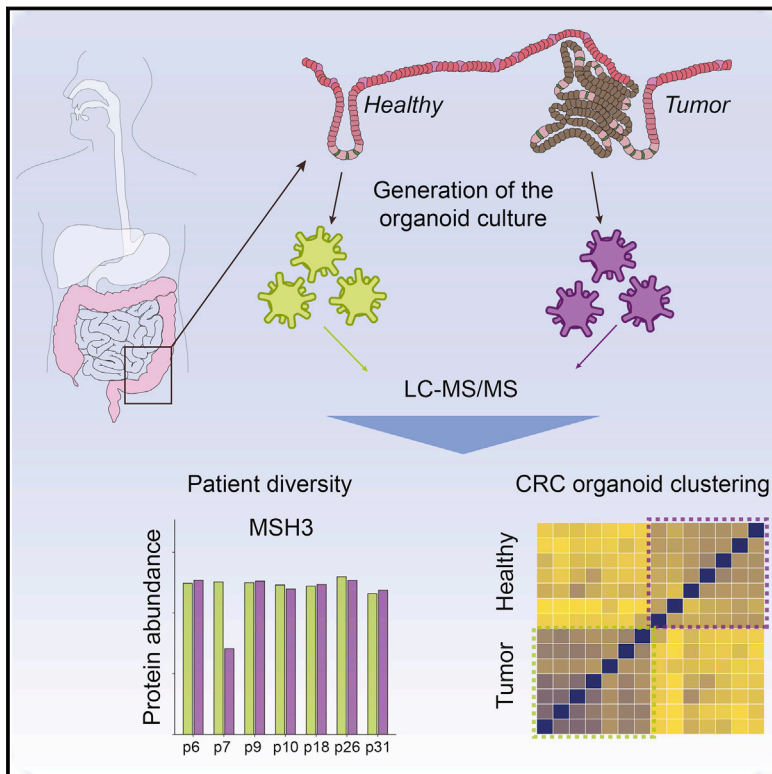# Cell Reports

Personalized Proteome Profiles of Healthy and Tumor Human Colon Organoids Reveal Both Individual Diversity and Basic Features of Colorectal Cancer

Graphical Abstract

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In Brief

Cristobal et al. perform deep proteome profiling of organoids derived from healthy as well as tumorous tissue in colorectal cancer patients. Analysis highlights that patient organoids recapitulate diversity among patients as well as common colorectal cancer features.

Highlights

- Deep proteomic profiling of human colon organoids

- Common colorectal cancer characteristics were present in all the tumor samples

- Patient-specific features can be identified that correlate with clinical diagnosis

- Organoids have the potential to be an excellent model to aid precision treatment

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Personalized Proteome Profiles of Healthy and Tumor Human Colon Organoids Reveal Both Individual Diversity and Basic Features of Colorectal Cancer

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SUMMARY

Diseases at the molecular level are complex and patient dependent, necessitating development of strategies that enable precision treatment to optimize clinical outcomes. Organoid technology has recently been shown to have the potential to recapitulate the in vivo characteristics of the original individual's tissue in a three-dimensional in vitro culture system. Here, we present a quantitative mass-spectrometry-based proteomic analysis and a comparative transcriptomic analysis of human colorectal tumor and healthy organoids derived, in parallel, from seven patients. Although gene and protein signatures can be derived to distinguish the tumor organoid population from healthy organoids, our data clearly reveal that each patient possesses a distinct organoid signature at the proteomic level. We demonstrate that a personalized patient-specific organoid proteome profile can be related to the diagnosis of a patient and with future development contribute to the generation of personalized therapies.

INTRODUCTION

Colorectal cancer (CRC) is one of the leading causes of death in adults. CRC usually develops from a benign precursor lesion, an adenoma, which is visible on the mucosal surface of the colon. The combination of mutational activation of oncogenes and mutational inactivation of tumor suppressor genes gradually leads to advanced adenoma and subsequently to an invasive cancer. Activation of the Wnt signaling pathway, through mutations in the APC gene (Powell et al., 1992) or β-catenin (Morin et al., 1997), is regarded as the initiating event in colorectal cancer. The following steps include mutations of the Kirsten rat

sarcoma viral oncogene homolog (KRAS) (Bos et al., 1987) and phosphatidylinositol 3-kinase (PI3K) oncogenes (Bardelli et al., 2003). These events are continued by accumulation of additional mutations that inactivate the transforming growth factor β (TGF-β) response (Markowitz et al., 1995) and the p53 pathway (Baker et al., 1989; Rodrigues et al., 1990). Although the alterations often occur in this order, the accumulation of the changes is likely more important than the order of occurrence (Fearon and Vogelstein 1990). The mechanisms underlying CRC development appear to be complex and heterogeneous and may entail patient-specific features (Fearon 2011; Markowitz and Bertagnolli 2009). To develop superior treatments, the molecular mechanisms underlying intestinal biology and colorectal cancer need to be thoroughly investigated. Previous work using model cell lines and organisms has helped to provide an understanding of both intestinal biology and cancer (Marshman et al., 2002; Barker et al., 2012). The Wnt pathway is the dominant force behind this high proliferative activity, and by studying the target genes of this signaling pathway, it was found that some genes appeared to be restricted to a limited number of cells in the crypts. One of these genes, Lgr5, was identified as a stem cell marker in both the small intestine and the colon (van de Wetering et al., 2002; Barker et al., 2007). A better understanding of the conditions maintaining these adult stem cells (Barker et al., 2008, 2009; Muñoz et al., 2012) in combination with analysis of three-dimensional cultures (Abbott 2003; Tanner and Gottesman 2015) has allowed the establishment of organoids, a promising preclinical model. Organoids are ever-expanding three-dimensional epithelial structures with all the hallmarks of in vivo epithelial tissue (Sato et al., 2009). Matrigel, which resembles the complex extracellular environment found in many tissues, is required for the culture of these organoids as well as a cocktail of growth factors (WNT, R-spondin1, Noggin, and EGF) (Sato et al., 2011). The phenotype and karyotype of these organoids remain unchanged over time, thus representing a valuable model to study the biology of stem cells and for evaluating their contribution to tissue homeostasis and disease. Organoids have

numerous valuable applications and may be used to advance our knowledge of disease mechanisms, regenerative medicine, and personalized precision medicine (Ohta and Sato 2014; Leushacke and Barker 2014). A detailed investigation, by us, into genotype-to-phenotype correlations was reported using several patient-derived organoids (van de Wetering et al., 2015). Furthermore, a comprehensive overview of the range of available organoids as well as all potential applications was recently published and emphasizes how rapidly the organoid field is developing (Clevers 2016). The closer the used preclinical model is to the affected patients, the more it will be effective for translation to the patient.

Mass spectrometry (MS)-based proteomics has become the preferred choice for system-wide protein characterization thanks to advances in every stage of the proteomics workflow, although improvements at the MS instrumentation level deserve praise (Yates 2013; Richards et al., 2015). Proteomics has developed to the point at which it is an indispensable tool for molecular and cellular biology as well as for systems biology (Cravatt et al., 2007; Larance and Lamond 2015; Altelaar et al., 2013; Cox and Mann 2011). In the context of colorectal cancer, several noteworthy proteomics studies have recently been published. Most relevant to the work described here, Mann and coworkers performed a comparative proteomics analysis of micro-dissected tissue from normal colon and adenocarcinoma (Wiśniewski et al., 2012). Slebos and coworkers characterized APC-driven proteomic differences using specific stable cell lines (Halvey et al., 2012). Jimenez and coworkers have done extensive studies on colorectal cancer proteomics, such as in a genomic context (Jimenez and Fijneman 2015), and have studied the tissue secretome in order to identify CRC candidate biomarkers (de Wit et al., 2014). Aebersold and coworkers have recently proposed a set of non-invasive prognostic biomarkers by targeted proteomics experiments (Surinova et al., 2015a) based on circulating plasma proteins (Surinova et al., 2015b). Roche and coworkers investigated the phosphoproteome of mouse xenografts in order to elucidate the contribution of the non-receptor tyrosine kinase SRC to colorectal cancer (Sirvent et al., 2012).

Notwithstanding the fact that such studies provided insight into some of the mechanisms underlying CRC, they did not address the bio-variability that may be present in individuals. Given the huge prospect of patient-derived organoids and given that differences in phenotype are closely related to changes in the proteome, we set out to evaluate personalized proteomes (adding transcriptome data for reference). Matched healthy and colorectal-tumor-derived organoids of seven individual patients with well-characterized, albeit distinct cancer-related mutational backgrounds were analyzed. Next to a proof of concept on whether personalized proteome profiling can be achieved using patient-derived organoids, an important aim of our study was to monitor how mutational differences in individual patients are reflected in each personalized proteome.

RESULTS AND DISCUSSION

Background on Human Colon Organoids
As organoids may constitute an accurate representation of the patients from whom they are derived, we set out to analyze their proteomes in order to decipher the differences between healthy and tumor colon at the protein level between a number of patients. Among the seven patients, three were female and four male, with an age range between 59 and 81 years. Biopsy specimens were taken from the ascending colon or the sigmoid colon, and adjacent healthy tissue was also obtained in order to grow matched "healthy" organoids. Information about the patients can be found in the Table S1. Earlier deep sequencing of these samples had revealed that each of the patients had a distinct mutational profile as summarized in Figure S1 (van de Wetering et al., 2015). The samples selected for the proteomic study were based on the mutation profile of the most common tumor driver genes in CRC (APC, TP53, KRAS, and PIK3CA). A representative population of the different CRC mutational profiles found in all the patients is present in the chosen organoids. These differences in mutational state may be reflected in differences in disease state and development and possibly also differences in the proteomes. The samples were named based on a previous publication (van de Wetering et al., 2015) in order to facilitate the correlation of the results.

Personalized Proteomics Profiling of Human Colon Organoids
We applied a quantitative mass spectrometrybased proteomics workflow to evaluate the protein expression in the organoids to derive so-called personalized proteomes. A schematic representation of this workflow is given in Figure 1. Following lysis, proteins from seven matched healthy and tumor-derived patient organoids were extracted. After enzymatic digestion, the resulting peptides were "dimethyl" labeled to allow accurate quantification (Boersema et al., 2009), in which peptides from the healthy organoids were labeled with light isotopes and peptides belonging to the tumor organoids with heavy isotopes. These organoids contain all the cell types of the tissue, constituting a somewhat complex (in terms of peptide population) sample. Therefore, after mixing the light- and medium-labeled peptides, a pre-fractionation step, by strong cation exchange (SCX), was included to reduce the sample complexity. Furthermore, in order to gain an in-depth analysis of the proteome, two complementary mass spectrometers were utilized, allowing two peptide identification strategies: electron transfer dissociation (ETD)-enabled Elite and a Q-Exactive with HCD. SCX allows fractionation based on charge and so fractions could be a priori chosen that would be optimal for ETD- or HCD-based sequencing.

Some experimental challenges needed to be addressed in the proteome analysis, such as those caused by missing values in one of the two labeling channels. In order to include proteins that are expressed only in either tumor or healthy organoids in our analysis ("on-off" proteins), we used missing value substitution, as described in detail in Experimental Procedures. Initial filtering provided ~7,600 identified proteins for each matched (tumor-healthy) organoid pair, and an average of 6,340 proteins could be quantified per patient. Hierarchical clustering of the identified peptides across the patients was performed, revealing a patient-centric pattern, as can be seen in Figure S2A. By applying an additional filter of considering only proteins with at least two peptides identified generated an average number of 5,323 quantified proteins per patient. A "Significance B" analysis
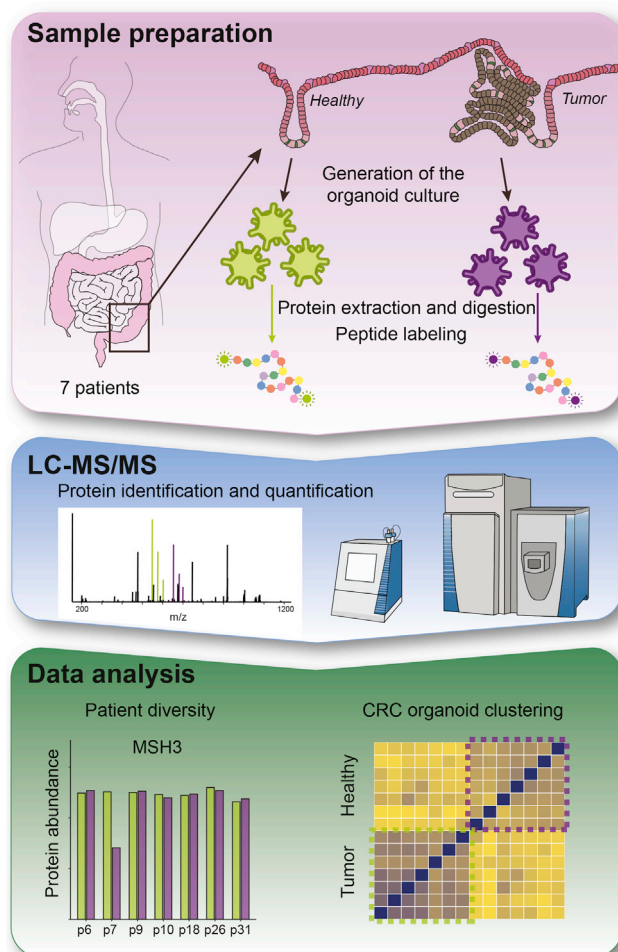
Figure 1. Experimental Design
Surgically resected tissue was obtained from previously untreated colorectal cancer patients. Organoids from tumor and normal tissue were cultured by isolating the crypts and resuspended in Matrigel. Tumor and normal organoids were lysed, and proteins were extracted and subsequently digested into peptides. The resulting peptides were differentially labeled using a dimethyl labeling strategy, mixed and pre-fractionated by strong cation exchange chromatography, and analyzed by LC-MS/MS. The MS results were analyzed using Proteome Discoverer, and statistical analysis was performed in R. From the data analysis, a combination of patient diversity and general features were extracted, as can be seen in the real data presented in the bottom part of the figure. See also Figure S1.

(Cox and Mann 2008) was performed to discern the differentially expressed proteins in each patient. On average, ∼400 proteins were assigned as differentially expressed in each individual patient. A representation of these data can be found in Figure S2B, and a detailed description of the proteins can be found in Table S2. Additionally, we decided to check how consistently these proteins were changing across all patients. From Figure 2, in which the differentially expressed proteins of each patient are highlighted in the other patients, the high diversity present in these CRC patients can be clearly observed. There are substantial differences in the protein populations that are significantly

different between healthy and tumor organoids of each patient. Additionally, we re-analyzed a quantitative microarray experiment performed on organoids obtained from the same patients. A total of 21,681 gene mRNA transcripts were quantified across the seven patients. These 21,681 transcript genes translate to 16,861 proteins. Following a similar statistical treatment as used for the proteomics data, an average of 130 transcripts were significantly changing at the transcriptome level across the seven patients (Figure S2C). From the, on average, 400 significantly changing proteins at the proteome level, less than 80 were recapitulated in the respective transcriptomes, demonstrating the additional perspective provided by the study of the proteome (Table S2). This limited overlap is not unusual considering the abundance of post-transcriptional and post-translational regulatory mechanisms and has been observed in several studies (Zhang et al., 2014; Schwanhäusser et al., 2011).

Organoids from Individuals Exhibit Distinct Personalized Proteomics Profiles
Microsatellite Instability

The DNA mismatch repair protein MSH3 showed a strong downregulation in the tumor organoid of patient 7 (female, 81 years old, whereby the biopsy specimen was taken from the ascending colon) while remaining unchanged in all other patients (Figure 3A). MSH3 is a component of the post-replicate DNA mismatch repair (MMR) system, which maintains genomic stability. The deficiency of this protein has already been related to colorectal cancer, and a loss of its expression has been shown to be frequent in MLH1-deficient colorectal cancers (Haugen et al., 2008; Plaschke et al., 2004). MLH1, one of the four mismatch repair genes (Wheeler et al., 2000; Hemminki et al., 1994), was also found downregulated exclusively for this patient in our dataset and furthermore, a strong downregulation was present at the transcriptome level as highlighted by van de Wetering et al. (van de Wetering et al., 2015). Moreover, we were pleased to find a frameshift deletion on MSH3 for the patient 7 in the mutational study, which confirmed our findings from the proteomics dataset (as summarized in Figure S1).

Genomic instability is a characteristic of all intestinal malignancies. As we described vide supra the majority of the colorectal cancers follow the classical adenoma-adenocarcinoma sequence and display chromosomal instability. Another form of genomic instability contributing to colorectal cancer is microsatellite instability (MSI). Approximately 15% of the colorectal cancer cases present high-frequency MSI, a hypermutatable phenotype caused by defects in the DNA MMR system. Apart from MSH3 and MLH1, there are other proteins showing a strong downregulation specifically in this patient, such as PMS2, RPL22, and MARCKS, that all have been linked to microsatellite instability. Expression data representing these proteins can be found in Figure S3A. For instance, PMS2, together with MLH1, is one of the four mismatch repair genes, and it has been shown to be mutated in hereditary non-polyposis colorectal cancer, leading to the production of an abnormally short or inactive PMS2 protein (Hampel et al., 2005; Hendriks et al., 2006). In our dataset, this protein was quantified in four patients, and it only shows a downregulation in patient 7. Another example is RPL22, for which a high mutation frequency of this gene has been reported
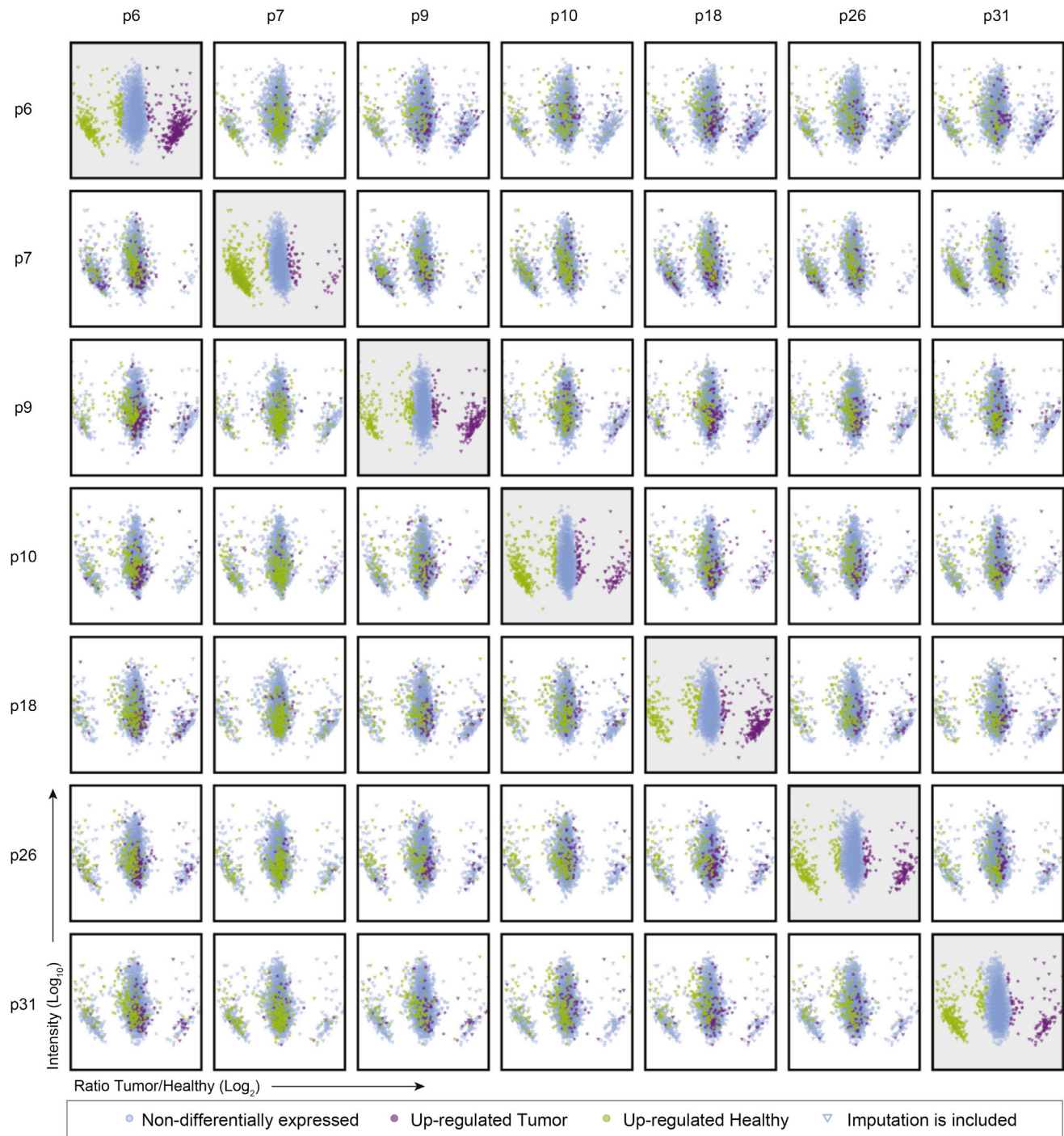
Figure 2. Personalized Proteomics Profiles

MA plots of the seven patients. The protein ratio ($\log_2$) is plotted against its intensity ($\log_{10}$). To visualize the heterogeneity of the samples, "Significance B" analysis was performed on the ratios of each patient. See also Figure S2.

in microsatellite-instable colorectal cancer (Ferreira et al., 2014). We were able to quantify RPL22 in all of the patients, finding exclusively a strong downregulation in patient 7. This is in agreement with a frameshift deletion found in the sequencing data only for patient 7. MARCKS is another protein only downregulated in

the tumor organoid of patient 7, while in the other patients, no change between healthy and tumor organoid was observed. MARCKS has already been identified in colorectal cancer as a major target of inactivation through coding microsatellite instability (Woerner et al., 2005; Kim et al., 2002). The valuable insight
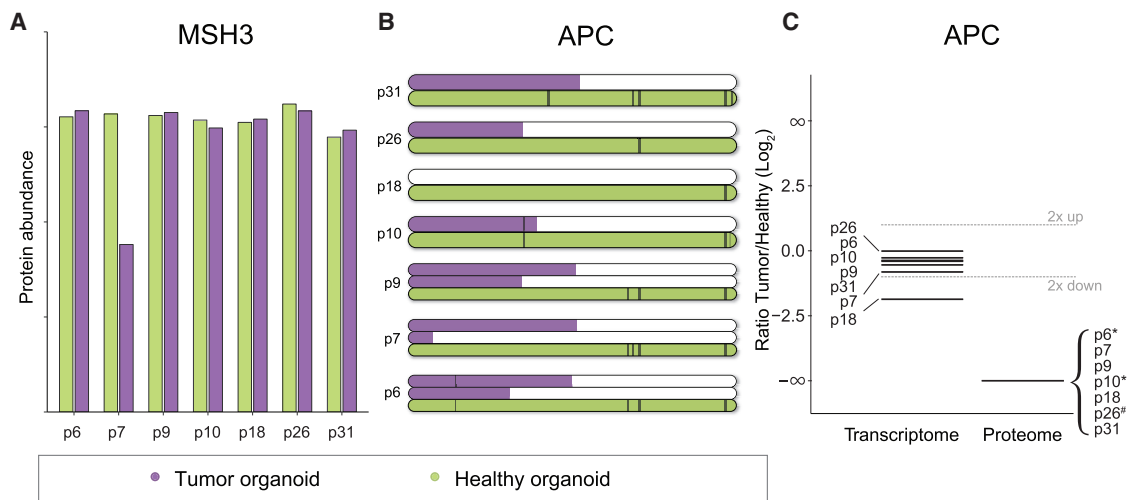
Figure 3. Overview of Patient Diversity

(A) Plot representing the protein abundance for MSH3 in healthy and tumor organoids across different patients. Green is used for healthy organoids, and purple is used for tumor organoids. A significant downregulation is observed exclusively in the tumor organoid from patient 7.

(B) Patient expression values of APC. The mutation pattern of the protein APC based on previously published exome sequencing data (van de Wetering et al., 2015) for the different patients is represented with the quantified peptides highlighted with black bars. The top purple line(s) represents the protein(s) found in the tumor organoid, and the green line represents the protein found in the healthy organoid. The length of the purple line depends on in frameshift mutations or nonsense mutations.

(C) The ratio between tumor and healthy organoids found at the transcriptome and proteome levels for the different patients is shown. *To be in line with the rest of our analyses a ratio is determined for "on-off" situations. #Used to include an expression value based only in one peptide.

See also Figure S3.

of the proteomics data can be clearly seen in this case, in which these four proteins were found significantly changing at the proteome level only for this patient, but only MARCKS appears to be significantly changing at the transcriptome level.

Due to the difference in the mutational pathway between the two genomic instability types, distinct and diverging clinical outcomes can be obtained. Patients with MSI tend to have longer survival and improved clinical outcome compared to those with microsatellite stability (MSS) (Gryfe et al., 2000). Distinct responses to cancer drugs is also observed between MSI and MSS, which makes recognition of molecular subtypes of colorectal cancer essential for future personalized treatment (Sinicrope and Sargent 2012).

The Wnt Signaling Pathway in Patients' Healthy and Tumor Organoids

Activation of the Wnt signaling pathway is a principal event in the development of colorectal cancer. Constitutively activated β-catenin signaling, due to APC deficiency or β-catenin mutations that prevent its degradation, leads to unbalanced stem cell generation, maintaining the cells in a progenitor state (van de Wetering et al., 2002). We detected APC in all seven patients, with peptides associated (almost exclusively) with the healthy organoids leading to extreme "on-off" ratios for this protein. The vast majority of detectable peptides originated from the C-terminal part, which is truncated in the tumor organoids (Figure 3B). In five of the seven patients, APC was only detected in the healthy organoids because C-terminal peptides were exclusively observed, and these would be missing in the truncated APC present in the tumor organoids. In one of the patients, a

single peptide was detected in a part of the APC that should be present in both healthy and tumor organoids, yet only signal was detected in the healthy organoid. Since we mix the samples before MS detection, we generate no bias between the healthy and tumor organoid with respect to protein detection. Nevertheless, this result requires both the transcript and protein information for an accurate interpretation of the data and highlights the need for a proteogenomic approach. A representation of the expression values found at the proteome and transcriptome level for this protein are displayed in Figure 3C. Mutational inactivation of APC leads to the inappropriate stabilization of β-catenin (Rubinfeld et al., 1993). β-Catenin (CTNNB1) was detected in all seven patients (Figure S3B); however, it only showed a light upregulation in one of the patients (patient 10), while in the rest of the patients, there was no difference between the healthy and tumor organoids. Stabilized β-catenin translocates to the cell nucleus and interacts with transcription factors of the transcription factor (TCF)/lymphoid enhancer-binding factor (LEF) family, leading to the transcription of Wnt target genes. Groucho/TLE proteins are transcriptional co-repressors that interact with proteins from the LEF/TCF family in the absence of a Wnt signal (and therefore β-catenin), keeping the pathway in an inactive state (Roose et al., 1998; Daniels and Weis 2005). However, in the presence of Wnt signal and if TLE1 is absent, we hypothesize that when β-catenin does not have to compete with TLE1, the signal might be stronger. TLE1 was quantified in our dataset in six out of seven patients with a strong downregulation in most of the tumor organoids, which is in concordance with the transcriptomics experiment. A representation of the expression

A

B
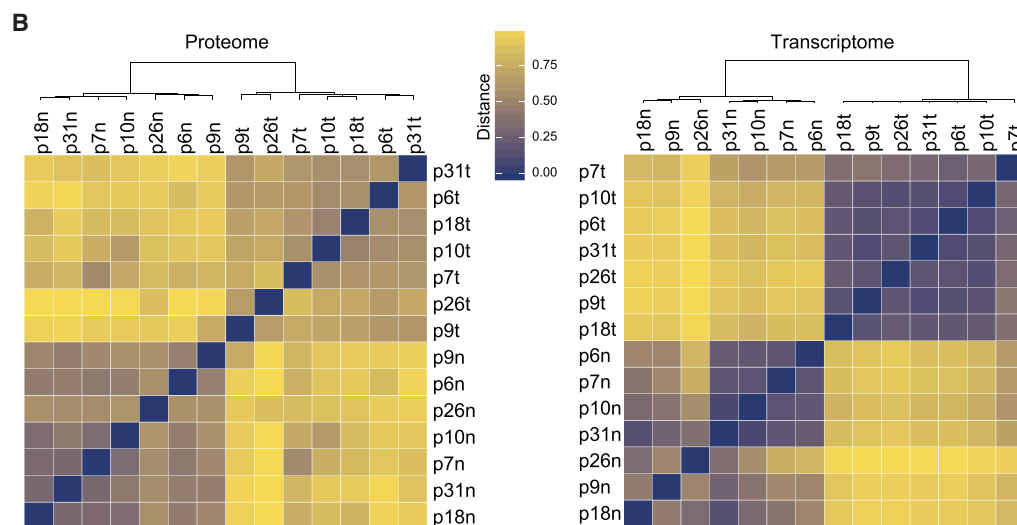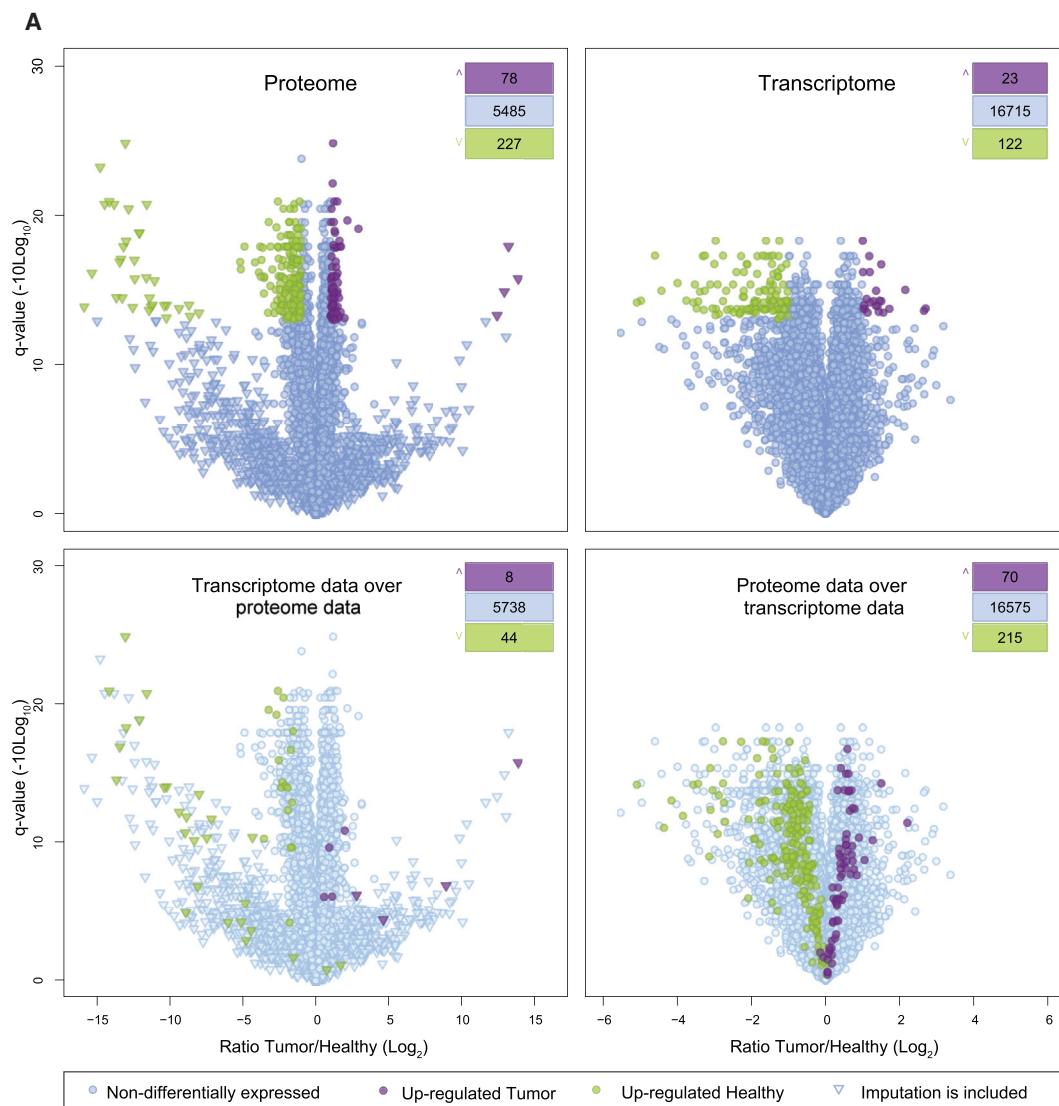
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values found at the proteome and transcriptome levels can be found in Figure S3B.

Above, we largely focused on the patient-specific differences observed in the proteome of healthy and tumor organoids, describing patient-specific features. These relevant patient-specific differences were not always present at the transcriptome level. To demonstrate the additional insight provided by proteomics, we performed immunohistochemistry (IHC) for the proteins MUC5A, MGMT, AMACR, and PHLDB2 on the organoids for patients 9, 18, and 26. We chose these proteins and patients because they only show expression changes at the protein level. The IHC confirmed our findings (Figure S3C), demonstrating the value of proteomics for any biological material.

Establishing a Human CRC Organoid Expression Profile by Quantitative Proteomics and Transcriptomics

Across all seven CRC patients, we were able to determine expression levels of 8,510 proteins. For the subsequent analysis, proteins with at least two peptides identified and quantified in at least three out of seven patients were used, which diminishes the total number to 5,790 proteins (Figure S4A). Certain proteins may present an "on-off" situation between tumor and healthy organoids. Since these proteins may be (most) important in the tumor state, we used imputed values from Proteome Discoverer, albeit only when necessary to obtain a ratio. We found a limited number of changes, constituting 5.3% of the detected proteins, which correspond to 78 proteins upregulated in the tumor organoids and 227 downregulated. A representation of these data can be seen in the top left panel of Figure 4A. On closer inspection of the quantified proteins, it is pleasing to note that several proteins previously described to be specific for colorectal cancer, such as PALM3 and GPR56 (Wiśniewski et al., 2012), are also observed in our data. The two proteins showed an upregulation in both datasets; namely, in the micro-dissected tumor tissue and our tumor organoids. Moreover, when comparing our whole dataset obtained from the colon organoids with a recent proteomics study on colorectal cancer from The Cancer Genome Atlas (TCGA) using human tumor tissue from 90 patients (Zhang et al., 2014), a high overlap is obtained (see Figure S4B). The TCGA data allowed the authors to determine five CRC subtypes based on the expression level of ~1,600 proteins. Although our choice of patients was based on the mutation profile of the most common tumor driver genes in CRC (APC, TP53, KRAS, and PIK3CA), we organized our data according to these subtypes and found a broad agreement (Table S3). Taking into consideration the overlap obtained between our study and previous studies about the characterization of the organoids (van

de Wetering et al., 2015), we can consider organoids as a promising proteomic model with multiple applications.

Among the 305 significantly changing proteins found in our dataset, several have already been related to CRC. MYO1C was found downregulated in the tumor organoids, and it has been recently suggested as a tumor suppressor gene candidate related to Tp53 (Hedberg Oldfors et al., 2015). Desmocollin-2 has been previously reported as being decreased or absent in CRC studies (Funakoshi et al., 2008; Khan et al., 2006) and was also downregulated in our dataset. UBE2C, also known as UBCH10, is essential for cell progression, and it has been related to several cancers (Xie et al., 2014) and was observed with an overexpression in the tumor organoids. Using CRC cell lines, depletion of this protein resulted in suppression of cellular growth, whereas overexpression promoted cell proliferation and oncogenic cellular growth (Fujita et al., 2009). The possibilities of inhibiting UBE2C for the treatment of CRC have also been studied (Chen et al., 2010). HspBP1 is a co-chaperone that binds to and inhibits the activity of Hsp70 and was also found to be overexpressed in the tumor organoids. High expression values of Hsp70 had been previously reported for several tumors, and the level of HspBP1 has also been reported as elevated in some tumors (Raynes et al., 2004).

Following a statistical treatment similar to that used for the proteomics data, 23 transcripts were found to be upregulated and 122 were downregulated in tumor versus healthy organoids consistently in the seven patients. A representation of these data is summarized in the top right panel of Figure 4A, and a detailed description of the proteins can be found in Table S3.

Next, we used hierarchical clustering for the differentially expressed proteins of both techniques, to classify all organoids. Figure 4B shows the correlation heatmaps at the proteome and the transcriptome level, respectively and in both cases the samples cluster by tumor versus healthy. For 5,320 proteins quantified in our proteome data we obtained corresponding data at the transcriptome level. Focusing first on the earlier defined differentially expressed proteins, only 22 proteins showed similar behavior at the mRNA level, as can be seen in Figure 5. Out of these 22 proteins only one, synaptotagmin 7 (SYT7), shows an upregulation in both datasets. SYT7 is also known as prostate cancer-associated protein 7 (Walker et al., 1999); however, a direct relation with CRC has not yet been shown. Among the other 21 downregulated proteins were CEAM7, mucin12, and gelsolin. Carcinoembryonic antigen-related cell adhesion molecule 7 (CEAM7) is also known as carcinoembryonic antigen CGM2 and is a member of the CEA family. CEA is a classical tumor marker for several types of cancer (Ballesta et al., 1995). CEAM7 is known to be expressed in

Figure 4. CRC Organoids Overview

(A) Volcano plots in which the average protein ratio ($\log_2$) against the q value ($-10\log_{10}$) are plotted. In the top panel, the data obtained from the proteomics experiment are presented on the left, whereas the data from the transcriptomics experiment are on the right. From the proteomics data, a total of 78 upregulated and 227 downregulated proteins are found. Regarding the transcriptome data, a total of 23 upregulated and 122 downregulated transcripts are found. In the bottom panel, a direct representation of the differentially expressed proteins from the transcriptomic analysis in the proteomic dataset is presented on the left and vice versa on the right. Although q values do not correlate, ratios do correspond well between the two technologies.

(B) Correlation heatmaps of the differentially expressed proteins from the proteomics (left) and transcriptomics (right) datasets. Tumor and healthy organoids are separated by hierarchical clustering.

See also Figure S4.
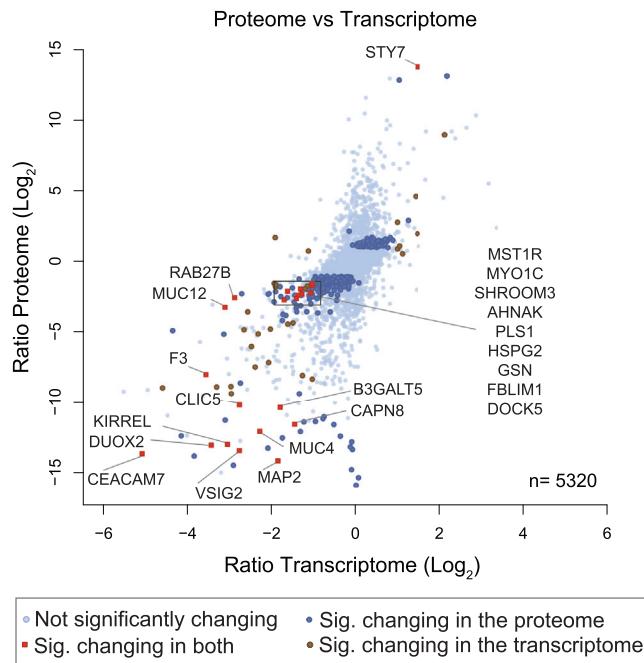
Figure 5. Overlap between the Proteome and the Transcriptome
Comparison of expression ratios between the proteins and their corresponding transcripts (5,320 protein-transcript pairs). Focusing on the differential proteins, 22 proteins showed alike behavior at the transcript level and only one of them is upregulated, while the rest are downregulated in both datasets. See also Figure S5.

normal colon and rectum and downregulated in colorectal adenocarcinomas (Thompson et al., 1994, 1997), which is in agreement with our results. Mucin12 is a transmembrane mucin that was downregulated in our dataset, which is in concordance with previous publications (Williams et al., 1999; Matsuyama et al., 2010; Packer et al., 2004). Gelsolin (GSN), a protein with structural molecule activity, is a multifunctional actin-binding protein that is downregulated in CRC. It has been suggested that the loss of this protein is a general mechanism associated with the transition from the noninvasive stage to the late invasive stage of the neoplastic process (Gay et al., 2008). A detailed description of the overlapping proteins can be found in Table S4.

We suspected that the limited overlap between both techniques (Figure S5A) might have occurred because our filtering criteria were too stringent. To test our hypothesis, we decided to color-annotate all differentially expressed proteins on the measured transcript data and vice versa. A consistent trend in expression among the proteome and mRNA datasets is observed; however, at the defined stringency level, the correlation is not high, as displayed in the bottom panels of Figure 4A. In order to broaden the stringency, without being overly tolerant, we decided to include the proteins found differentially expressed in one of the techniques that could be observed changing in the same direction using the other technique. A Venn diagram representing this profile with the overlapping proteins highlighted, constituting the CRC organoid profile, in red is shown in Figure S5B. A total of 308 pro-

teins are included in this dataset. The main molecular function of these proteins can be divided into proteins with catalytic activity (35%), binding proteins (28%), and proteins with structural molecule activity (12%). A pie chart with the representation of the molecular function obtained in Panther can be found in Figure S5C. Among the binding proteins, gastrotropin (FABP6) is found with an overexpression in the tumor organoids. Gastrotropin, also called fatty-acid-binding protein 6, has already been reported as being related to colon carcinogenesis, presenting a higher expression value in cancer tissue compared to normal tissue (Ohmachi et al., 2006). Proteins related to other cancers are also found in this dataset, such as BCAS1, which has been described as a strong candidate oncogene for breast cancer (Collins et al., 1998). Surprisingly, compared to breast cancer, in which upregulation is observed for this protein, an opposite trend is found in colorectal cancer (Correa et al., 2000). The same trend is found in our study; once again, proteomics data of organoids is recapitulating observations made with biopsy specimens.

Conclusions
Clinical models, such as cultured cancer cell lines and animal models, have been used for many years to investigate the mechanisms of diseases and design promising treatment strategies. It is well known that these model systems all have their limitations, and ideally, studies should be performed, in vivo, on patient material; however, this is currently not always realistic. Recently, a promising research technology has been developed, so-called organoids, that allows the continuous culture of a variety of healthy and diseased human tissues. These organoids provide a potentially unlimited supply of well-characterized patient material, circumventing some of the limitations of current models (i.e., lack of genetically stable cell lines and need of extensive colonies of animals). Several studies have already highlighted the potential for applications of organoid technology in the study of human cancer (Weeber et al., 2015; Boj et al., 2015; Gao et al., 2014; Karthaus et al., 2014; Drost et al., 2015; van de Wetering et al., 2015). The amenability of this technology for genomic and functional analysis as well as for high-throughput drug screening is beginning to be explored. Here, we set out to make a proteomic characterization of healthy and tumor colon organoids. Although sample amount is a genuine limiting factor in proteomics, we demonstrate here the feasibility of generating personalized human proteome profiles of human healthy and tumor colon-derived organoids. Fourteen organoid samples (seven tumor and seven healthy) from seven patients were quantitatively analyzed. System-wide data analysis revealed strong patient-specific features. A single patient with a hypermutated phenotype in colorectal cancer characterized by microsatellite instability clearly stood out, evidenced by several patient-specific proteins that have already been linked to microsatellite instability. Patients with microsatellite instability have a different clinical outcome compared to those exhibiting chromosomal instability (the most frequently occurring genomic instability in colorectal cancer), illustrating why personalized treatment is essential for the survival of individual patients.

Although we observed that patient proteomes exhibit a clear personalized profile, we were still able to extract some

tumor-specific characteristics across all patients from the proteomics data by focusing, for instance, on one of the most frequently mutated signaling pathways in colorectal cancer, Wnt signaling. Expression levels of several proteins belonging to this pathway changed significantly and consistently when tumor and healthy organoid proteomes were compared, which is consistent with the fact that several of these proteins were previously reported to be colorectal cancer biomarker proteins.

The present study further highlights the potential of organoids as model systems for personalized cancer research, demonstrating that advanced proteomics analysis is nowadays feasible and meaningful. A better categorization of CRC would be possible by further extending the work presented in this paper by investigating a larger number of patients and/or the proteome profiles of organoids when treated with anti-cancer drugs. With proteomics becoming ever more sensitive and faster, these goals may be achieved in the near future, expanding the technical toolbox for optimizing personalized cancer treatment.

EXPERIMENTAL PROCEDURES

Patient Material Background and Sample Preparation

Organoids were prepared as previously described (van de Wetering et al., 2015), and the samples were named based on this previous publication in order to facilitate the correlation of the results. Organoids were lysed and digested with Lys-C and trypsin. The resulting peptides were chemically labeled using stable isotope dimethyl labeling as described before (Boersema et al., 2009). Prior to the MS analysis, samples were fractionated to reduce the complexity using as SCX system. A detailed description of the sample preparation can be found in Supplemental Experimental Procedures.

Nano-UHPLC and Mass Spectrometry

The SCX fractions containing doubly and triply charged peptides (25 fractions from each SCX) were reconstituted in 10% formic acid and analyzed using a nanolitre-ultra high performance liquid chromatography (nano-UHPLC) Proxeon system (Easy-nLC 1000, Thermo Scientific) coupled to different mass spectrometers. Different amounts of sample were injected based on the SCX UV trace. The injected samples were first trapped on an in-house packed trap column (ReproSil-Pur C18-AQ, 3 μm [Dr. Maisch GmbH], 2 cm × 100 μm) before being separated in an in-house-made analytical column (Zorbax SB-C18, 1.8 μm [Agilent Technologies], 50 cm × 50 μm) at a constant temperature of 40°C. The buffers used were solvent A (containing 0.1 M acetic acid in water) and solvent B (containing 0.1 M acetic acid in 80% acetonitrile).

Peptides were loaded into the trap column at a constant pressure of 800 bars with 30 μL solvent A and chromatographically separated in the analytical column at a flow rate of 100 nL/min. LC methods of a duration of 120 min or 180 min were used: 7%–30% solvent B within 91 or 151 min, 30%–100% solvent B within 3 min, 100% solvent B for 2 min, and 13 min 100% solvent A. The column effluent was directly connected to an in-house pulled and gold-coated fused silica needle (with a 5 μm outer diamater [o.d.] tip).

For the LTQ-Orbitrap Elite (Thermo Scientific), a voltage of 1.7 kV was applied to the needle. The survey scan was recorded with a 350- to 1,500-m/z scan range at a resolution of 30,000, and for the tandem mass spectrum (MS2), the resolution was set to 7,500. The ten most intense precursors were selected for subsequent fragmentation in a data-dependent acquisition mode as described before (Frese et al., 2011) using collision induced dissociation (CID) and electron transfer dissociation ion trap detection (ETD-IT) activation techniques.

For the Q-Exactive (Thermo Scientific), a voltage of 1.7 kV was applied to the needle. The survey scan was recorded with the same scan range as for the LTQ-Orbitrap Elite but at a resolution of 35,000, and for the MS2, the resolution was set to 17,500. The 20 most intense precursors were selected for subsequent fragmentation using HCD as the activation technique.

Data Analysis

The raw files obtained from both instruments (Elite and Q-Exactive) were processed with Proteome Discoverer (PD, version 1.4, Thermo Scientific). The spectra were searched against a modified SwissProt human database (version 56.2) to which Matrigel proteins that were determined separately using a triplicate proteomics experiment were added. Searching was done with Mascot (version 2.5.1, Matrix Science), with the following parameters: trypsin digestion with up to two allowed missed cleavages, cysteine carbamidomethylation as fixed modification, oxidation of methionine, dimethyl labeling (light and intermediate) of lysine residues, and the peptide N termini as dynamic modifications. Peptide tolerance was set to 50 ppm for both instruments, and MS/MS tolerances were set to 0.6 Da for ETD-IT and 0.05 Da for electron transfer dissociation fourier transform orbitrap detection (ETD-FT) and HCD. The quantification protocol for PD was double dimethyl labeling, with a mass precision of 2 ppm for consecutive precursor mass scans.

The results were filtered using Percolator (Käll et al., 2007; Spivak et al., 2009) to a false discovery rate (FDR) below 1%. We further only accepted peptides with at least six amino acid residues, a Mascot ion score of at least 20, and search engine rank of 1.

All further analyses were performed in RStudio 0.98.1103, with R version 3.1.2.

To obtain a global picture of protein levels for the tumor versus healthy organoids, we performed t tests on the log2-transformed protein ratios, considering the different tissue donors as biological replicate measurements. Only proteins identified by more than two peptides were considered. Tests were performed whenever at least three measurements (i.e., patient ratios) were available for a protein. A detailed description of the complete analyses can be found in Supplemental Experimental Procedures.

ACCESSION NUMBERS

The accession number for the raw data and result files reported in this paper is ProteomeXchange Consortium PRIDE: PXD004149 (Vizcaíno et al., 2016).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.12.016.

AUTHOR CONTRIBUTIONS

A.C., M.v.d.W., H.C., A.J.R.H., and S.M. designed the study. A.C. performed all proteomics experiments. M.v.d.W. prepared all organoid samples, and carried out the transcriptome analysis. H.W.P.v.d.T. carried out and supervised the computational analysis. All authors helped interpret the results and together wrote the manuscript.

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