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RasGRP1 is a potential biomarker to stratify anti-EGFR therapy response in colorectal cancer

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Abstract

Colorectal cancer (CRC) is the third most frequent neoplastic disorder and is a main cause of tumor-related mortality as many patients progress to stage IV metastatic CRC. Standard care consists of combination chemotherapy (FOLFIRI or FOLFOX). Patients with WT KRAS typing are eligible to receive anti-EGFR therapy combined with chemotherapy. Unfortunately, predicting efficacy of CRC anti-EGFR therapy has remained challenging. Here we uncover that the EGFR-pathway component RasGRP1 acts as CRC tumor suppressor in the context of aberrant Wnt signaling. We find that RasGRP1 suppresses EGF-driven proliferation of colonic epithelial organoids. Having established that RasGRP1 dosage levels impacts biology, we focused on CRC patients next. Mining five different data platforms, we establish that RasGRP1 expression levels decrease with CRC progression and predict poor clinical outcome of patients. Lastly, deletion of one or two Rasgrp1 alleles makes CRC spheroids more susceptible to EGFR inhibition. Retrospective analysis of the CALGB80203 clinical trial shows that addition of anti-EGFR therapy to chemotherapy significantly improves outcome for CRC patients when tumors express low RasGRP1 suppressor levels. In sum, RasGRP1 is a unique biomarker positioned in the EGFR pathway and of potential relevance to anti-EGFR therapy for CRC patients.
Introduction

Colorectal cancer (CRC) represents the third most frequent neoplastic disorder worldwide and one of the main causes of tumor-related mortality (1). CRC patients receive combination chemotherapy such as FOLFIRI (leucovorin, fluorouracil, and irinotecan) and FOLFOX (leucovorin, fluorouracil, and oxaliplatin) and these have become the main chemotherapeutic treatment options for CRC (2, 3). Thirty five percent of CRC patients are diagnosed as stage IV metastatic CRC and a large proportion of stage II and III CRC patients progress to stage IV at some point during their course of disease (4). Unfortunately, 5-year survival rate for stage IV metastatic CRC patients is only approximately 13 percent (https://www.cancer.org/). EGFR (Epidermal Growth Factor Receptor) expression is widespread in CRC (5). Use of targeted therapies that inhibit the action of the EGFR, such as cetuximab an antibody that binds and interferes with the EGFR, have been approved in combination with chemotherapy for metastatic CRC (6). Multiple randomized trials including both chemo-refractory and chemo-naive patients with metastatic CRC have established the overall survival (OS) benefits of the anti-EGFR blocking antibodies cetuximab and panitumumab (5). Examples are the CALGB80203 trial with metastatic CRC patients on FOLFIRI/FOLFOX therapy that was closed early (7) and the CALGB80405 trial that compares FOLFIRI/FOLFOX therapy with bevacizumab (anti-VEGF) or with cetuximab (CALGB stands for Cancer and Leukemia Group B, now part of Alliance for Clinical Trials in Oncology).
 Nonetheless, it has been challenging to predict success with these antibodies or with EGFR kinase inhibitors (erlotinib) in CRC. After introduction of cetuximab and panitumumab into the clinic, evidence soon arose that tumors harboring activating mutations in $KRAS$ are insensitive to EGFR inhibition. Somatic mutations in $KRAS$ (8, 9) occur in roughly forty per cent of CRC patients (10) and these mutations in $KRAS$ have also been implicated as mediators of acquired resistance to anti-EGFR therapy (11, 12). Furthermore, roughly four percent of CRC patients reveal mutations in $NRAS$ (13). These notions led to systematic, US Food and Drug Administration (FDA) approved typing for $RAS$ as an accompanying diagnostic and since 2012 anti-EGFR therapy is restricted to patients without detectible $KRAS$ and $NRAS$ mutations (5). Still, there remains a significant gap between available analytical tools used to assess therapeutic benefit or risk, the likelihood of response or progression, and actual patient clinical outcome and it is clear that $KRAS$ and $NRAS$ status are not the only determinants. Further, anti-EGFR therapy is costly and can be toxic; thus, the need to better understand the role of EGFR signals in CRC and to identify additional predictive markers is clear.

In the intestine, Wnt ligands signal to ensure self-renewal of stem cells in the crypts regions that produce daughter cells (14). Wnt signals support stem cell function in many organs and enable the generation of organoids that can be perpetuated in Matrigel in vitro (15, 16). Binding of the ligand R-spondin to the receptor Lgr5 (Leu-rich repeat-
containing receptor 5) enables sustained Wnt signals (17) and R-Spondin and surrogate Wnt ligands are highly effective in sustaining the growth of organoids (18). Stem- and progenitor- cells in the intestine also get exposed to EGFR signals (14) and Ras-MAPK signals are observed in human progenitor cells in normal intestinal crypts as well (19). Deletion of the egfr in mice leads to disorganized crypts (20) and fine-tuning of EGFR signaling is critical for balanced proliferation in the intestinal stem cell niche (21, 22). Deletion of Kras has no effect on the adult intestinal epithelium in mice (23), but expression of an oncogenic form of Kras, Kras\textsuperscript{G12D}, in the murine colonic epithelium leads to hyperproliferation in a Ras-MAP kinase dependent manner (24). The above-mentioned studies imply that EGFR-Ras signals balance proliferation and differentiation in intestinal progenitor cells (19, 21, 22). Better understanding of how EGFR-Ras signaling nuances impact CRC and particularly responses to anti-EGFR therapy could improve the clinical uses of EGFR-targeting agents.

The EGFR couples to Ras guanine nucleotide exchange factors (RasGEFs) that can activate the small GTPase Ras (25). We previously established that two RasGEFs, RasGRP1 and SOS1, are structurally and biochemically different (26-29). Both RasGRP1 and SOS lie downstream of the EGFR (30) and we demonstrated that these RasGEFs play opposing roles in the intestine. Whereas SOS1 stimulates proliferation of cancer cells (30, 31), RasGRP1 opposes EGFR-SOS1 signals and as such suppresses proliferation (30). Rasgrp1 deficiency results in hyperproliferation of non-transformed intestinal epithelial cells and leads to exacerbation of CRC when this epithelium also
carries oncogenic mutations in *KRas* or *APC* (adenomatous polyposis coli) (30, 32, 33).

Here, we established that RasGRP1 features as a tumor suppressor in the colon and suppresses EGF-driven proliferation of colonic organoid cultures. Decreases in RasGRP1 levels are prognostic of poor clinical outcome for CRC patients. Lastly, spheroid assays and clinical trial data reveal that RasGRP1 expression levels predict efficacy of anti-EGFR therapy in CRC.
Results

*RasGRP1 is a tumor suppressor in the colonic epithelium of Apc\textsuperscript{Min/+} mice*

Given that RasGRP1 opposes proliferative EGFR-SOS1 signals in CRC (30), we performed a more detailed analysis of the expression levels of RasGRP1 and tested our hypothesis that deletion of only one Rasgr1 allele may already have a biological impact.

We first mined published gene expression data deposited in the GSE49355 database on sets of trio samples from individual patients with advanced colorectal cancer patients receiving FOLFIRI (34). This platform revealed that the levels of RasGRP1 expression in epithelium from the same patient is lower in primary colorectal cancer compared to normal tissue. There was an additional decrease in RasGRP1 expression in the comparison between primary CRC and CRC metastasis in the liver (Figure 1A), suggesting that levels of RasGRP1 could play a role in CRC progression.

We next determined Rasgrp1 expression levels along five segments of the intestinal track in the mouse (Figure 1B). We utilized the intestine from Rag1-deficient mice that do not have T- or B- cells, to avoid potential sample contamination with these lymphocyte subsets that express high levels of Rasgrp1 (35). We observed that Rasgrp1 protein levels are highest in the duodenum and more moderate in the other parts along the intestinal track (Figure 1C and Supplemental Figure 1), a pattern that was mirrored by Rasgr1 mRNA expression data (Figure 1D). By contrast, the mRNA
expression levels of the RasGEF Sos1, pro-proliferative in its function (30, 31), were relatively similar throughout the intestinal tract (Figure 1E).

In mouse models, heterozygosity for Rasgrp1 result in roughly half the Rasgrp1 protein expression (36). To test the implications of reduced levels of RasGRP1 expression in the context of CRC, we capitalized on the Apc\(^{Min/+}\) mouse model with aberrant Wnt signaling (37) that is widely used to mimic human CRC (38-40). Complete deficiency in Rasgrp1 results in reduced survival of Apc\(^{Min/+}\) mice due to increased EGFR-SOS1 signals driving proliferation (30). Here, we uncovered that Rasgrp1 has stereotypical features of a tumor suppressor; Apc\(^{Min/+}\) mice succumb sooner to disease when these mice harbor only one allele of Rasgrp1 (Figure 1F and Supplemental Figure 2A). Apc\(^{Min/+}\) mice develop frequent tumors in the small intestine (SI) but rarely in the colon (38-40), a limitation of this murine model for human CRC. Deficiency or heterozygosity for Rasgrp1 in the context of Apc\(^{Min/+}\) did not result in altered numbers or size of SI tumors (Supplemental Figure 2B and Depeille et al. (30)); instead there is an impact on the colon. Strikingly, there is a prominent increase in the frequency of tumors in the distal part of the colon when Rasgrp1 is deleted (Figures 1G and H). Individual tumors heterozygous for Rasgrp1 grew to a larger size before we had to euthanize Apc\(^{Min/+}:Rasgrp1^{WT/-}\) mice while the absolute number of tumors was not different from those in Apc\(^{Min/+}\) mice (Figure 1I and Supplemental Figure 2C). It should be noted that tumor data in Figure 1G-I and Supplemental Figure 2C originated from mice of different ages and that we had to euthanize Apc\(^{Min/+}:Rasgrp1^{WT/-}\) and Apc\(^{Min/+}:Rasgrp1^{-/-}\)
mice at earlier age. Of note, the effects on colonic tumors were not caused by T cell defects; first, \textit{Rasgrp1}^{WT/-} have normal T cell development (41) and, secondly, when we grafted \textit{Apc}^{Min/+}::\textit{Rasgrp1}^{-/-} mice with \textit{Apc}^{Min/+} bone marrow to circumvent the known T cell deficiency in \textit{Rasgrp1}^{-/-} mice (41) we also observed colonic tumors (Supplemental Figure 3). In sum, these results reveal that \textit{Rasgrp1} is a tumor suppressor gene in colonic epithelium with aberrant Wnt signaling.

\textit{Rasgrp1 suppresses EGFR-driven growth of colonic organoids}

The features of increased number and size of colonic tumors in \textit{Apc}^{Min/+} mice caused by decreases in \textit{Rasgrp1} alleles share similarities with \textit{Apc}^{Min/+} mice that are deleted for \textit{lrig1} (22). \textit{Lrig1} (Leucine-rich repeats and immunoglobulin-like domains 1) is a transmembrane molecule and an intestinal stem cell marker (22). Tet-induced expression of \textit{Lrig1} provides negative feedback to EGFR signaling (42) to balance intestinal stem cell homeostasis (21). Because of these similarities, we next investigated the functional impact of \textit{Rasgrp1} deletion in the normal colonic epithelium, by optimizing the generation of colonic epithelial organoids (Figure 2A).

Colonic epithelial organoids were originally described by Sato et al. (43). We capitalized on R-Spondin and Wnt surrogate ligands that trigger Lgr5 and LRP/Frizzled receptors to phenocopy sustained, canonical Wnt signaling (18) (Figure 2B). These Rspo-Wnt ligands allowed for efficient generation of colonic epithelium organoids (termed colonic organoids here), from both young WT and young \textit{Rasgrp1}^{-/-} mice (Figure 2C). WT and
Rasgrp1−/− colonic organoids reveal similar levels of Egfr and Sos1 expression (Figure 2D). Rasgrp1−/− colonic organoids proliferated more vigorously than WT counterparts in Rspo-Wnt surrogate-supplemented Matrigel (Figure 2E). We subsequently analyzed growth of organoids at day 3, a time-point of exponential growth phase. The growth rate was drastically reduced in both WT and Rasgrp1−/− organoids when these were subjected to EGFR kinase inhibitor erlotinib (Figure 2F), arguing that proliferation is EGFR signaling-dependent. In sum, Rasgrp1 limits proliferation of colonic organoids, and organoid proliferation requires uninhibited EGFR signaling.

RasGRP1 is a prognostic marker for clinical outcome of CRC

Given our findings on Rasgrp1’s role in mouse colonic organoids, we next explored RasGRP1 expression related to clinical outcome in samples from colorectal cancer patients using four different platforms. We first interrogated The Cancer Genome Atlas (TCGA). Mutations in APC, the tumor suppressor P53, and in KRAS are the three most frequent genetic events leading to colorectal cancer (44). Using TCGA data, we found that RasGRP1 expression levels are lower in patients with mutations in the APC or TP53 tumor suppressors, when compared to patients with wildtype APC or P53 alleles in their tumors (Figure 3A and 3B). We did not observe a significant difference in RasGRP1 expression when tumors are stratified solely on KRAS status (Figure 3C). However, in agreement with the pattern of decreasing RasGRP1 expression when CRC advances in Figure 1A, we observe
progressively lower \textit{RasGRP1} levels when tumors accumulate more mutations in the three main drivers \textit{APC}, \textit{P53}, or \textit{KRAS} (\textit{Figure 3D}). By contrast, \textit{SOS1} expression levels did not reveal any alterations as a function of mutational load for \textit{APC}, \textit{P53}, or \textit{KRAS} (\textit{Figure 3E}).

Second, we capitalized on our own collection of resected, bio-banked liver metastases from stage IV colorectal cancer patients to establish that there is a range of \textit{RasGRP1} mRNA expression in tumor samples that presents itself in a continuum, both for \textit{KRAS}^{WT} and \textit{KRAS}^{MUT} tumors (\textit{Figure 3F}). By and large, the levels of \textit{RasGRP1} protein, determined by immunohistochemistry with a \textit{RasGRP1}-specific monoclonal antibody (\textit{Figure 3G}), paralleled the mRNA levels.

Third, to assess if \textit{RasGRP1} levels may have prognostic value for clinical outcome of CRC, we first explored the correlation between \textit{RasGRP1} expression and survival of 752 CRC patients who were enrolled in the PETACC3 trial (Pan European Trial Adjuvant Colon Cancer). PETACC3 (45) includes stage II and stage III patients and was designed to investigate whether disease-free status improved when irinotecan was added as adjuvant to infusional FU/LV combination (5-Fluorouracil/Leucovorin). Instead of applying more commonly used cut-off finder software, we divided 752 patients without any bias in exact 50/50 groups (\textit{RasGRP1}^{HIGH} and \textit{RasGRP1}^{LOW}) based solely on \textit{RasGRP1} expression level in their tumors determined on the ALMAC Colorectal Cancer DSA platform (Craigavon, North Ireland) (45, 46). We next correlated \textit{RasGRP1}^{HIGH} and \textit{RasGRP1}^{LOW} to overall survival. This unbiased 50/50 split revealed that \textit{RasGRP1} has
prognostic value as a single marker on 282 stage II and stage III patients with $KRAS^{MUT}$, with the 141 RasGRP1$^{\text{HIGH}}$ patients showing significantly prolonged survival (Figure 3H). When all 752 stage II and stage III patients were analyzed in one group, RasGRP1 did not have prognostic value as a single marker (Supplemental Figure 4). Dividing patients in SOS1$^{\text{HIGH}}$/SOS1$^{\text{LOW}}$ did not correlate with survival, irrespective of $KRAS$ status of the tumor (Supplemental Figure 4).

There are various features in CRC that have been described to correlate with clinical outcome. One of these is microsatellite stability or instability. Microsatellite instability (MSI) generally correlates with better clinical outcome (47, 48), and in agreement with this notion, we observed better clinical outcome in the 75 PETACC3 patients who demonstrated MSI (Supplemental Figure 5A). On average, these 75 MSI-positive PETACC3 patients had higher RasGRP1 levels than the 603 MSS (Microsatellite stable) patients (Supplemental Figure 5B). Combining both RasGRP1 expression and MSI or MSS for the patient group analyzed in Figure 3H revealed that MSI or MSS does not add additional discriminatory power for prediction of clinical outcome on the basis of RasGRP1 expression (Supplemental Figure 5C).

Lastly, we explored a fourth platform with focus on metastatic CRC. We assessed RasGRP1 and SOS1 expression levels, using primary CRC with patient-matched liver metastases from the MOSAIC study (Multicenter International Study of Oxaliplatin/5-Fluorouracil/Leucovorin in the Adjuvant Treatment of Colon Cancer with stage II and III CRC). Paired differential analysis revealed that RasGRP1 levels significantly decrease in liver metastases compared to the primary tumors (Figure 3I, blue colored lines). By
contrast, SOS1 expression levels are not significantly altered (Figure 3I, red and blue lines). In sum, expression levels of RasGRP1 – but not of SOS1 – decrease when CRC tumors become more aggressive and RasGRP1 shows potential as a future prognostic marker for CRC clinical outcome in several platforms.

Murine CRC spheroids with less Rasgrp1 are more sensitive to anti-EGFR therapy

Given the challenge to identify which individual patient will benefit most from anti-EGFR therapy and the clinical and financial implications hereof in CRC, we wanted to explore if RasGRP1 might be a valuable biomarker for the efficacy of anti-EGFR therapy.

To first systematically test this idea that was based on RasGRP1’s suppressive actions, we generated organoids from murine colonic tumors (named spheroids here) (Figure 4A). Spheroids from tumors found in the distal portion of the colon of Apc\textsuperscript{Min/+}, Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{+/-}, and Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{-/-} mice were established with equal efficiency (data not shown), manifested the stereotypic sphere appearance (Figure 4B). In agreement with increased Wnt signals when Apc function is perturbed (37), Apc\textsuperscript{Min/+} and Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{-/-} spheroids proliferated spontaneously without addition of R-Spondin and Wnt surrogate ligands (Figure 4C).

EGFR signaling is known to critically contribute to Apc\textsuperscript{Min/+}-driven pathology; a hypomorphic Egfr\textsuperscript{wa2} allele crossed into Apc\textsuperscript{Min/+} results in a 90% reduction of intestinal
polyps (49). Addition of exogenous EGF to the Matrigel revealed that this growth factor drove proliferation in all spheroids but more extensively in $Apc^{\text{Min/}+} : Rasgrp1^{-/-}$ spheroids that lack suppression by Rasgrp1 (Figure 4D). Furthermore, inclusion of erlotinib in the media submerging the Matrigel droplet (Figure 4A) reduced the viability of $Apc^{\text{Min/}+}$ spheroids in a dose dependent manner and with the highest efficacy when one or two alleles for Rasgrp1 were absent ($Apc^{\text{Min/}+} : Rasgrp1^{-/-}$ and $Apc^{\text{Min/}+} : Rasgrp1^{\text{WT/}+}$ Figure 4E). This platform of CRC spheroids from well-defined genetically engineered mouse models reveals that anti-EGFR therapy is most effective when expression levels of the suppressor Rasgrp1 are low or absent.

**RasGRP1 is a marker that stratifies anti-EGFR therapy efficacy**

Lastly, we collaborated with the Alliance for Clinical Trials in Oncology and performed a retrospective analysis of colorectal cancer patients who were enrolled in the national CALGB80203 trial of FOLFOX/FOLFIRI versus FOLFOX/FOLFIRI with the anti-EGFR blocking antibody cetuximab (7). 238 patients were enrolled in CALGB80203 (Figure 5A, consort overview of the FOLFIRI and FOLFOX groups) and this is currently the only trial available for data analysis. The follow-up CALGB80405 trial is still actively ongoing and no data is liberated yet. In addition, the CALGB80405 trial compares cetuximab and/or bevacizumab (anti-VEGF therapy – vascular endothelial growth factor) together with combination-chemotherapy and there is no combination-chemotherapy alone arm. Alliance-banked RNA from patients was tested for **RasGRP1** mRNA levels by Taqman
and RasGRP1 RNA levels could be reliably assayed in 84 patient tumors for which KRAS typing was available as well (Figure 5B).

Taqman results revealed a gradual range of RasGRP1 mRNA expression levels (Figure 5C) with distribution that approximated a Gaussian curve over eight groups of RasGRP1 expression (Figure 5D). Since the RasGRP1 mRNA expression levels in the midrange level are close together, we next divided patient tumors into the 25% highest and 25% lowest RasGRP1 expression quartiles (Figures 5C and D) and compared the clinical outcome between FOLFOX/FOLFIRI alone or FOLFOX/FOLFIRI in combination with cetuximab. We evaluated the distribution of overall survival and RasGRP1 within patients having KRAS$^{WT}$ tumors (52 patients). Patients having low tumor expression of RasGRP1 experienced prolonged survival, when cetuximab was added to FOLFOX/FOLFIRI (median of 40.8 months vs 20.0 months, p=0.03) (Figure 5E). This effect was not observed in patients expressing high levels of RasGRP1 (p=0.85) (Figure 5F and Supplemental Figure 6). Since CALGB 80203 was conducted before KRAS testing became part of standard of care, we had available data for some KRAS$^{MUT}$ (n=32) patients (Figure 5B). Our limited analysis of patients with KRAS$^{MUT}$ tumors suggests that there is no benefit of cetuximab addition to FOLFOX/FOLFIRI when CRC patients are stratified on the basis of RasGRP1 expression (Supplemental Figures 5B and 5C). It should be noted that we did not observe significant differences in patient survival when we divided patient tumors into the 50% highest and 50% lowest RasGRP1 expression halves (Figures 5C and D and data not shown).
In sum, the murine CRC spheroid assays in Figure 4 and retrospective analysis of CALGB 80203 data are consistent with the notion that RasGRP1 is a tumor-suppressor gene in colonic epithelium with aberrant Wnt signaling and an unique marker positioned in the EGFR pathway that could be of relevance in the clinic as both a prognostic and potential predictive factor for efficacy of anti-EGFR therapy for CRC patients.

Discussion

Here we demonstrate that RasGRP1 is a tumor suppressor in colonic epithelium with aberrant Wnt signaling that has value as a prognostic marker to monitor clinical progression of CRC. Moreover, capitalizing on murine CRC spheroids and CALGB 80203 clinical trial CRC patient data, we uncover that RasGRP1 is a biomarker that could be relevant to predict efficacy of anti-EGFR therapy for CRC patients. It is of interest to note that RasGRP1 itself is activated by EGF-EGFR signals (32). This feature may make RasGRP1 particularly useful as prognostic and as accompanying biomarker as the predictive potential is unambiguously coupled to EGFR signals and RasGRP1’s biology and additional testing in randomized trials of EGFR-targeting agents should be considered to confirm tumor expression of RasGRP1 as a predictive biomarker.

Data mining of five bioinformatics platforms shows that decrease of RasGRP1 expression correlates with tumor aggressiveness and poor clinical outcome in human
colorectal cancer, whereas analysis of \textit{SOS1} did not reveal any particular patterns. The parallels between Rasgrp1 and Lrig1 we mentioned above are intriguing. Tet-induced expression of the transmembrane molecule Lrig1 dampens EGFR signaling (42) and establishes balanced intestinal stem cell homeostasis (21). Analysis of the TCGA database (colorectal, lung, glioblastoma and ovarian) reveals that the \textit{LRIG1} locus is rarely lost or mutated (50). Nevertheless, LRIG1 expression analysis helps define the tumor type and stage in clear cell renal cell carcinoma (51) and low level expression of Lrig1 correlates with poor differentiation of squamous cell carcinomas and worse clinical outcome (52). In breast cancer, high expression of Lrig1 has been described in cells with best clinical outcome and high Lrig1 correlates with a greater chance for relapse-free survival (50). To date, little is known about regulation of LRIG1 expression levels. We have not observed increases in RasGRP1 expression upon EGFR signaling (data not shown), implying that RasGRP1’s negative feedback on EGFR signaling is constant. LRIG1 as a marker in CRC deserves future studies, possibly motivated by our study here that RasGRP1 expression declines when CRC progresses and becomes more aggressive. How RasGRP1 expression levels decrease or disappear needs to be explored in depth.

Roughly 95 per cent of pancreatic ductal carcinomas (PDAC) contain \textit{KRAS} mutations, yet, EGFR signalling still appears essential for \textit{KRAS^{MUT}}-driven PDAC in mice (53, 54) and in the clinic erlotinib is beneficial for some PDAC patients (55). These results in
PDAC suggest that anti-EGFR therapy should perhaps not be totally discarded when tumors have $RAS$ mutations. Organoid technology has revolutionized cell biological studies on stem cell compartments (15, 16) and is also starting to emerge as a versatile and reliable platform for therapy testing (56-58). We have employed organoids here combined with R-Spondin and Wnt surrogate ligands, EGF growth factors, EGFR inhibitors, and genetic deletion of $Rasgrp1$ since Rasgrp1 inhibitors are not available to date. How to overcome the challenge on oncogenic $RAS$ mutations is an on-going challenge, but future organoid and spheroid assays, in the 3D space of the Matrigel droplet that mimics a 3D tissue, should allow for multi-dimensional testing of parameters as RasGRP1 and LRIG1 expression levels, $KRAS$ status, as well as multiple therapies, including but not limited to anti-EGFR therapy.
Methods

Bioinformatics and TCGA datamining

For analysis of RasGRP1 mRNA expression in correlation with APC, KRAS and TP53 mutation statues, the Cancer Genome Atlas (TCGA) Colorectal Adenocarcinoma database was obtained and analyzed with cbioPortal (http://www.cbioportal.org/) that is embedded in the TCGA database (https://tcga-data.nci.nih.gov/tcga) as we previously described (30).

To evaluate the correlation for RasGRP1 expression in 18 normal colon, 20 primary colonic tumor, and 19 liver metastases, the GSE49355 (34) were downloaded from NCBI GEO database (National Center for Biotechnology Information, Gene Expression Omnibus). The gene expression values were normalized and annotated by GeneSpring GX 12 software (Agilent Technologies, USA). One-way ANOVA statistical analysis with Bonferroni corrections was carried out by using SPSS 17.0 (Chicago, IL, USA).

For RasGRP1 as a marker for survival of metastatic colorectal cancer (mCRC) patients, we used the clinical trial Pan European Trial in Adjuvant Colon Cancer (PETACC-3, ArrayExpress E-MTAB-990) (45). The PETACC-3 dataset gathers 752 FFPE samples from CRC patients and is a randomized, multicenter, adjuvant therapy clinical trial that evaluated 5-Fluorouracil and Leucovorin alone or combined with Irinotecan in curatively resected stage II and stage III colon cancer. To assess whether RasGRP1 expression level affects survival of mCRC patients, we split mCRC patients from PETACC3 into two
subgroups: one group of patients with high expression of RasGRP1; one group of patients with low RasGRP1 expression. The threshold used to split the data was the median RasGRP1 expression value, an arbitrary value leading to equal number of patients in each subgroup. With same strategy we also assessed separately the effect of RasGRP1 expression level on survival for $\text{KRAS}^{\text{WT}}$ and $\text{KRAS}^{\text{MUT}}$ patients. Survival analyses were done under the R programming language (version 3.3.1) using functions from the ‘survival’ R package. Survival curves were drawn following the Kaplan-Meier method and compared via the log rank test. A Cox regression model was fitted on the data so to compute the hazard ratios between the two RasGRP1 groups.

For the MOSAIC study; RNA quality was assessed using the Agilent Bioanalyzer and only the samples displaying acceptable RNA integrity (RIN > 7) were kept for sequencing, corresponding to 42 duplets (primary tumors + liver metastases) 8 triplets (normal colon + primary tumors + liver metastases) and 2 unmatched metastases. RNA sequencing libraries were prepared using the Illumina TruSeq Stranded Total RNA reagents (Catalog number RS-122-2201; Illumina; San Diego, USA) according to the protocol supplied by the manufacturer and using 400ng of total RNA. We used the Ribo-Zero Human kit (Catalog number MRZH11124; Illumina; San Diego, USA) in order to get rid of cytoplasmic ribosomal RNA. Cluster generation was performed with the libraries using the Illumina HiSeq PE Cluster Kit v4 cBot reagents (Catalog number PE-401-400) and sequenced on the Illumina HiSeq 2500 using HiSeq SBS Kit V4 reagents (Catalog number FC-401-4002). Sequencing data were processed using the Illumina Pipeline Software version 1.84. Initial number of reads averaged 123 ± 29 (standard
deviation (s.d.)) million per sample. Reads were first trimmed to remove polyA and Illumina TruSeq adapter sequences using cutadapt (59), and aligned to the human reference hGRC37 genome using the STAR aligner (60). The number of counts was summarized at the gene level using featureCounts (61). Reads that uniquely mapped to the reference genome averaged 86% ± 14% (s.d.). A non-negligible part of the sequences (30.4% ± 13.8% (s.d.)) mapped to intronic regions and thus were filtered out for subsequent analyses. After filtering out intronic reads, the rate of reads mapping to ribosomal RNA averaged 8.1% ± 15.7% (s.d.) while 88.2% ± 4.1% (s.d.) mapped to exonic protein-coding sequences, corresponding to a final read number of 59 ± 19 million reads per sample. Read counts were normalized into reads per kilobase per million (RPKM) and log2 transformed after addition of a pseudocount value of 0.001. Two patients were filtered out based on a principal component analysis, which resulted in a final gene expression matrix of 14112 genes and 96 samples (corresponding to primary tumors and paired liver metastases of 48 patients).

Antibodies and reagents

Antibodies were obtained from the following sources and used at indicated concentration: Sos1 (1:1000) from BD Bioscience (610095, clone 25/SOS1); EGFR (1:1000, #4267) and beta-actin (1:1000; #4970) from Cell Signaling Technology; murine Rasgrp1 (m199) (1:500) generated by our laboratory together with Epitomic. Matrigel
Growth Factor Reduced (GFR) Basement Membrane Matrix (ref. 354230) from Corning.
Erlotinib HCl (OSI-744) from Selleckchem.

**Mice**

Apc\textsuperscript{Min/+} mice were crossed to Rasgrp1\textsuperscript{-/-} mice (provided by Jim Stone, University of Alberta, Canada) to generate a mouse expressing Apc\textsuperscript{Min/+} in the context of 1 and 2 Rasgrp1 alleles deleted Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{WT/-} and Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{-/-} respectively (30). Rag knock-out mice were kindly provided by Dr Averil Ma (UCSF, San Francisco). Primers used for genotyping of Rasgrp1 and Apc\textsuperscript{Min} were described previously (30).

**CALGB 80203 Sample Collection and RNA Isolation**

Formalin-fixed, paraffin-embedded (FFPE) baseline tumor samples were collected from patients participating in CALGB 80203 (62). RNA was extracted from an FFPE block of primary colon or rectum tumor as previously described (63).

**CALGB 80203 qRT-PCR Analysis**

Taqman quantitative PCR was performed for RasGRP1 using the StepOne Real Time PCR System (Applied Biosystems-Life Technologies, Foster City, CA, USA). The relative amounts of RasGRP1 mRNA expression were normalized to β-actin mRNA and
expressed as $\Delta C_T = C_T(\text{RasGRP1}) - C_T(\beta\text{-actin})$, where $C_T$ is the threshold cycle. The RasGRP1 and actin assays were provided by the Roose lab (Depeille et al. (30)). The Taqman $\beta$-actin assay was Hs00357333_g1; RasGRP1 was Hs0096734_m1. All assays were performed in duplicate. Duplicate samples with $C_T$ standard deviation $>0.5$ cycles and average $C_T <35$ were re-run for improved qPCR reproducibility. The second run was used if the standard deviation was $<0.5$ cycles. Samples with a final standard deviation $>0.7$ cycles were excluded. A final total of 84 samples were used for this analysis.

**Mouse organoids; preparation, culture, and imaging**

Mouse (6-8 week old) colon was isolated and washed in cold PBS. Distal colon was cut longitudinally and into small pieces before being incubated in isolation buffer (PBS 3mM EDTA) for 60min rotating at 4°C. Pieces were incubated in 20ml of cold buffer and shake for 4min. Suspension was collected and process was repeated until suspension clear (3-4 times). All suspensions were pooled, centrifuged at 500g for 10 min at 4°C and resuspended in cold PBS-10% FBS. After counting and centrifugation 5min, 700g at 4°C crypts were resuspended in matrigel to a concentration of 200-500 crypts per 50μl matrigel drop. Murine organoids were cultured in media (adapted from Sato et al. (43)) DMEM/F12 with Glutamax (Gibco-Life Technologies, Grand Island, NY, USA) Penicillin/Streptomycin (100μg/ml), N2 Supplement (1X), B27 Supplement minus Vitamin A (1X) (Gibco-Life Technologies), HEPES (10mM) (UCSF Cell Culture Facility,
San Francisco, CA), Murine Recombinant Noggin (100ng/ml) (PeproTech, Rocky Hill, NJ, USA), N-Acetylcysteine (1mM) (Matrix Scientific, Columbia, SC), R-Spondin2 (25nM), Wnt Surrogate ligand (0.3nM) (K. Christopher Garcia Laboratory, Stanford, CA) (18) and supplemented or not by Murine Recombinant EGF (50ng/ml) (PeproTech).

Images were taken at day 11 using inverted microscope Keyence BZ-X700 with CDD cooling camera Keyence and software BZ-X analyzer.

**Mouse colorectal cancer organoids.**

Colon was dissected and flushed with cold PBS. Colonic adenomas were collected and incubated in cold PBS 5-10min for gentle wash. As previously described (64), tumors were incubated in chelation buffer (2 mM EDTA (Corning), 5.6 mmol/L Na₂HPO₄, 8.0 mmol/L KH₂PO₄, 96.2 mmol/L NaCl, 1.6 mmol/L KCl, 43.4 mmol/L sucrose, 54.9 mmol/L D-sorbitol, 0.5 mmol/L DL-dithiothreitol (Sigma-Aldrich) in distilled water) for 60 min on ice. Chelation buffer was then washed with cold PBS and tumors cut in pieces were incubated in digestion buffer (2.5% fetal bovine serum (Omega Scientific Inc., Tarzana, CA), 1 unit/ml of penicillin, 1 μg/ml of streptomycin, and 2.5 ng/ml of amphotericin B (Sigma Aldrich, St. Louis, MO), 200 U/ml type IV collagenase (Gibco-Life Technologies), 125 μg/ml type II dispase (Sigma Aldrich) in Dulbecco's Modified Eagle Medium) for 2 hr at 37 °C and shake regularly. Cells were then filtered, counted and seeded with matrigel for up to 15000 cells/ 50 μl matrigel drop. Culture media was then added to the well after 5-10 min polymerization at 37°C. Murine spheroids were cultured were cultured in
DMEM/F12 with Glutamax Penicillin/Streptomycin (100μg/ml), HEPES (10mM), N2 Supplement (1X), B27 Supplement minus Vitamin A (1X), N-Acetylcysteine (1mM), Amphotericin B (2.5ng/ml) and supplemented by Murine Recombinant EGF (50ng/ml) (64).

**RNA extraction and Real time PCR.**

Total RNA was isolated from human tissue using RNeasy kit (Qiagen). RNA was reverse-transcribed with random primers (Invitrogen) and Moloney murine leukaemia virus reverse transcriptase. Mouse RNA extraction from IEC was done using polyDT primers for reverse transcription. Real-time PCR was performed in triplicate using Eppendorf RealPlex2. Gene expression was normalized to GAPDH (human) or beta-actin (mouse) and quantified with the comparative CT method according to the manufacturer’s instructions. Probes and primers of RasGRP1 and GAPDH were obtained at Applied Bio System.

**Western blot**

Cells were lysed with ice-cold 1% NP40 supplemented with protease and phosphatase inhibitors [10 mM sodium fluoride, 2 mM sodium orthovanadate, 0.5 mM EDTA, 2 mM phenyl- methylsulfonyl fluoride, 1 mM sodium molybdate, aprotonin (10mg.ml⁻¹), leupeptin (10mg.ml⁻¹), pepstatin (1mg.ml⁻¹)]. For colonic organoids, protein extraction
was done in 2% NP40. After 30min on ice, lysates were centrifuged and supernatant were mix with 4X LDS sample buffer (Invitrogen). Protein lysates were separated on pre-cast gel tris glycine 4-12% (Invitrogen), transferred on PVDF membrane and incubated with primary antibodies of interest. Western blots were visualized with enhanced chemo-luminescence and imaging on a Fuji LAS 4000 image station (GE Healthcare).

**Extraction of intestinal epithelial cells.**

Intestinal epithelial cells were isolated as previously described (65). Briefly, small intestine and colon of mouse (12-13 weeks old) were dissected, washed with cold PBS, cut in three identical pieces (2 cm), duodenum, jejunum and ileum and two pieces for colon, proximal and distal. After cleaning, pieces were incubated on ice in 10ml of cold isolation buffer (2.7 mM KCl; 1 M KCl; 150 mM NaCl; 1.2 mM KH$_2$PO$_4$; 680 mM Na$_2$HPO$_4$; 1.5 mM EDTA; 0.5 mM DTT (added fresh) in 1 L of ddH$_2$O. After 15min incubation, suspension was discarded; tissues were vortexed in 5ml PBS. Suspension was collected in another tube and isolation buffer was added to tissue and incubated 15min on ice. The process was repeated 3 times. Suspension of each repeat was pooled in the collecting tube and centrifuge at 1000rpm, 4°C for 10min. Cells were resuspended in PBS and separated for protein extraction (2/3 of suspension) or RNA extraction (1/3 of suspension).
Viability assays.

Organoids were seed in 96-well clear flat bottom plate in mixture matrigel/media for 24h at 37°C. Organoids were then incubated with increased concentration of Erlotinib with 4 wells per condition for 72h at 37°C. Organoids viability was evaluated using CellTiter Glo 3D as described by manufacturer (Promega Corporation). Luminescence was read on SpectraMax M5 (Molecular Devices, USA). Values were normalized to untreated condition after removing background.

Immunohistochemistry.

Human tissues were collected, fixed in 4% PFA and paraffin-embedded. Then 5-µm-thick sections were de-waxed in Histo-Clear (National Diagnostics) and rehydrated in graded alcohol baths. Antigen retrieval was performed in pressure cooker for 10 min in 10 mM sodium citrate buffer, pH 6.0. Endogenous peroxidase activity was inhibited with 1.5% H2O2 in methanol for 20 min and washed in PBS. Nonspecific binding sites were blocked in blocking buffer (PBS, pH 7.4, 3% goat serum, 1% BSA, and 0.1% Tween) for 60 min at RT. Sections were then incubated with primary antibodies diluted in blocking buffer overnight at 4°C. Slides were then washed twice with 0.1% PBS-Tween before incubation in Universal Immuno-peroxydase polymer anti-rabbit Histofine® (Nichirei Biosciences, Japan) used as a secondary reagent. Stainings were visualized with DAB
(3, 3'-diaminobenzidine, Sigma-Aldrich) and a hematoxylin counterstain (Shandon Instant Hematoxylin) was performed before dehydration. After dehydration, sections were mounted in Cytoseal 60 (Thermo Scientific). To study the structure of tissue and cell types, Haematoxylin & Eosin (H&E) was performed. Images were acquired using Imager M2 Zeiss microscope equipped with an AxioCam MRs (Zeiss).

Statistics.

Pixel values were transformed in metric values using microscope scale. All data were represented as mean ± SEM. All specific statistical analyses and number of repeats are mentioned in the respective figure legend paragraphs. Unpaired t-test analysis was used to compare two groups such as figures 3A-C and supplemental figure 6B. For all other experiments, with multiple comparisons, a one-way and two-ways ANOVA followed by a Bonferoni or Dunnett’s post hoc test were used for comparisons of three or more groups. Log-rank (Mantel-Cox) Test was used for Kaplan–Meier survival curve. For all tests, a P value of < 0.05 was considered statistically significant. Analyses and graphs were done using GraphPad Prism 6.

Study approval.

Mice were handled according to the Institutional Animal Care and Use Committee regulations, described in the Roose laboratory University of California, San Francisco (UCSF) mouse protocol AN084051 “Ras Signal Transduction in Lymphocytes and Cancer”. Decoded CRC patient samples (UCSF samples) Taqman analysis were
obtained from Dr. Warren and analysed for RasGRP1 expression (CHR approval - Study Title: RasGRP1 in Human T cell lymphoma and colorectal cancer IRB #: 12-09467). For CALGB 80203, approved by Duke University Health System IRB under protocol ID Pro00018430 and titled “Markers of Efficacy and Resistance to Cetuximab Treatment in Metastatic Colorectal Cancer: A Correlative Sciences Study of Tissues from CALGB Protocol 80203”. Patient and ethics approval for this study was obtained from the PETACC-3 Translational Research Working Party (PTRW). For the “MOSAIC” study, bulk RNAseq data previously generated by the Colorectal Cancer Metastases Working Group (Dr. Sabine Tejpar, unpublished) was examined to compare the expression of RasGRP1 in primary colorectal carcinomas and their matched liver metastases in previously untreated patients. All tissue was collected under local IRB-approved tissue banking projects at 11 sites within Europe and the United States.
**Author Contributions**

OMG and PD performed the majority of all experiments. CB assisted with in vivo experiments and analyses. MM and RSW provided RNA of patient specimens. AJH assisted with *RasGRP1* expression in 80203 trial. CYW, MRM, ABN, DG, MDL and ST assisted with bioinformatics approaches. YM and KCG provided innovative reagents. PD and JPR conceived the study, analyzed data, and wrote the manuscript. CB, DG and CYW contributed equally to the manuscript and authorship order has been assigned by alphabetic order. All authors commented on the manuscript.

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**Competing financial interests**

PD, RSW, and JPR are holders of a patent PCT/US2016/025334 PROGNOSTIC AND DIAGNOSTIC METHODS FOR COLORECTAL CANCER and OMG, PD, RSW, and JPR hold stock in SealBiosciences, Inc..
References


A

\[ p = 0.006 \]

Relative RasGR1 level

Normal 18
Primary 20
Liver Metastasis 19

B

Rag^-/-

C

Rasgrp1
\[ B\text{-actin} \]

D

**NS**

Relative Rasgrp1 mRNA level

Small intestine 1 2 3 4 5
Colon

E

NS

Relative Sos1 mRNA level

Small intestine 1 2 3 4 5
Colon

F

\[ p < 0.05 \]

Survival

Days

G

****

Number of tumors (colon)

H

**NS**

Tumor size / genotype (%)

I

Gbenedio et al. Figure 1
Figure 1. RasGRP1 acts as a tumor suppressor in Apc\textsuperscript{Min} mice.

(A) Box and whiskers plot of a representation of normalized RasGRP1 mRNA levels analyzed with GeneSpring GX software. Normal colon (n = 18), primary colonic tumor (n = 20) and Liver Metastasis (n = 19). One-way ANOVA statistical analysis with Bonferroni corrections was carried out using SPSS 17.0 (Chicago, IL, USA). Post hoc t-tests using SPSS Bonferroni adjusted p-values. (B, C) Detection of Rasgrp1 expression by western blot in different sections of small intestine (duodenum (1), jejunum (2) and ileum (3)) and colon (proximal (4) and distal (5)). Western blot panel is a representative example of 3 independent experiments. β-Actin serves as protein loading control. Protein lysate from CD4-positive mouse cells (C57Bl6 mouse) is used as positive control for Rasgrp1. Western blot from 2 more extraction can be seen in Supplemental Figure 1. (D, E) Rasgrp1 (D) and Sos1 (E) mRNA levels determined by Taqman PCR on distinct portions of intestinal tract. mRNA expression for one Duodenum portion was used as an arbitrary reference and set at 1.0 and other samples values were related to that one. Data are depicted as fold difference compared to the value of 1.0 in Duodenum and data is plotted from n=3 independent experiments (n = 3 mice). Each point is an average of 2 wells. Each column was compared to others. NS not significant, **p < 0.001 (One way Anova, Bonferroni’s multiple comparison test). (F) Kaplan–Meier curves of Apc\textsuperscript{Min/+} mouse survival with different copies of Rasgrp1 alleles. Statistical analysis was performed on Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{WT/-} (n = 37) and compared to Apc\textsuperscript{Min/+} mice (n = 25). * p < 0.05, Log-rank (Mantel-Cox) Test, which means that the two groups are
significantly different from each other. (G) Quantification of colonic tumor incidence in Apc\textsuperscript{Min/+}, Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{WT/-} and Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{-/-} mice (n = 16, 25 and 19 respectively; NS not significant, **p < 0.05, ***p < 0.001 (One way Anova, Bonferroni’s multiple comparison test). (H) Image of the distal colon of a Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{-/-} mouse bearing colonic adenomas. (I) Percentage of colonic tumor sizes in Apc\textsuperscript{Min/+}, Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{WT/-} and Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{-/-} mice (n = 10, 15 and 19 respectively). Additional data is displayed in Supplemental Figure 2C.
Figure 2

A

B

C

RSPO

WT (1)

WT (2)

Rasgrp1

-/-

Rasgrp1

-/-

(D1)

(D2)

Day 0.2

Day 0.5

Day 2

Erlotinib (µM)

1

3

5

1

3

5

0

2

4

6

8

Proliferation rate (normalized to untreated)

Erasgp1

-/-

WT

Mouse 1

Mouse 2

Mouse 3

Rspo + Wnt surrogate ligand

Sustained canonical Wnt signaling

D

Erasgp1

-/-

WT

Sos1

Egfr

B-Actin

Proliferation rate (normalized to untreated)

ER cobedio et al. Figure 2
Figure 2. RasGRP1 is a critical suppressor of EGF-induced growth in the colon.

(A) Pipeline of colonic organoid generation. (B) Cartoon representation of Wnt surrogate ligands simultaneously triggering LRP5/6 (Low-density lipoprotein receptor-related protein) and FZD (Frizzled) as well as R-Spondin (RSPO) contributing to sustained, canonical Wnt signalling. (C) Representative images of colonic organoids from individual WT and Rasgrp1−/− mice. Each image is a representative example of 12 wells of organoids per genotype from 3 independent experiments (n = 3 mice per genotype; n = 9 total). Scale bars, 200µm. (D). Detection of Egfr and Sos1 expression by western blot in growing organoids from WT and Rasgrp1−/− mice (n = 2 mice per genotype). β-Actin serves as protein loading control. Panels are representative of two independent organoid experiments. (E) Proliferation of developing organoids (represented in panel C) evaluated in growth media containing R-spondin and Wnt surrogate ligand over 5 days. Each point represents average of 2 wells in triplicate (n= 3 mice per genotype). (F) Organoids were treated with erlotinib at different doses (0.2, 0.5, 2 µM) for 1, 3 and 5 days. All values were normalized to untreated of similar developing day. Graph is representative of 3 independent experiments (n = 3 mice per genotype) with 2 wells per condition. We analyzed growth of organoids at day 3 when these are in exponential growth phase. *p < 0.05, **p < 0.01, ****p < 0.01, NS not significant (One-way Anova, Bonferroni’s multiple comparison test). Data are mean± SEM.
Figure 3. RasGRP1 is a prognostic marker for CRC.

(A-C) RasGRP1 expression in human colorectal cancer patients from TCGA Colorectal Adenocarcinoma data sets. Panels show expression of RasGRP1 patients with either APC wild type (WT) versus Mutant (A), TP53 WT versus mutant (B), and KRAS WT versus mutant (C). Statistical analyses were performed using unpaired t-test and the P-value < 0.05 was considered significant. NS; not significant. (D) Analysis of relative RasGRP1 expression using TCGA data as in panels A-C, now organized by cumulative mutations in APC, TP53, and KRAS. *p < 0.05 (Dunnett’s test). NS; not significant. (E) Analysis of relative SOS1 expression using TCGA data as in panels B-D, now organized by cumulative mutations in APC, TP53, and KRAS. NS; not significant. (F) RasGRP1 mRNA expression determined by Taqman PCR on liver metastasis samples surgically removed from 124 patients with metastatic colorectal cancer. KRAS\textsuperscript{MUT} (n=87) and KRAS\textsuperscript{WT} (n=37). (G) Immunohistochemistry for RasGRP1 (brown staining) on liver metastasis patient samples selected from panel G with either low or high RasGRP1 levels. Scale bars, 20μm. (H) Kaplan–Meier overall survival curve for stage II and III PETACC3 CRC patients (n=282 patients) carrying-KRAS mutation divided in two equal groups of 141 patients expression high levels (red line) or low levels of RasGRP1 (*p = 0.02, Log-rank (Mantel-Cox) Test). (I) Analysis of RasGRP1- and SOS1 expression in matched primary- and metastatic- tumors from patients with metastatic CRC in the MOSAIC program. Gene expression levels (in log2 of the RPKM) for RasGRP1 and SOS1 in Metastases (Red dots, right side) were compared to levels in primary tumors (blue dots, left side). Red lines and blue lines show increase and decrease of
expression level, respectively. Student’s t-test was performed to assess the statistical significance between comparisons. NS, not significant; * $p< 0.05$.

Figure 4. Deletion of Rasgrp1 alleles makes CRC spheroids susceptible to EGFR inhibition.

(A) Scheme of murine CRC organoid generation from colonic tumors. (B) Representative images of CRC organoids from $Apc^{Min/+}$, $Apc^{Min/+}:Rasgrp1^{WT/-}$ and $Apc^{Min/+}:Rasgrp1^{-/-}$ colonic adenomas. Each image is a representative example of 40 or
more wells of tumor spheroids; 8 wells per mouse tumor and 5 or more mice per genotype. Scale bars, 200μm. (C and D) Murine Apc\textsuperscript{Min/+} CRC organoids (blue square), Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{−/−} CRC organoids (red square) were incubated in plain growth media (C) or with exogenous EGF (50 ng/ml) (D). Proliferation rate was evaluated on day 1, 3 and 5 after plating. Data are mean± SEM. and were normalized to day 1. A total of 6 wells for each condition from 2 independent experiments was evaluated (n= 3 Apc\textsuperscript{Min/+}, n=3 Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{−/−} mice). **** p < 0.0001 (Two way Anova, Bonferroni’s multiple comparison test). (E) Apc\textsuperscript{Min/+} CRC organoids (blue bars), Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{−/−} CRC organoids (red bars), and Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{WT/+} CRC organoids (grey bars) were treated with different doses of erlotinib for 3 days. DMSO was used as control. All data were normalized to untreated condition. A total of 4-5 independent experiments with 4 wells per condition (16-20 wells total) were evaluated. Each point represents average of 4 wells (n= 5 Apc\textsuperscript{Min/+}, n=4 Apc\textsuperscript{Min/+}:Rasgrp1\textsuperscript{−/−} mice). NS, not significant; *p < 0.05, **p < 0.01 (Dunnett’s test).
238 CRC patients on 80203

FOLFIRI (n=61)
FOLFOX (n=60)
FOLFOX + Cetuximab (n=58)

20 w/ sample
28 w/ sample
23 w/ sample

19 consented
27 consented
21 consented

KRAS

KRAS

Patients (consented) Taqman RasGRP1 expression

FOLFOX / FOLFIRI (n=15)
FOLFOX / FOLFIRI + Cetuximab (n=17)

FOLFOX / FOLFIRI (n=25)
FOLFOX / FOLFIRI + Cetuximab (n=27)

RasGRP1 Low

RasGRP1 High

Patient samples (n=84)

Average of Ct per group

RasGRP1 Low

RasGRP1 High

FOLFOX / FOLFIRI (n=7; 20.02 months)
FOLFOX / FOLFIRI + cetuximab (n=6; med. 40.8 months)

FOLFOX / FOLFIRI (n=7; med. 24.46 months)
FOLFOX / FOLFIRI + cetuximab (n=7; med. 25.03 months)

p = 0.03

p = 0.85

Gbenedio et al. Figure 5
Figure 5. RasGRP1 as a biomarker marker for anti-EGFR therapy.

(A) Consort overview of CALG80203 trial patients receiving the indicated therapy, provided by the Alliance for Clinical Trials in Oncology. (B) Scheme of CALG80203 trial patients analysed for RasGRP1 expression by Taqman and organized on KRAS status. (C) RasGRP1 mRNA levels in 84 patients of the CALGB80203 clinical trial. Graph shows quartile of patients with highest RasGRP1 expression in their tumors ("RasGRP1 High" in blue) as well as the quartile of patients with lowest RasGRP1 expression in their tumors ("RasGRP1 Low" in red). Groups were identified by calculating average of Ct. Standard deviation (SEM) represent value of repeat of same samples. (D) Patients were divided into 8 groups of step-wise, increasing Ct values. Nonlinear regression (curve fit) showing difference between quartile patients high and low. Blue bars represent quartile of patients with high expression of RasGRP1 and red bars represent quartile of patients with low expression of RasGRP1. (E) Comparison of FOLFOX/FOLFIRI chemotherapy (dashed lines) versus FOLFOX/FOLFIRI with cetuximab (solid lines) for the quartile of patients with lowest RasGRP1 expression in their tumors ("RasGRP1 Low"). Overall survival was graphed in a retrospective analysis of KRASWT colorectal cancer patients enrolled in the CALG80203 trial. Median (med.) survival is indicated for the two groups in months, p=0.03. (F) As in 5C, but the comparison between FOLFOX/FOLFIRI (dashed lines) and FOLFOX/FOLFIRI with cetuximab (solid lines) is now made for the quartile of patients with highest RasGRP1 expression in their tumors ("RasGRP1 High"). Median (med.) survival was calculated for each group in months, p=0.85