
SLC4A7	Solute carrier family 4, sodium bicarbonate cotransporter, member 7	-2,242
CA9	Carbonic anhydrase IX	-2,242
OSMR	Oncostatin M receptor	-2,232
ARID3A	AT rich interactive domain 3A (BRIGHT-like)	-2,221
ABCC1	ATP-binding cassette, sub-family C (CFTR/MRP), member 1	-2,195
MARK1	MAP/microtubule affinity-regulating kinase 1	-2,193
HMGA2	High mobility group AT-hook 2	-2,193
MFNG	MFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	-2,180
P4HA2	Prolyl 4-hydroxylase, alpha polypeptide II	-2,177
MT2A	Metallothionein 2A	-2,177
ARL2BP	ADP-ribosylation factor-like 2 binding protein	-2,174
OAF	OAF homolog (Drosophila)	-2,171
KARS	Lysyl-tRNA synthetase	-2,166
OBFC1	Oligonucleotide/oligosaccharide-binding fold containing 1	-2,159
CBLC	Cas-Br-M (murine) ecotropic retroviral transforming sequence c	-2,157
FEZ1	Fasciculation and elongation protein zeta 1 (zygin I)	-2,156

Table II. Continued.

ID	Entrez gene name	Δ
HCFC1R1	Host cell factor C1 regulator 1 (XPO1 dependent)	-2,156
GCLM	Glutamate-cysteine ligase, modifier subunit	-2,151
AP1S1	Adaptor-related protein complex 1, sigma 1 subunit	-2,150
LMO4	LIM domain only 4	-2,141
WASF3	WAS protein family, member 3	-2,130
ADSSL1	Adenylosuccinate synthase like 1	-2,128
GPT2	Glutamic pyruvate transaminase (alanine aminotransferase) 2	-2,127
DEPDC7	DEP domain containing 7	-2,116
SDC1	Syndecan 1	-2,098
HIST2H2BE	Histone cluster 2, H2be	-2,095
NOL3	Nucleolar protein 3 (apoptosis repressor with CARD domain)	-2,094
PRPS1	Phosphoribosyl pyrophosphate synthetase 1	-2,077
CDKN2A	Cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	-2,070
FAM3C	Family with sequence similarity 3, member C	-2,067
CENTD3	ArfGAP with RhoGAP domain, ankyrin repeat and PH domain 3	-2,038
BG108194	Hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix transcription factor)	-2,033

POOL2). Tables I and II summarize gene transcripts up- and down-regulated, respectively, in the overlapping 'cetuximab unresponsiveness-transcriptomic signature'.

Second, the 'core analysis' function included in IPA software was employed to interpret the above data in the context of biological processes, networks, and pathways. Networks of up- and down-regulated functionally-related annotated genes were algorithmically generated by IPA based on their connectivity and assigned a score (i.e., a numerical value that takes into consideration both the number of focus genes in a network and the size of the network to approximate how relevant this network could be to the original list of focus genes). IPA analysis revealed 14 gene networks significantly up-regulated and 12 gene networks significantly down-regulated (scores of ≥3) within the 'cetuximab unresponsiveness-transcriptomic signature'. Table III summarizes the IPA-recognized gene networks with the ten highest scores of overexpressed (bold) and down-regulated (underlined) transcripts. Third, 'canonical pathways analysis' identified the pathways, from the IPA library of canonical pathways, which were most significant to the input data set. The significance of the association between the data set and the canonical pathway was determined based on two parameters: a) a p-value calculated using Fischer's exact test determining the probability that the association between the genes and the canonical pathway is due to chance alone, and b) a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway. Table IV lists the top 5 canonical pathways that were up- and down-regulated within the 'cetuximab unresponsiveness-transcriptomic signature'.

Pathway-based analysis of genome-wide transcriptomic data reveals that signaling cascades related to 'inflammatory responses' are the crucial gene network functions over-activated in cetuximab-unresponsive KRAS WT tumor cells. Fig. 2 (top panels) illustrates graphically the 3 top gene network functions significantly up-regulated within the 'cetuximab unresponsiveness-transcriptomic signature'. Top functions of up-regulated gene networks were related to: a) inflammatory response, nucleic acid metabolism, antigen presentation (Fig. 2a), including genes coding for members of the tumor necrosis factor receptor superfamily (*TNFRS9, TNFRS14, TNFRS21*) that have crucial roles in both innate and adaptive immunity (21), several interferon (IFN)-related inflammatory markers such as genes encoding for members of the 2'-5'oligoAdenylate Synthetase family (*OAS1, OAS2, OAS3*) (22,23), and several keratins involved in epidermal cell differentiation/keratinization (*KRT13, KRT14, KRT15, KRT19*) (24). This gene network was identified around the major pro-inflammatory cytokine tumor necrosis factor (TNF)-α; b) anti-microbial response, antigen presentation, cell-to-cell signaling and interaction (Fig. 2b), including baculoviral IAP (inhibitor of apoptosis protein) repeat-containing 3 (*BIRC3*) (25), a homologous of c-IAP2 (cellular inhibitor of apoptosis protein 2) that plays a critical role in resistance to toll-like receptor 3 (*TLR3*)-mediated apoptosis (26-28) as well as members of the TNF cytokine superfamily including TNF and lymphotoxins alpha and beta (*LTA, LTB*) (29). This gene network was identified around TNF/LT-regulated anti-apoptotic members of the NF-κ family; c) cellular growth and proliferation, hepatic system development and function, liver proliferation (Fig. 2c), a gene network that was identified around IFN-γ and included several IFN-induced transcripts (e.g., the *IFI6*/*IFI27* anti-apoptotic genes and the member of the HERC family of ubiquitin ligases *HERC6*) (30-32).

When IPA was used to determine canonical pathway analysis enrichment categories 'interferon signaling' (33-36) was the most statistically significant map modulated by the up-regulated genes within the 'cetuximab unresponsiveness-transcriptomic signature' (Fig. 3a). Cetuximab-accommodated

Table III. Top 10 IPA-recognized gene network functions in cetuximab unresponsiveness-transcriptomic signature.

ID	Molecules in network	Score	Focus molecules	Top functions
1	**ABR, AQP3, BCL2L14, C10ORF10,** Cytokeratin, **DUSP10,** I kappa b kinase, **IFI6, IFI44L,** Ifn gamma, Ifn gamma receptor, **IFNGR1, IL17RA,** IRG, **KRT13, KRT14, KRT15, KRT19, LAMP3, LGALS9, MUC20,** Oas, **OAS1, OAS2, OAS3 (includes EG:4940), OPTN, SLC1A3, TM4SF1, TNF, TNFRSF9, TNFRSF14, TNFRSF21, TWIST2, TYMP, UBD**	56	29	Inflammatory response, nucleic acid metabolism, antigen presentation
2	**BIRC3, C1R, CARD6, CD82,** CFB, CHEMOKINE, Ferritin, HLA-DR, Ifn, IFN alpha/beta, IFN TYPE 1, **IL23A,** ISGF3, **LCN2, LTB,** lymphotoxin-alpha1-beta2, **MYD88, NCOA7,** NFκB (complex), NFκB (family), NfκB-RelA, **PIM3, PLK2,** Pro-inflammatory Cytokine, **RSAD2, SAA1, SAA2, SLPI,** Tlr, **TLR3,** Tnf, Tnf receptor, **TRAF, TXNIP, UBA7**	31	19	Antimicrobial response, antigen presentation, cell-to-cell signaling and interaction
3	ANKS1A, **C1ORF38,** CD7, CDKN2A, CEBP-AP-1, CEBPA, CPB2, CTSZ (includes EG:1522), CYP2C11L, DEFB3 (includes EG:27358), **FA2H, FAM111A,** fatty acid, FOS, **GLIPR2,** GPR109B, **GTSE1, HERC6,** HNF1A, **IFI6, IFI27,** IFNG, **KRT33A, MGAT4A,** MMRN1, NFYB, **OAS2, OPTN,** PLA2G16, **RAB20, RTP4, SAMD9, SECTM1, TBC1D22A, TEF**	29	18	Cellular growth and proliferation, hepatic system development and function, liver proliferation
4	20s proteasome, 26s proteasome, Akt, Ampa receptor, CaMKII, **CARD11,** Caspase, **CGN,** cldn, **CLDN1, CLDN4, CXCL1, EPPK1,** F actin, Fascin, Hsp27, Hsp70, **ID1, ID3,** Ikb, IKK (complex), Ikk (family), **LMO2, MAP3K8,** MHC Class I (family), **MLLT4,** NFκB1-RelA, **NFKBIA, OCLN, PLAC8, PVRL4,** Smad, **TJP2, TPM1,** Ubiquitin	27	17	Cardiovascular system development and function, cellular movement, gene expression
5	**APOL3 (includes EG:80833),** Calcineurin protein(s), **CCL5, CIITA, CSF1, CTSS,** Cytochrome c, Dynamin, Gm-csf, **GPRC5A, HLA-F, HSH2D (includes EG:84941),** IFN Beta, Ige, IgG, IL12 (complex), IL12 (family), Immunoglobulin, **INPP5D,** Interferon alpha, JAK, Jnk, Laminin1, MARCO, Mek, MHC Class II (complex), PARP, **PARP14, PPL,** STAT5a/b, **TACSTD2, TNFSF10, TRAF1, TRIM16, TRIM22**	27	17	Cell death, cell morphology, connective tissue development and function
6	**ARHGDIB,** Caspase 3/7, CD3, Collagen Alpha1, **CST6,** Growth hormone, Hdac, HISTONE, Histone h4, **IFI27, IFIT1, IFITM1, IGFBP3, IL7,** Importin alpha, **ING3,** Interferon-α Induced, **IRF9, JDP2,** MHC Class I (complex), **MUC4, MX1, MX2,** N-cor, Nfat (family), **NUP50,** P38 MAPK, SAA, **SAA4, STAT1,** Stat1-Stat2, **TYMS, USP18,** VitaminD3-VDR-RXR, **WNT4**	25	19	Infection mechanism, organismal injury and abnormalities, cancer
7	**APOL6,** CABC1, **CABLES1,** CASP3, CCAR1, **CLMN,** CLTCL1, **CMPK2, EMILIN2, EPC2, GGT6,** HNF4A, IFNA2, LTA4H, **MAL2,** MGST2, MT1B, MT1L, MT1X, **NAD+, OAS3 (includes EG:4940),** ORM2, **PEG10,** PEPD, **REC8,** REG1A, RNASE4, **RNF213,** RUVBL2, **SLC15A3,** TOPBP1, TP53, TP73, **TRIM14,** ZBTB11	21	15	Cell cycle, gene expression, infection mechanism
8	Calpain, collagen, Collagen type I, Collagen type IV, Collagen(s), Cpla2, Elastase, **ELF3,** Eotaxin, ERK1/2, Fibrin, Fibrinogen, Focal adhesion kinase, GC-GCR dimer, **GPER, IL32,** Integrin, Integrin alpha 3 beta 1, Integrin β, **ITGAV, ITGB2, ITGB8,** Laminin, Lfa-1, **MDK,** Mmp, **MMP7, PLAT, PPAP2B, RBM5, S100A10,** Tgf beta, **THBS1, TIMP2, VWF**	20	15	Tissue development, hematological system development and function, neurological disease

Table III. Continued.

ID	Molecules in network	Score	Focus molecules	Top functions
9	5430435G22RIK, ACP5, **AMOT,** AMOTL1, beta-estradiol, CHUK, **GBP2, GBP3,** GBP1 (includes EG:14468), **GBP4 (includes EG:115361), IFI6,** IFI35, IFIT1B, IFNA17, Interferon Regulatory Factor, IRF3, IRF7, IRF3-IRF7, **MX1, MX2,** NFRKB, **OAS2, OVOL1, PDZK1IP1, PLEKHA6,** PLG, SARM1, **SIGIRR,** TGFB3, TLR4, TLR12, **TSPAN2,** TSPAN5, **WFDC2, ZNF143**	20	15	Inflammatory response antimicrobial response infection mechanism
10	Actin, Alp, Ap1, **APOBEC3B, ATP2B4, BID,** Calmodulin, Cbp/p300, **CD24,** Ck2, Cyclin A, **ENC1, FGFR2,** FSH, **GBP2,** hCG, Histone h3, Hsp90, **HTT, JPH2,** LDL, Lh, **NR2F2, PAFAH1B1,** peptidase, Pka, **PRR15L,** Ras homolog, Retinoic acid-RAR-RXR, RNA polymerase II, TCR, Tubulin, **UBE2L3,** Vegf, **ZNF268**	16	14	Gastrointestinal disease, embryonic development, organismal development
1	AIM2, APOC1, BDKRB1, BEX2, CBLB, CSRP2, CTH, CXCR7, CYP27B1, DUSP6, FABP6, FXR ligand-FXR-Retinoic acid-RXRα, HDL, HMGA2, HOXD9, IER3, MLXIPL, NCOR-LXR-Oxysterol-RXR-9 cis RA, NFκB (complex), p85 (pik3r), Pak, Pdgf Ab, PDGF BB, PDGFA, Pdgfr, PEPCK, PHLDA1, PLC gamma, PLTP, Rxr, SCD, THBD, VAV, VAV3, VitaminD3-VDR-RXR	37	21	Lipid metabolism, molecular transport, small molecule biochemistry
2	ADA, CA9, Calcineurin protein(s), Caspase, CDKN2A, Cyclin A, Cytochrome c, E2f, FEZ1, GPX2, GSPT1, hCG, HIF1A, Histone h3, Histone h4, HOXD10, Hsp90, Lh, Mek, MT1G, NOL3, P38 MAPK, P4HA2, PFKFB4, PMEPA1, Rb, RNF128, SLC20A1, SLC7A11, SNAI1, STAR, STC2, STEAP1, TP63, Vegf	33	21	Gene expression, cellular development, connective tissue development and function
3	Akt, C5ORF13, CBLC, Collagen type I, Collagen(s), DUSP4, EFNB1, EFNB2, EGFR, ERK, Fibrinogen, Focal adhesion kinase, Gpcr, Growth hormone, Ige, IL1RAP, Integrin, ITGA5, JAK, JAK3, Mapk, Metallo-protease, MT2A, NUPR1, OSMR, PI3K, PREX1, PTPRE, Rac, Shc, STAT, STAT5a/b, TNS4, VEGFA, VSNL1	28	17	Cancer, lymphoid tissue structure and development, organismal development
4	ANKRD57, ANKS1A, ARID3A, ARID3B, CAPZA2, CLTCL1, cyclic AMP, DLG4, EFCAB1, ERBB2, FAM3C, GNAQ, GNG4, GPSM1, HMGB1 (includes EG:3146), HNF4A, HNRNPA0, HOXD11, HPCAL1, IFNA2, LANCL2, MIR204 (includes EG:406987), MT1B, MT1L, OAS3 (includes EG:4940), ORM2, PMEPA1, PRKCDBP, PRR7, RB1, TGFBI, THBD, TOPBP1, TP53, ZBTB11	28	18	Cell cycle, infection mechanism, cellular development
5	ADSSL1, ANKH, ARAP3, BCHE, beta-estradiol, CA2, CAPRIN2, CDK18, CLDN4, CSTA, FLOT1, GARS, GSTT1, HIST2H2BE, HOXA10, KRT7, KRT83, KRT86, KRT81 (includes EG:3887), MAOA, MFNG, MIR26B (includes EG:407017), MSGN1, PDZK1IP1, phosphatidylinositol-3,4,5-triphosphate, progesterone, SLC27A2, SMPDL3A, TACSTD2, TFCP2L1, TRH, TSPAN5, WFDC2, WISP2, YWHAZ	26	16	Dermatological diseases and conditions, genetic disorder, drug metabolism

Table III. Continued.

ID	Molecules in network	Score	Focus molecules	Top functions
6	26s proteasome, ACP5, ACPL2, Actin, Alp, Calmodulin, Ck2, Creb, DBN1, FSCN1, FSH, FUS, GJA1, Hsp70, IgG, IL12 (complex), Immunoglobulin, Interferon alpha, KCTD12, LMO4, MDFI (includes EG:4188), MIR1, MT1H, MT1L, MT1X, Pka, Pkc(s), RNA polymerase II, SGSM3, SH3PXD2A, SOX2, TCOF1, TCR, TINAGL1, Ubiquitin	25	16	Cell-to-cell signaling and interaction, connective tissue development and function, skeletal and muscular system development and function
7	AREG, Cbp/p300, Collagen Alpha1, Collagen type IV, CYR61, EGFR ligand, ERBB, ERBB4 ligand, EREG, ERK1/2, GNAZ, HAS3, HEY1, ITGB5, Laminin, LAPTM5, Ltbp, LTBP1, LTBP2, LTBP4, Mlc, NRG, NRG1, NRG2, Pdgf, RUNX2, SERPINE1, Smad, SMAD7, Smad1/5/8, Smad2/3, Smad2/3-Smad4, SPHK1, Tgf beta, TGFBI	25	18	Cellular movement, organismal functions, cardiovascular system development and function
8	14-3-3, ABCC1, Adaptor protein 1, AMPK, ANKH, Ap1, AP1S1, CA2, CPE, ERF, Estrogen Receptor, ETV4, GCLM, GCNT1, IKK (complex), IL1, IL1A, Insulin, Jnk, LAMB3, LDL, Mmp, Nfat (family), NGF, NT5E, p70 S6k, PC, Pi3-kinase, PPARG, PPARGC1A, Ras, Ras homolog, SDC1, SPP1, Trypsin	24	17	Gene expression, cellular development, connective tissue development and function
9	ALB, AMIGO2, AMY1A, AREG, C6ORF1, COL17A1, CSF1, EFS, EN1, FAM113B, FGF1, FHOD3 (includes EG:80206), FOXA2, GBP2, Gelatinase, GPR68, HCFC1R1, HMGCS2, KRT14, MMP9, NR3C1, PCSK6, Pdgf, PDGFA, POSTN, SLC29A1, SORBS3, TEAD4, TFEB, THBD, TNFSF14, TPP1, VAV3, WASF3, WT1	23	15	Connective tissue development and function, skeletal and muscular system development and function, tissue development
10	ACP5, CFDP1, COL6A1, DUSP4, Ecm, FCAR, GBP2, GPT2, GYLTL1B, HADH, HMGN5, HOXA2, IDI1, IL5, KCNG1, LIMK2, MIR124-1 (includes EG:406907), NEUROG3, NOMO1, OAF, PORCN, PRDX4, PRPS1, PYCR1, SLC5A5, SMAD3, SMURF2, SNAI2, TGFB1, TMED1, TPSAB1, TWIST2, WISP1, WNT4, WNT5B	21	14	Cell death, gene expression, cellular development

The composite score of the networks represents the negative log of the p-value for the likelihood that network molecules would be found together by chance. A higher score indicates greater statistical significance that molecules depicted in the network are interconnected.

Table IV. Top 5 IPA-recognized canonical pathways in cetuximab unresponsiveness-transcriptomic signature.

Name	p-value	Ratio
Interferon signaling (Fig. 3a)	7,33E-08	7/30 (0.233)
Role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis	5,44E-06	16/359 (0.045)
Role of pattern recognition receptors in recognition of bacteria and viruses	1,23E-05	7/82 (0.085)
Acute phase response signaling	6,97E-05	10/178 (0.056)
Death receptor signaling	8,81E-05	6/64 (0.094)
FXR/RXR activation	1,54E-05	8/103 (0.078)
Neuregulin signaling (Fig. 3b)	6,95E-04	6/103 (0.058)
VDR/RXR activation	1,91E-03	5/80 (0.062)
Ephrin receptor signaling	3,71E-03	7/198 (0.035)
Oncostatin M signaling	6,61E-03	3/35 (0.086)

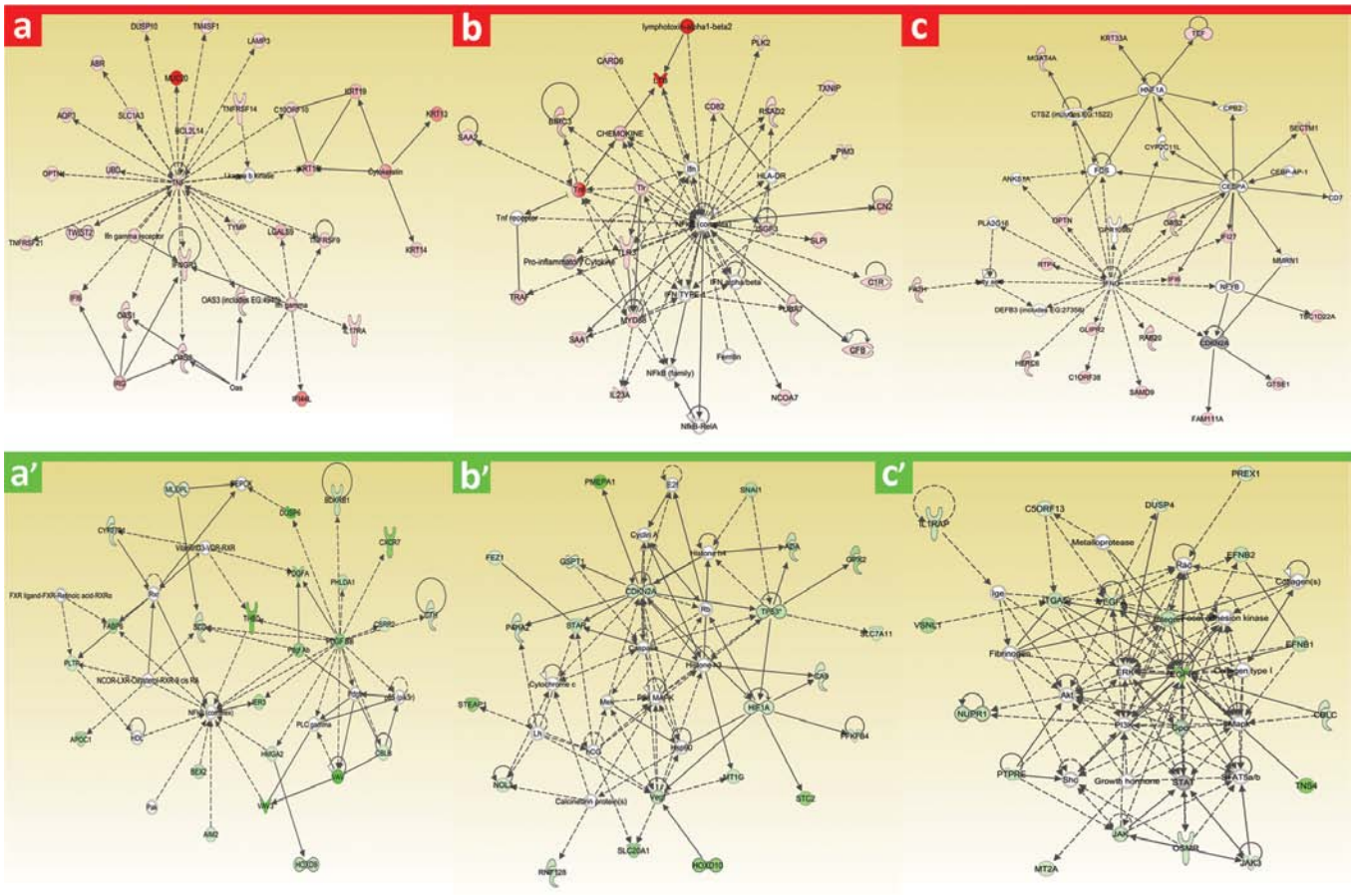

Figure 2. Network analysis of differentially-expressed genes in *KRAS* WT A431 tumor cell populations chronically adapted to grow in the presence of cetuximab. A dataset containing the differentially-expressed genes, called the *focus molecules* (n=473), between LT-cetuximab adapted A431 POOLs and cetuximab-naïve A431 parental cells was overlaid onto a global molecular network developed from information contained in the ingenuity pathway (IPA) knowledge base. Networks of these focus molecules were then algorithmically generated based on their connectivity. Figure shows up- and down-regulated networks (upper and bottom panels, respectively) with the three highest IPA score (a composite measure that indicates statistical significance that molecules depicted in the network are interconnected; Table III). Focus molecules are colored according to gene expression (fold-change) value; red gene symbols indicate up-regulation and green gene symbols indicate down-regulation. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges with dashed lines show indirect interactions, while a continuous line represents direct interactions.

cell populations appear to up-regulate members of the interferon signaling-input layer [i.e., the interferon gamma receptor (*IFNGR1*, ↑2.31-fold)], of the interferon signal-processing layer [i.e., the transcription factor signal transducer and activator of transcription 1 (STAT1, ↑2.1-fold)], which binds to the cytoplasmic portion of ligand-activated IFN-γ receptor and is phosphorylated by JAKS to cause STAT1/STAT1 and/or STAT1/STAT2 dimerization, nuclear translocation and binding of STAT1 to its cognate promoter DNA, the gamma interferon-activated site (GAS), and of the interferon signaling-output layer [i.e., STAT1-dependent transcription of the interferon-inducible genes *IFIT1* (↑2.26-fold), *IFITM1* (↑2.48-fold), *IRF9* (↑4.1-fold), *MX-1* (↑6.69-fold), *MX-2* (↑6.44-fold), *OAS1* (↑2.89-fold), *OAS2* (↑2.81-fold), and *OAS3* (↑2.27-fold), which are markers of enhanced interferon bioactivity].

Pathway-based analysis of genome-wide transcriptomic data reveals that 'retinoic acid' and 'neuregulin' signaling cascades are the crucial gene network functions down-regulated in cetuximab-unresponsive KRAS WT tumor cells. Fig. 2 (bottom panels) illustrates graphically the 3 top gene network functions significantly down-regulated within the 'cetuximab unresponsiveness-transcriptomic signature'. Top functions of up-regulated gene networks were related to: a) lipid metabolism, molecular transport, small molecule biochemistry (Fig. 2a'), including genes implicated in fatty acid synthesis, uptake, transported and metabolism such as stearoyl-coenzyme A (*SCD*) (37), apolipoprotein C1 (*APOC1*) (38) and fatty acid binding protein-6 (*FABP6*) (39) as well as genes coding for isoforms of the platelet-derived growth factors (*PDGFA, PDGFB*) and the guanosine nucleotide exchange factors (GEFs) *Vav* and *Vav2*, two members of the *Vav* family of proto-oncogenes that couple activation of multiple cell surface receptors (EGFR, PDGFR) to actin cytoskeletal rearrangements and transcriptional alterations during chemotactic-cell migration responses (40-42). This gene network, which also included the regulator of PDGF-induced activation of ERK (MAPK) *DUSP6* (also called *MKP3*) (43), was identified around PDGFBB and NF-κB complex. b) Gene expression, cellular development, connective tissue development and function (Fig. 2b'), including the cell cycle regulators *CDKN2A* (also called *MTS-1* or *p16*) and *TP63* (44,45) as well as the angiogenic/metastatic factors hypoxia-inducible factor 1 alpha (*HIF1A*) and vascular endothelial growth factor (*VEGF*) (46,47). This gene network, which included also EMT-related genes such as *SNAI2* (often called *SLUG*) - a pivotal transcription factor that initiate EMT transitions throughout development

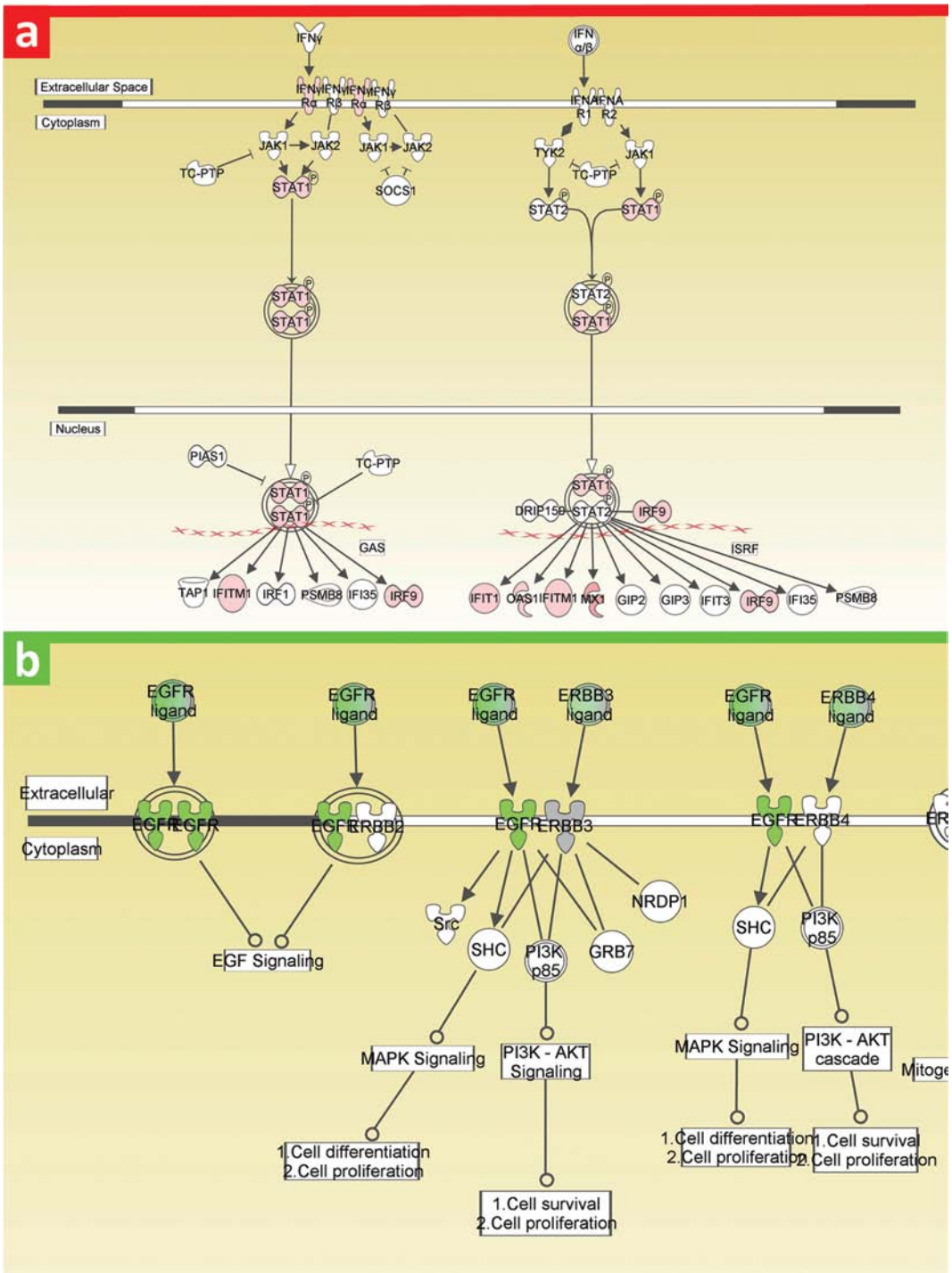

Figure 3. Canonical pathway analysis of differentially-expressed genes in *KRAS* WT A431 tumor cell populations chronically adapted to grow in the presence of cetuximab. Using the 'canonical pathways' feature of IPA global functional analysis, we were able to identify cetuximab response-related pathways that were significantly affected in LT-cetuximab adapted A431 POOLs when compared to cetuximab-naïve A431 parental cells. Figure shows two selected-high level canonical pathways up-regulated (top panel) and down-regulated (bottom panel) pathways differentially affected upon acquired resistance to cetuximab-induced cell growth inhibition (Table IV).

and in cancer metastasis (48), and prostate transmembrane protein, androgen induced 1 (*PMEPA1*), a TGFβ-induced marker of terminal colonocyte differentiation whose expression is maintained in primary and metastatic colon cancer (49), was identified around p38 MAPK. c) Cancer, lymphoid tissue structure and development, organismal development (Fig. 2c'), including genes closely related to cell adhesion/migration and angiogenesis such as *VEGFA*, members of the integrin family (*ITGA5*) (50), and the B1 and B2 members of the Ephrin receptor family (*EFBN1, EFBN2*) (51,52). This gene network was identified around the gene coding for the cetuximab target itself (i.e., *EGFR*).

When IPA was used to determine canonical pathway analysis enrichment categories '*FXR/RXR activation*' and '*neuregulin* signaling' (Fig. 3a) were the most statistically significant maps modulated by the up-regulated genes within the 'cetuximab unresponsiveness-transcriptomic signature'. Cetuximab-accommodated cell populations appear to speci-

fically down-regulate several members of the *neuregulin* signaling-*input layer* (53-55) including the HER1 (EGFR) ligand *AREG* (↓8.38-fold), the HER1/HER4 ligand *EREG* (↓5.69-fold), the HER3/HER4 ligand *NRG1* (neuregulin-1, ↓4.75-fold) and the HER1 receptor itself (*EGFR,* ↓8.745-fold).

Discussion

Early clinical studies demonstrated that immunohistochemistry-based assays measuring expression of the cetuximab target EGFR does not serve as a robust predictor for response (i.e., CRC patients can exhibit major objective response to cetuximab despite the absence of measurable EGFR) (56). In the same regard, no mutations in *EGFR* gene have been identified to date that are reliably predictive for response to cetuximab-based EGFR therapies (57). Although *KRAS* and *BRAF* mutations in primary CRC are predictive of nonresponse to cetuximab in patients with mCRC, an intuitive mechanism of primary resistance to cetuximab may relate to the presence of *KRAS* or *BRAF* mutations only in metastases. However, several recent studies have concluded that acquisition by metastases of a *KRAS* or a *BRAF* mutation that was not originally present in the primary tumor is a rare event that occurs solely in up to 5% of cases of mCRC (58-60). These studies have confirmed that a high concordance between the mutation status of *KRAS* and *BRAF* occurs in primary CRC and in corresponding metastases, therefore suggesting that other molecular mechanisms should exist that modulate intrinsic (primary) and/or acquired (secondary) resistance to cetuximab. De Reyniès *et al*, when testing the performance of the top genes of the cetuximab response signature for their ability to separate *KRAS* WT from mutant tumors, obtained clusters of samples highly associated to mutation in *KRAS* and in the serine-threonine kinase *BRAF* - the principal effector of *KRAS* (61). Given that *BRAF* WT is required also for response to cetuximab and could be used to select mCRC patients who are eligible for treatment (62), *KRAS* mutation signatures in mCRC significantly overlaps with the cetuximab response signature. In this study, the expression signature of the response to cetuximab monotherapy was obtained in a disease control population significantly enriched for *KRAS* WT patients relative to the nonresponder population. Therefore, it is reasonable to argue that we should expect a significant overlapping between set of genes that are associated with disease control after cetuximab treatment and sets of genes that are differentially-expressed as a function of *KRAS* mutational status (61). Indeed, there is a significantly higher expression of the mRNAs of *DUSP4* and *DUSP6* in *KRAS* mutant compared to WT mCRC treated with cetuximab and high *DUSP4* expressing WT *KRAS* tumors may have undetected *KRAS* or *BRAF* mutations (14). Further supporting this notion of an inherent overlap between *KRAS* mutation and cetuximab response signatures, the EGFR ligands *EREG*, *HB-EGF* (heparin-binding EGF-like growth factor) and *BTC* (betacellulin) as well as the MAPK phosphatases *DUSP4* and *DUSP6*, have been all found to be co-up-regulated among the multiple genes induced by several activating *KRAS* mutations (including those in the *KRAS* hotspots codons 12 and 13 and a novel ones outwith hotspot codons) in NIH-3T3 fibroblasts (63).

Because molecular mechanisms other than activating *KRAS* mutations should modulate the occurrence of either weaker or stronger responses to cetuximab in tumors with an intact regulation of *RAS* signaling, we here envisioned that *KRAS* WT tumor cell-line models chronically exposed to cetuximab can be interrogated to obtain novel exploratory biomarkers for cetuximab efficacy. By tracking the evolution of transcriptomic changes during cetuximab-induced chronic prevention of EGFR activation and long-term deactivation of EGFR/RAS/MAPK pathway as determined by pathway-based functional analysis of whole human-genome microarray data, we confirmed that cetuximab is significantly more effective at inhibiting the growth of EGFR-positive *KRAS* WT tumor cells overproducing the EGFR ligands (e.g., *AREG, EREG*) compared with those producing low amounts of EGFR ligands. Yonesaka *et al* pioneeringly focused on ligand-mediated activation of EGFR and identified autocrine production of AREG as an important biomarker associated with growth inhibition by cetuximab in non-small cell lung cancer (NSCLC) cell lines and patients (64). In their hands, cell lines overproducing AREG were significantly more likely to be growth inhibited by cetuximab than those that produced minimal or no AREG. Our current study shows that expression of *AREG* and *EREG* as assessed by microarray analyses may be an appropriate biomarker for identifying patients who will benefit from treated with cetuximab. Indeed, it is reasonable to suggest that, in terms of EGFR ligands and *neuregulin* signaling, the most significant distinction between primary and secondary refractoriness to cetuximab appears to be just the timing of detection. *AREG* and *EREG* mRNAs have been considered beacons of an activated EGFR pathway as part of a necessary positive feedback loop for cetuximab efficacy. Thus, EGFR ligands such as AREG stimulate the growth of cetuximab-sensitive cells whereas inhibition of the EGFR ligand expression alone (either by a neutralizing antibody, or by siRNA) is sufficient to inhibit cell growth (64). Given that acute treatment with cetuximab rapidly down-regulates the mRNA expression levels of *AREG* and *EPI* in cetuximab-naïve tumor cells including A431 parental cells (9; data not shown), it is reasonable to suggest that the occurrence of AREG/EREG-mediated autocrine activation of EGFR is sufficient and necessary to allow functioning of cetuximab in EGFR-positive *KRAS* WT tumor cells. We can hypothesize that chronic exposure to cetuximab negatively select initially dominant (cetuximab-sensitive) AREG/EREG-positive clones and promote selection of (cetuximab-refractory) tumor clones constitutively bearing a down-regulated *neuregulin* signaling cascade (Fig. 4). The ability of accurately establish an '*AREG*/*EREG* mRNA threshold' that clinically predicts functioning of cetuximab may indirectly inform about the intra-tumor clonal heterogeneity of *AREG*/*EREG* mRNA status during tumor progression and treatment. This strategy may help select *KRAS* WT mCRC patients before initiation of cetuximab-based treatments to avoid the rapid selection of existing cetuximab-resistant AREG/EREG-negative clones.

Given that IFN/STAT1 signaling has traditionally been connected with pro-apoptotic tumor-suppressor functions (30, 31,33-36,65), an adaptive scenario analogous to that described above for the down-regulated *AREG*/*EREG* mRNA expression status may account for the counterintuitive up-regulation of the IFN/STAT1 signaling in *KRAS* WT tumor cell populations chronically adapted to grow in the presence of cetuximab. Emerging data begin to reveal that, in certain cellular contexts, the IFN/STAT1 pathway may mediate tumor cell growth, meta-

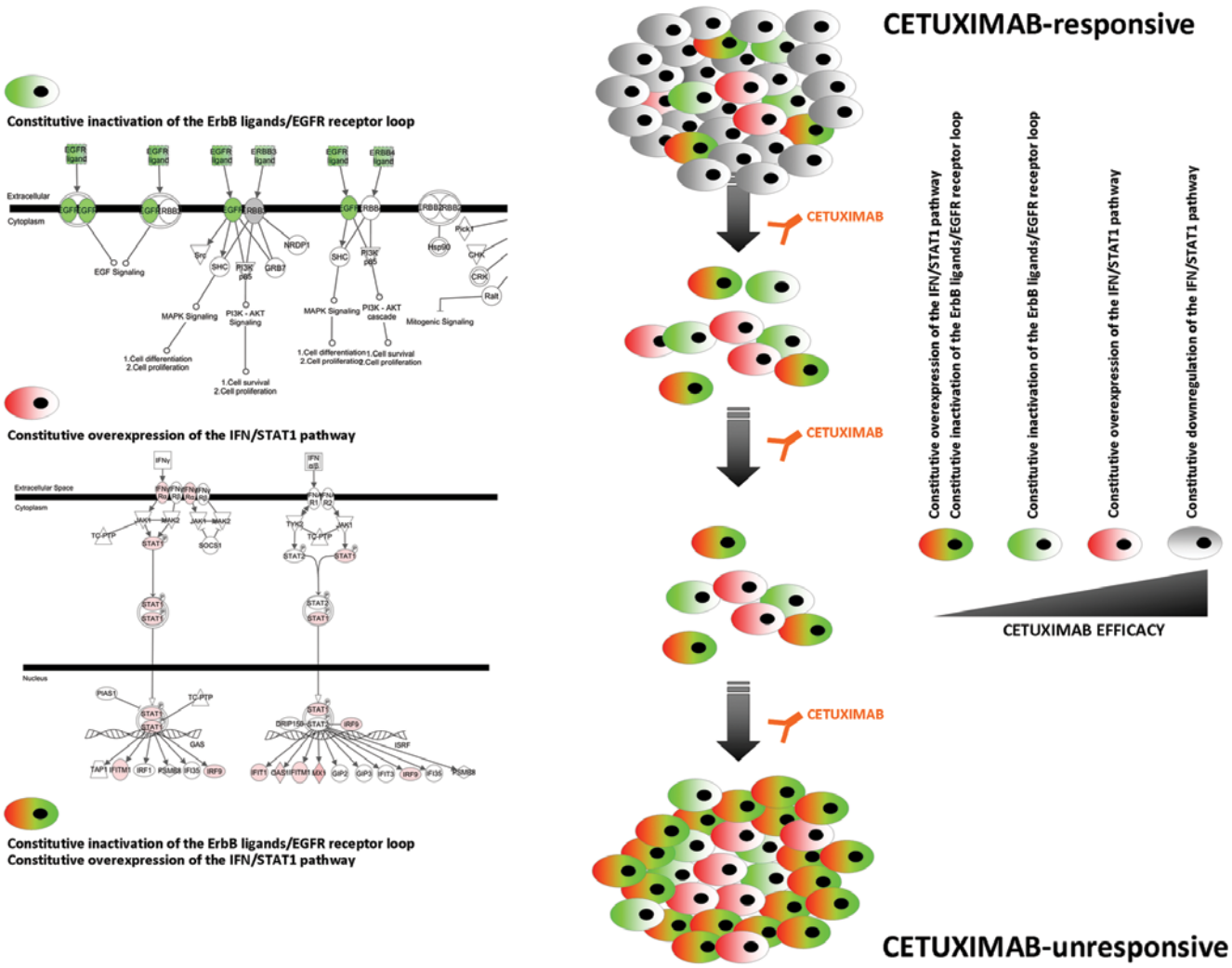

Figure 4. A schematic depicting the molecular events (i.e., signaling pathways) required for EGFR-dependent *KRAS* WT A431 tumor cell populations to grow upon chronic cetuximab-induced inhibition of EGFR signaling.

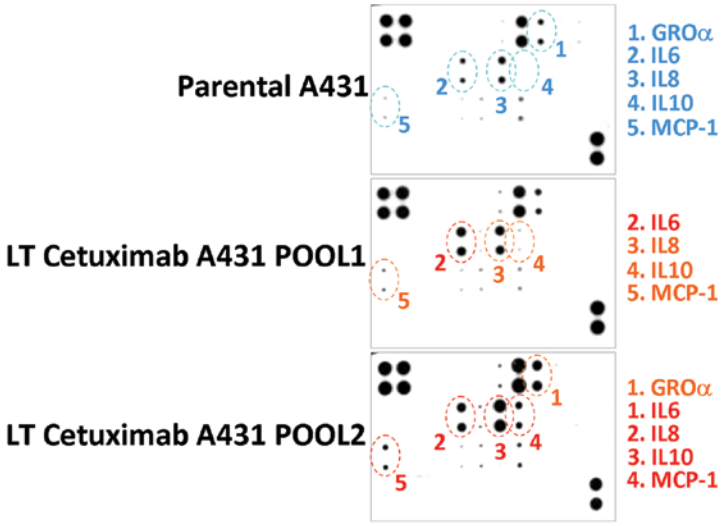

Figure 5. Cytokine microarray analysis of conditioned media from *KRAS* WT A431 tumor cell populations chronically adapted to grow in the presence of cetuximab. Comparison of cytokine microarray membranes from parental A431 cells and LT-cetuximab adapted A431 POOLs. The cytokines altered in POOL1 or POOL2 cells compared with untreated A431 parental cells are marked and numbered on the membranes and correspond to: 1, GROα; 2, IL6; 3, IL-8; 4, IL-10; 5, MCP-1. Densitometric data were arbitrarily expressed as orange for moderate to high (from 2-fold up to 5-fold increase) or red for extremely high (>5-fold increase) when compared to baseline (blue) levels found in parental A431 control cells.

static potential and resistance to certain therapies. Khodarev *et al* pioneeringly revealed that a radioresistant tumor that was selected against fractionated ionizing radiation from a radiosensitive parental tumor (and resistant to IFN-mediated cytotoxicity) constitutively overexpressed the IFN/STAT1 pathway (66,67). Recent studies have confirmed that constitutive induction of the STAT1 signaling positively associates with protection of tumor cells from genotoxic stress following treatment with fludarabine (68), doxorubicin (69), cisplatin (70), and the combination of ionizing radiation with doxorubicin (71). Our current findings confirm and expand further the notion that IFN/STAT1 pathway overexpression not only relates to resistance to cytotoxic therapy including radiation and chemotherapy but also to molecularly targeted treatments such as the anti-EGFR monoclonal antibody cetuximab. Khodarev *et al* have recently demonstrated that tumor clones that constitutively overexpress the IFN/STAT1 pathway appear to be positively selected by tumor microenvironment due to a resistance to STAT1-dependent cytotoxicity and demonstrate increased metastatic ability combined with increased resistance to IFN and genotoxic stress relative to parental, low-expressors of the IFN/STAT1 signaling pathway (72). In our hands, acute treatment of A431 parental cells with high-dose cetuximab (i.e., 100 μg/ml) was found to notably up-regulate gene expression for the IFN/JAK/STAT1 pathway as part of the transcriptomic signature associated with cetuximab-induced cell growth inhibition (data not shown). Upon chronic exposure to cetuximab, therefore, activation of the IFN/STAT1 pathway appears to paradoxically switch from 'growth inhibitory' to 'pro-survival' in response to cetuximab-induced cell injuries. This molecular switch can be explained when considering the pre-existence of clones inherently resistant to the cytotoxic effectors of the IFN/STAT1 signaling pathway that overexpress STAT1-regulated genes with pro-survival functions [e.g., *IFITM1* (73)] and they are intrinsically resistant to a wide variety of tumoricidal insults including blockade of EGFR-driven tumor growth promoting signaling. We can hypothesize that chronic exposure to cetuximab negatively select initially dominant IFN-sensitive clones and promotes selection of IFN-refractory tumor clones constitutively bearing an up-regulated *IFN/STAT1* signaling cascade (Fig. 4).

KRAS mutation status is being used as a biomarker to predict cetuximab therapeutic efficiency but it is not sensitive enough to determine the absolute indication of cetuximab in mCRC with an intact *KRAS* signaling pathway. The expression (or lack thereof) of other biomarkers should, therefore, actively regulate weaker or stronger responses to cetuximab. Because molecular mechanisms other than activating *KRAS* mutations should modulate intrinsic (*de novo*) and secondary (acquired) resistance to cetuximab in tumors with an intact regulation of RAS signaling, pre-clinical development and characterization of cultured cetuximab-resistant *KRAS* WT tumor cells might elucidate molecular features equivalent to those expected in the important sub-group of *KRAS* WT mCRC patients that do not benefit from cetuximab. In this regard, an ever-growing body of research with cultured cancer cells has substantially enhanced our current understanding of the molecular mechanisms implicated in resistance to the anti-EGFR antibody cetuximab including (reviewed in ref. 74): a) overexpression of the EGFR ligand transforming growth factor-α (TGF-α) (75); b) overexpression/overactivation of the cetuximab target (EGFR; HER1) itself and of other HER family members (HER2, HER3) (76,77); c) dysregulation of EGFR internalization or degradation upon alteration of EGFR ubiquitination (76-78); d) subcellular distribution of EGFR and/or altered EGFR trafficking (79,80); enhanced expression of VEFG/VEGFR (81-83); and occurrence of epithelial-to-mesenchymal transition (EMT) (84). Obviously, this evolving list that has been generated using pre-clinical models may provide new candidate molecules capable to predict resistance to cetuximab, but all of them lack a definitive validation as clinically useful predictive biomarkers. Our current findings on *neuregulin* and *IFN/STAT1* signaling are also hypothesis-generating and need to be further validated in prospective clinical studies and furthermore in paired primary and metastatic tumor specimens from cancer patients that have not been treated with cetuximab to determine the prognostic and/or predictive nature of these exploratory biomarkers. It should be noted, however, that unlike *EGFR* mutations, which are almost uniformly restricted to NSCLC (85) and *KRAS* mutations, which are informative of cetuximab inefficacy solely in mCRC (86,87), the expression status of mRNAs coding for EGFR ligands (e.g., *AREG* and *EREG*) and INF-related genes may be appropriate biomarkers for identifying patients who will benefit from treated with cetuximab in cancers other than CRC [e.g., for locally/regionally advanced head and neck squamous cell carcinoma (HNSCC) in combination with radiotherapy and as monotherapy for recurrent/metastatic HNSCC after failing platinum-based chemotherapy] in which this EGFR-targeted antibody is widely in clinical use (88-90).

In conclusion, the feasibility of monitoring the expression status of *neuregulin* and *INF/STAT1* signaling pathways by performing serial fine-needle aspirations and/or core biopsies to provide enough material for mRNA-based profiling studies might be explored in future studies as it may confirm *AREG, EREG* and/or IFN/STAT1-regulated genes as important predictors of response during and after cetuximab treatment in patients bearing *KRAS* WT tumors. Provided that cancer cells are shed into the circulation proportional to their clonal frequency, quantitative real-time RT-PCR analyses of *AREG, EREG* and/or *IFN/STAT1* mRNA markers originating from circulating tumor cells (CTC) during cetuximab therapy may provide direct evidence of positive or negative clonal selection (91-94), thus allowing early modification or adjustment of the therapeutic strategy before clinical progression to cetuximab will occur. Alternatively, the identification of soluble factors specifically secreted upon activation of pathway signatures using ELISAs or microarrays may predict the efficacy of cetuximab. For instance, activation of the IFN/STAT1 pathway leads to the induction of multiple pro-survival cytokines and radioresistance of STAT1-overexpressing tumor cells has been associated with increased production and secretion of interleukin-6 (IL6) and interleukin-8 (IL8) (73), two genes which expression can be controlled through direct interaction of STAT1 with *IL6* and *IL8* gene promoters (95,96). Experiments in our laboratory using human cytokine antibody arrays to determine the profile of cytokines in conditioned media (97) from cetuximab-naïve A431 parental cells and from LT-cetuximab adapted A431 POOL1 and POOL2 confirmed that IFN/STAT1-overexpressing cetuximab-refractory cells likewise secrete significantly higher amounts of IL6 and IL8 as well as of IL10, GROα and MCP-1 (Fig. 5). These findings

suggest that circulating levels of IL6 and/or IL8 might represent unexpected novel biomarkers for monitoring tumor responses to cetuximab-based therapies. Obviously, our current identification of functional gene expression signatures as surrogate markers for understanding the efficacy of cetuximab at the molecular level should be integrated with parallel whole-transcriptomic analyses of clinical trial tissues to validate the robustness of these signatures to improve the identification of cetuximab responders and to predict therapeutic outcome of *KRAS* WT squamous carcinoma patients being treated with cetuximab-containing regimens (98,99).

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