Sorbitol dehydrogenase overexpression and other aspects of dysregulated protein expression in human precancerous colorectal neoplasms: a quantitative proteomics study

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Abstract: Colorectal adenomas are cancer precursor lesions of the large bowel. A multitude of genomic and epigenomic changes have been documented in these preinvasive lesions, but their impact on the protein effectors of biological function has not been comprehensively explored. Using shotgun quantitative MS, we exhaustively investigated the proteome of 30 colorectal adenomas and paired samples of normal mucosa. Total protein extracts were prepared from these tissues (prospectively collected during colonoscopy) and from normal (HCEC) and cancerous (SW480, SW620, Caco2, HT29, CX1) colon epithelial cell lines. Peptides were labeled with isobaric tags (iTRAQ 8-plex), separated via OFFGEL electrophoresis, and analyzed by means of LC-MS/MS. Nonredundant protein families (4325 in tissues, 2017 in cell lines) were identified and quantified. Principal component analysis of the results clearly distinguished adenomas from normal mucosal samples and cancer cell lines from HCEC cells. Two hundred and twelve proteins displayed significant adenoma-related expression changes (q-value < 0.02, mean fold change versus normal mucosa ±1.4), which correlated (r = 0.74) with similar changes previously identified by our group at the transcriptome level. Fifty-one (25%) proteins displayed directionally similar expression changes in colorectal cancer cells (versus HCEC cells) and were therefore attributed to the epithelial component of adenomas. Although benign, adenomas already exhibited cancer-associated proteomic changes: 69 (91%) of the 76 protein up-regulations identified in these lesions have already been reported in cancers. One of the most striking changes involved sorbitol dehydrogenase, a key enzyme in the polyol pathway. Validation studies revealed dramatically increased sorbitol dehydrogenase concentrations and activity in adenomas and cancer cell lines, along with important changes in the expression of other enzymes in the same (AKR1B1) and related (KHK) pathways. Dysregulated polyol metabolism might represent a novel facet of metabolome remodeling associated with tumorigenesis.

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Proteomic profiling of colorectal adenomas

SORD overexpression and other aspects of dysregulated protein expression in human precancerous colorectal neoplasms: A quantitative proteomics study

Running title: Proteomic profiling of colorectal adenomas

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Non-standard abbreviations (alphabetical):

2-DE: 2-dimensional gel electrophoresis; AGC: automatic gain control; FDR: false discovery rate; GC-TOF-MS: gas chromatography-time of flight-mass spectrometry; GO: gene ontology; HCD: higher-energy collisional dissociation; HPA: Human Protein Atlas; INHAT: inhibitor of acetyltransferases; iTRAQ: isobaric tags for relative and absolute
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quantification; LTQ: linear trap quadrupole; MGF: Mascot generic format; MS/MS: tandem mass spectrometry; PCA: principal component analysis; PSM: peptide spectra match; SORD: sorbitol dehydrogenase; TBST: tris-buffered saline with Tween 20.

Key words: colorectal adenomas; sorbitol dehydrogenase; mass spectrometry; quantitative proteomics; iTRAQ 8-plex.

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SUMMARY

Colorectal adenomas are cancer precursor lesions of the large bowel. A multitude of genomic and epigenomic changes have been documented in these preinvasive lesions, but their impact on the protein effectors of biological function has not been comprehensively explored. Using shotgun quantitative MS, we exhaustively investigated the proteome of 30 colorectal adenomas and paired samples of normal mucosa. Total protein extracts were prepared from these tissues (prospectively collected during colonoscopy) and from normal (HCEC) and cancerous (SW480, SW620, Caco2, HT29, CX1) colon epithelial cell lines. Peptides were labeled with isobaric tags (iTRAQ 8-plex), separated by OFFGEL electrophoresis, and analyzed by LC-coupled tandem MS. Non-redundant protein families (4325 in tissues, 2017 in cell lines) were identified and quantified. Principal component analysis of the results clearly distinguished adenomas from normal mucosal samples, and cancer cell lines from HCEC cells. Two hundred twelve proteins displayed significant adenoma-related expression changes (q-value < 0.02, mean fold change vs. normal mucosa +/-1.4), which correlated (r=0.74) with similar changes previously identified by our group at the transcriptome level. Fifty-one (~25%) proteins displayed directionally similar expression changes in colorectal cancer cells (vs. HCEC cells) and were therefore attributed to the epithelial component of adenomas. Although benign, adenomas already exhibited cancer-associated proteomic changes: 69 (91%) of the 76 protein upregulations identified in these lesions have already been reported in cancers. One of the most striking changes involved sorbitol dehydrogenase (SORD), a key enzyme in the polyol pathway. Validation studies revealed dramatically increased SORD concentrations and activity in adenomas and cancer cell lines, along with important changes in the expression of other enzymes in the same (AKR1B1) and related (KHK)
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pathways. Dysregulated polyol metabolism may represent a novel facet of the metabolome remodeling associated with tumorigenesis.
INTRODUCTION

Colorectal cancer ranks third among the world’s high-incidence cancers and is a leading cause of cancer-related death among older adults (1, 2). In the United States alone, projections for 2013 include 102,480 new cases and 50,830 deaths (2). Cancerogenesis in the large bowel begins with the transformation of the epithelial cell lining of the gut. Molecular alterations, mainly involving the WNT signaling pathway, render these cells hyperproliferative, and they form benign adenomatous tumors. The neoplasms are initially noninvasive (3, 4), and the vast majority remain that way. But as genetic and epigenetic anomalies continue to accumulate, the tumor cells’ capacity for invasion and destruction of surrounding tissues increases. At some point, this process drives certain adenomas into the realm of frank malignancy, transforming them into adenocarcinomas.

Early diagnosis of colorectal tumors has been greatly facilitated by screening methods based on fecal analysis or colonoscopy, but both approaches have limitations (5-9). Better understanding of the molecular mechanisms underlying large bowel tumorigenesis could improve our chances of detecting these lesions in the adenomatous or localized adenocarcinomatous stage, when the chances of successful treatment are greater. Promising results for the detection and validation of potential cancer biomarkers are emerging from proteomic studies of cancer development (10). Compared with older gel electrophoresis-based approaches, shotgun proteomic methods, particularly those that include pre-MS OFFGEL electrophoretic peptide fractionation (11), enhance the sensitivity, robustness, and reproducibility of these studies (12) and expand the proteome coverage to include proteins that are less abundantly expressed (13-16). Furthermore, with the aid of isobaric-tag peptide labeling strategies, MS can also be used for relative quantification of protein expression levels within a series of multiple human tissue samples (12, 17-19).
Thus far, only a few MS-based proteomic studies have examined human colorectal adenomas (reviewed in refs. (9, 20)). We therefore decided to explore the proteome of a relatively large series of these precancerous lesions (each with a paired sample of normal colon mucosa) using quantitative shotgun MS with the widely used iTRAQ (isobaric tags for relative and absolute quantitation) peptide labeling technique (21, 22) and OFFGEL fractionation. Adenoma-related protein expression variations specific to the epithelial compartment of these lesions were identified with a novel approach, which involved comparing the human tissue proteome with that of colon epithelial cell lines. The results of these studies revealed several protein expression changes previously documented only in advanced colorectal cancers. They also disclosed several novel changes with potentially important roles in early-stage large bowel tumorigenesis, including the marked upregulation of a key enzyme in the polyol pathway.

EXPERIMENTAL PROCEDURES

Human tissue samples and cell lines

Human colorectal tissues were prospectively collected from patients undergoing colonoscopy in the Istituti Ospitalieri of Cremona, Italy. Local ethics committee approval was obtained, and tissues were used in accordance with the Declaration of Helsinki. Each donor provided written informed consent to sample collection, data analysis, and publication of the findings. Progressive numbers were assigned to each patient to protect human confidentiality. The series comprised 30 colorectal adenomas, each with a paired sample of normal mucosa from the same colon segment, >2 cm from the lesion. Tissues were collected endoscopically, promptly frozen in liquid nitrogen, and stored at -80 °C.

Five colorectal cancer cell lines (HT29, Caco2, CX1, SW480, SW620) were obtained from the Zurich Cancer Network's Cell Line Repository. All had been recently
Proteomic profiling of colorectal adenomas purchased from the American Tissue Culture Collection (Teddington, UK) and were certified to be mycoplasma infection-free. Caco2 and CX1 cells were cultured in Dulbecco's Modified Eagle Medium, HT29 cells in McCoy's medium, and SW480 and SW620 cells in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, and 1% penicillin-streptomycin (Sigma, St Louis, Missouri, USA). The recently established line of immortalized human colon epithelial cells (HCEC) was obtained from J. W. Shay and grown as described elsewhere (23).

Protein extraction from tissues and cell lines

For MS studies, frozen tissue samples were quickly weighed and homogenized on ice (1 min grinding, 1 min on ice, 1 min grinding) in a Wheaton glass borosilicate grinder containing a solution of 100 mM triethylammonium bicarbonate (Sigma, St Louis, MO, USA), 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche, Mannheim, Germany), 1 M urea, 5 mM β-glycerophosphate disodium salt hydrate, 1 mM sodium orthovanadate, and 5 mM sodium fluoride (Sigma). The efficiency of cell lysis was microscopically confirmed. The homogenates were then sonicated with a Bioruptor (Diagenode, Denville, NJ, USA) (high power, five 10''/10'' ON/OFF cycles) and centrifuged (16,000 g for 5 min at 4 °C). The supernatant containing the proteins was collected and stored at -80 °C.

Cells (grown to >80% confluence in 15 cm² dishes) were washed in PBS, covered with 250 µL of the buffer used for tissue sample homogenization (see above), detached from the dish with a cell scraper, and homogenized (25 passages through a 25G needle). The efficiency of cell lysis was microscopically confirmed. Sonication and centrifugation were repeated, as described above, and the protein concentration was determined by Bradford assay. Prior to MS analysis, a 5-µg sample of each protein extract was subjected to 1-dimensional gel electrophoresis on a 12% bisacrylamide gel to assess protein integrity and extraction protocol reproducibility. The entire proteomic workflow, from tissue/cell
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processing to statistical analysis, is summarized in Figure 1 and described in detail in the next five sections of Experimental procedures.

For sorbitol dehydrogenase (SORD) assays (see below), >80% confluent cells were washed in PBS and covered with a solution consisting of 100 mM triethanolamine (Sigma) and 1X Complete EDTA-free Protease Inhibitor Cocktail (Roche). (A simple buffer was used to reduce the risk of introducing anti-enzymatic substances into our extract.) Cells were then scraped and homogenized with 25 passages through a 25G needle. Tissue samples were weighed and homogenized in a Wheaton glass borosilicate grinder containing the buffer described above. After centrifugation (16,000 g, 4 °C, 5 min), the supernatant was aliquoted and stored at -80 °C. Protein concentration was measured by Bradford assay.

Protein digestion and iTRAQ 8-plex labeling

iTRAQ 8-plex experiments were performed to analyze tissue extracts (10 experiments) and cell-line extracts (1 experiment) (Figure 1). Labeling efficiency and relative quantitation accuracy were assessed with the aid of two reference protein extract mixtures: one for tissue samples (pooled extracts from 3 normal tissues and 3 adenomas) and one for cell lines (pooled aliquots of each of the six cell line extracts). Fifty micrograms of protein per sample were used for each iTRAQ channel. Tryptic digestion (10% w/w, sequencing-grade modified trypsin; Promega, Madison, WI, USA) and iTRAQ 8-plex labeling (AB Sciex, Framingham, MA, USA) were performed according to manufacturers’ instructions (2.5-hour incubation of samples with iTRAQ labels). For tissue experiments, two iTRAQ labels, 113 and 114, were chosen for the reference mixture, while labels 115/116, 117/118, and 119/121 were used for the 3 pairs of normal/adenomatous tissues included in each experiment. For the cell line experiment, labels 113 and 114 were used for the reference mixture, and labels 115-121 represented HCEC, HT29, Caco2, CX1, SW480,
Proteomic profiling of colorectal adenomas and SW620 cells, respectively (Figure 1). After iTRAQ labeling, the samples (for each experiment) were combined, desalted on 500 mg SepPak C18 columns (Millipore, Billerica, MA, USA), dried in a SpeedVac concentrator (Thermo Scientific, NC, USA), and subjected to peptide fractionation.

**OFFGEL electrophoresis**

Peptide fractionation was performed according to the manufacturer’s protocols with an Agilent 3100 OFFGEL fractionator and 12-well OFFGEL kit (both from Agilent Technologies, Santa Clara, CA, USA). Briefly, samples were resolubilized in 1.8 mL of 1x OFFGEL peptide stock solution containing carrier ampholytes (pH range 3–10), loaded into the wells (150 µL per well), and focused until 20 kV/h was reached with a maximum current of 50 µA. For each experiment, 12 fractions were collected. A 15-µL aliquot of each fraction was acidified with 1.5 µL of a 50% acetonitrile / 1% trifluoroacetic acid solution, desalted using ZipTip C18 (Millipore, Billerica, MA, USA), dried, resolubilized in 15 µL of a 0.1% formic acid / 3% acetonitrile solution, and analyzed with MS.

**Liquid Chromatography and Mass Spectrometry**

Peptide samples (4 µL) were analyzed on an LTQ-Orbitrap Velos mass spectrometer (Thermo Fischer Scientific, Bremen, Germany) coupled to a nano-HPLC system (Eksigent Technologies, Dublin, CA, USA). The solvent compositions were 0.2% formic acid and 1% acetonitrile for channel A and 0.2% formic acid and 80% acetonitrile for channel B. Peptides were loaded onto an in-house-made tip column (75 µm × 80 mm) packed with reverse-phase C18 material (AQ, 3 µm 200 Å, Bischoff GmbH, Leonberg, Germany) and eluted (flow rate, 250 nL/min; solvent B gradient: from 3% to 30% in 62 min, from 30% to 45% in 70 min, and 45% to 97% in 75 min).

Full-scan MS spectra (300–1700 m/z) were acquired at a resolution setting of 30000 at 400 m/z after accumulation to a target value of 1 x 10⁶. For the eight most intense
signals per cycle above a threshold of 1000, both CID (collision-induced dissociation) and HCD (higher-energy collisional dissociation) spectra were acquired in a data-dependent manner (Figure 1). CID scans were recorded in the ion-trap (settings: normalized collision energy, 35; maximum injection time, 50 ms; automatic gain control [AGC], $1 \times 10^4$ ions). For the HCD scans, spectra were recorded at a resolution setting of 7500 at 400m/z (normalized collision energy, 52; maximum injection time, 125 ms; AGC, $5 \times 10^4$ ions). Charge state screening was enabled, and singly charged states were rejected. Precursor masses previously selected for MS/MS were excluded from further selection for 60 s, and the exclusion window was set at 10 ppm. The maximum number of entries in the exclusion list was set at 500. All samples were analyzed in duplicate, and precursors selected in the first run were excluded from fragmentation in the second run. The exclusion list was set on a time window of 4 minutes and mass width of 10 ppm. Spectra were acquired using internal lock mass calibration on m/z 429.088735 and 445.120025.

**Peak list generation and database search**

As depicted in Figure 1, Mascot Distiller 2.4.3.3 (Matrix Science, Boston, MA, USA) was used to generate Mascot generic format (MGF) peak lists. De-isotoping and peak picking were not performed between 112.5 and 121.5 m/z (the range containing iTRAQ reporter ions), and the HCD and CID spectra were merged by summing. For each of the 11 experiments, the corresponding 24 MGF peak lists were concatenated and searched, with the aid of the Mascot Server 2.3.02 (Matrix Science), against a forward UniProtKB/Swiss-Prot database for human proteins concatenated to a reversed decoyed FASTA database. The concatenated database contained a total of 147,438 proteins with accessions in Gene Ontology-compatible format and 260 common MS contaminants (NCBI taxonomy ID 9606, release date 2011-12-13).
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Methylthio (C), iTRAQ 8-plex labeling at N-terminal and lysine were set as fixed modifications, while variable modifications consisted of methionine oxidation and iTRAQ 8-plex labeling of tyrosine. We used the iTRAQ 8-plex-vs114 (Applied Biosystems) quantitation method. The isotope and impurity correction factors used for each iTRAQ label were those provided by the manufacturer. Precursor and fragment tolerances were set at 10 ppm and 0.8 Da, respectively. Enzyme specificity was set to trypsin with an allowance of up to 1 missed cleavage. Using Mascot internal export scripts, we transformed Mascot DAT files into XML files and parsed them with in-house scripts so that peptide sequences, scores, and intensities of the individual reporter ion channels were reported. Confidently identified and quantified peptides were selected with the following filters: rank 1 (best spectra assignment); ion score: > 15; and presence of iTRAQ intensity values for reporter channels 113 and 114.

Quantification of relative protein abundance (steps described in the boxes of the lower half of Figure 1)

Peptide reporter channel intensities were summed for each protein individually using R-scripts. Ratios were built from summed channels (113/114 to 121/114) for all proteins identified in each iTRAQ experiment. False discovery rates (FDRs) (24) were determined at the spectrum, peptide, and protein levels. The results of individual experiments were then merged into one matrix, which was used for statistical analysis in R and Perseus (Version 1.2.7.4). All proteins identified with the same peptide(s) were grouped into families, each of which was identified by a unique protein family number. Ratios of the intensity of each ion channel to that of 114 were converted to base 2 logarithmic values and normalized respectively on the median (which was set at 0), resulting in ratios that followed a Gaussian distribution. Proteins identified on the basis of the same peptide(s) shared the same family number and were represented once in our
statistical analysis. The paired t-test was used to compare the expression of a given protein in each adenoma and that found in the corresponding sample of normal mucosa. To correct for multiple comparisons, the FDR was controlled with the Benjamini-Hochberg procedure. The average protein-expression fold change in adenomas, compared with the normal mucosa, was then calculated. For this, median normalized ratios for all proteins in each paired adenoma-normal mucosa sample were deconvoluted of the reference standard effects (114) to compute the adenoma vs. normal mucosa ratio per protein (deconvoluted fold change, i.e., \([116/114] / [115/114] = [116/115]\)) and the mean fold change per protein in all tissue pairs. The Mascot emPAI value for all proteins were included in XML exports for each experiment. Thereafter, the mean Mascot emPAI value was calculated for all proteins.

**Functional annotation of proteins**

Gene ontology (GO) annotations and GO terms for proteins in the UniProt/SwissProt database were sourced from http://www.ebi.ac.uk/. The Scaffold program (Version 3) was used to identify the *cellular localizations* and *biological processes* most represented in lists of proteins quantified in tissues and cell lines. The topGO Bioconductor software package in R was used to identify and screen for GO *biological process* categories displaying enrichment for proteins that were differentially regulated in adenomas (vs. normal mucosa) (25). First, we prepared a "Universe" comprising all the proteins quantified in our study, each matched to GO terms and annotations. This served as the "Background." The "Foreground" consisted of the list of significantly dysregulated proteins. The most significant GO terms were scored with the Eliminating Genes (*elim*) method (25).

**Western Blotting**

Proteins were separated on a 10% SDS-polyacrylamide gel and transferred to a hydrophobic polyvinylidene difluoride membrane (GE Healthcare, Amersham Hybond-P)
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PVDF membrane, Pittsburgh, PA, USA) according to standard protocols (26). After 1 h of blocking with 5% non-fat dry milk in TBS with 1% Tween 20 (milk-TBST), membranes were incubated overnight with the primary antibody (anti-SORD [HPA040260 Sigma]; anti-aldose reductase, AKR1B1 [GTX113381 GeneTex]; anti-ketohexokinase, KHK [GTX109591 GeneTex]) diluted 1:1000 in milk-TBST, washed once with milk-TBST (20 min) and twice with TBST (20 min). After 1 h of incubation in horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG, GE Healthcare) diluted 1:5000 in milk-TBST, membranes were washed once with milk (20 min) and twice with TBST (20 min). Enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech, Cat. No. RPN2106) was used to detect immunoreactive proteins.

**Immunostaining of cells and tissues**

HT29 and HCEC cells were seeded (3 x 10^5 per well) on 22 x 22 mm cover slips in 6-well plates and grown under standard conditions until cells reached 70-80% confluence. Cells were then washed twice with PBS and fixed in ethanol:methanol solution (50:50) for 10 min at room temperature. Fixed cells were permeabilized (10 min with 0.25% Triton X-100), blocked (30 min in 10% goat serum [X0907, Dako, Glostrup, Denmark]), and incubated with primary antibody (rabbit polyclonal anti-SORD, HPA040260, Sigma, 1:100) for 18 h at 4 °C. After three washes with PBS, the cells were incubated for 1 h with secondary antibody conjugated to polymer-HRP anti-rabbit (Dako, EnVision+ System-HRP; Cat. No. K4010). They were then washed three times in PBS and incubated for 15 min in the substrate-chromogen, 3,3-diaminobenzidine tetrahydrochloride (DAB, Dako, EnVision+ System- HRP; Cat. No. K4010). Cells were washed quickly with PBS and mounted on slides (EUKITT, O. Kindler, GmbH, Freiburg, Germany) for light microscopy (Leica Microsystems GmbH, Wetzlar, Germany). Images were examined and recorded with Leica Application Suite (V3.3.0) software.
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Sorbitol dehydrogenase immunohistochemistry was performed as previously described (27). Tissue sections (normal colon and ileum, colorectal adenomas and adenocarcinomas) were incubated for 24 h at 4 °C with primary antibody (rabbit polyclonal anti-SORD, HPA040261, Sigma) at a 1:100 dilution.

Measurement of SORD activity

Total protein was extracted from cell lines and tissues as described above. SORD catalyzes the reversible conversion of D-sorbitol to D-fructose, with β-NADH as a cofactor:

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\text{D-fructose + β-NADH} \rightleftharpoons \text{D-sorbitol + β-NAD} \]

SORD activity was quantified via continuous spectrophotometric rate measurement of the β-NAD formation rate (temperature 25 °C, pH 7.6, \(A_{340\text{nm}}\), light path of 1 cm) in a Cary 50 Scan UV-visible spectrophotometer using the Cary Kinetics Application (both from Varian Inc., Palo Alto, CA, USA) (28). The final reagent concentrations in a 1-mL cuvette were as follows: 78.33 mM triethanolamine, 183 mM D-fructose, 0.21 mM β-NADH, 0.033% (w/v) BSA. The absorbance reading was recorded when the enzyme was added. One unit of enzyme activity was defined as the amount of enzyme required per minute to convert 1.0 µmole of D-fructose to D-sorbitol at pH 7.6 at 25 °C. A mixture of reagents plus recombinant SORD was used as the positive control; negative controls consisted of the same reagent mixture with no recombinant SORD, with recombinant SORD but no D-fructose, or with recombinant SORD but no β-NADH.

Extraction and quantification of intracellular metabolites by targeted gas chromatography-coupled MS

Frozen tissue (50 to 100 mg) was homogenized in 250 µl ice-cold 80% methanol using a glass borosilicate grinder from Wheaton. The homogenate was microscopically examined to ensure that it was cell-free and then transferred to Eppendorf vials and left on
ice for 15 min to ensure efficient protein precipitation. After centrifugation (15,000 g for 3 min at 0 °C), the supernatant was snap-frozen and stored at -80 °C, while the protein content of the pellet was determined by the Bradford method.

For gas chromatography-coupled MS (GC-TOF-MS) analyses, 10 µL of supernatant was transferred to a 1.5-mL Eppendorf tube, and internal standards (13C1-sorbitol, 13C1-fructose, and 13C1-glucose—1.2 pmoles of each) were added. The samples were then dried overnight in a vacuum centrifuge (Concentrator 5301, Eppendorf AG, Germany). Methoxyamine hydrochloride and N-methyl-N-(trimethylsilyl)trifluoroacetamide were used as derivatization reagents (29).

The derivatized metabolites and internal standards were subjected to GC-TOF-MS (GC 7890A, Agilent Technologies, Santa Clara, CA, USA; GCT Premier Micromass, Waters, Manchester, UK) with a Rxi-5Sil MS Integra-Guard column (length: 30 meters, internal diameter: 0.25 mm) and a film thickness of 0.25 µm (Restek, USA). One microliter of each derivatized sample was injected in splitless mode on a baffled glass liner and transferred to the capillary column by rapid heating of the liner from 50 °C to 250 °C at a rate of 12 °C/sec. For the separation of the metabolites, helium was used at a flow rate of 1 ml/min and, after an initial hold time of 2 min, a temperature gradient from 80 °C to 320 °C (rate 8 °C/min) was applied. The TOF-MS was set to acquire centroided standard electron ionization mass spectra over a range of 50 to 600 m/z at a rate of 3 spectra/sec. The GC-MS transfer line was heated to 280 °C. Dynamic range enhancement was activated. C6ClF5 was used as lock mass compound.

The MassLynx and QuanLynx programs (Waters, UK) were used to review and analyze the acquired data. The absolute concentrations of D-sorbitol, α and β D-fructose, and α and β D-glucose were calculated on the basis of the ratio of the intensity of specific fragments originating from the unlabeled compound to that of the added labeled analogue
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These concentrations were used to estimate intracellular levels per milligram of tissue (adenoma vs. normal mucosa). The relative concentration of lactate was estimated from the ratio of the intensity of specific fragments originating from the unlabeled compound and that of the added $^{13}$C$_1$-sorbitol (internal standard).

RESULTS

Proteomic analysis of human colorectal tissues and colon cell lines.

We used a quantitative-MS-based discovery strategy to explore the proteome of human colorectal tissues and colon cell lines (normal and neoplastic). The characteristics of the precancerous colorectal lesions are listed in Table 1. Protein extracts from these tumors and their paired samples of normal mucosa (total, 60 samples) were analyzed using iTRAQ LC-MS/MS and the workflow described in Figure 1. The inclusion of two reference sample mixes allowed us to control for technical variability across the ten experiments on tissue samples since the reference sample was analyzed twice in each experiment. OFFGEL electrophoresis was used to obtain highly reproducible, pI-based, in-solution separation of pooled iTRAQ-labeled peptides. Furthermore, for relative quantification of proteins using iTRAQ reporter ions, we adopted a stringent FDR for protein spectra matches (PSMs), and high-confidence peptides for protein quantification were selected only if the reporter ions (113 and 114) were quantified in the reference sample mix (iTRAQ reporter channels 113 and 114). The data set generated with this approach was large and complex, but we developed a simplified analytical method that allowed us to work with and merge the large data files generated after MS/MS (Figure 1). High-resolution MS/MS spectra acquired on the LTQ-Orbitrap Velos spectrometer after duplicate analysis of OFFGEL tissue sample fractions produced a total of 240 raw files (10 experiments, 120 fractions, 2 replicates). A total of 37,184 (FDR = 0.9%) unique tryptic peptides were confidently identified and
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quantified from 285,929 unique PSMs (FDR = 0.2%) (Table 2, Supplementary Table 1). Ten thousand four hundred fifty-two proteins (FDR = 1.5%) were assembled from the quantified peptides. Proteins that were indistinguishable by MS/MS (i.e., two or more proteins identified on the basis of the same peptide sequence; see Experimental procedures for details) were represented as a single family. The result was a total of 4325 non-redundant protein families, two-thirds (2865, 66%) of which were relatively quantified in at least 9 normal mucosa/adenoma pairs and 1072 (25%) in all 30 pairs (Table 2, Supplementary Table 1).

To verify the efficiency of iTRAQ protein labeling, we repeated the database search with Methylthio (C) set as a fixed modification and iTRAQ8plex (N-term), iTRAQ8plex (K), iTRAQ8plex (Y), and Oxidation (M) set as variable modifications. (All other search parameters were unchanged.) The assigned PSMs were filtered, as described in Experimental procedures, and the average iTRAQ labeling efficiency achieved in each of the 10 tissue experiments was 96% (Supplementary Table 2). To ascertain the efficacy of including a standard sample mix as a reference for normalization, we compared combined Gaussian plots of log2-protein ratios of normal mucosa or adenoma samples with the respective reference channel per experiment (e.g., 115/114 vs. 113/114 for normal tissues, 116/114 vs. 113/114 for adenomas, see Figure 1). The ratios displayed normal distributions in all channels. For the reference channel (113/114), log2-ratios were largely centered around 0, whereas the distribution of adenoma and normal channel log2-ratios was broader and not always centered at 0 (data not shown).

Sample complexity is a common problem in the analysis of proteomic data from human colorectal tissues. It stems in part from contamination of the epithelial cell proteome by proteins from stromal cells (which were inevitably present in our specimens even though the endoscopic tissue sampling procedure we used yielded superficial specimens with
consistent high epithelial contents). Microdissection can be utilized to isolate subpopulations of cells, but it can diminish the quantity and quality of the proteins, rendering them suboptimal for some types of proteomic analysis. To avoid this problem, we adopted a novel strategy for preliminary identification of the proteomic alterations that were most likely to involve the epithelial-cell component of the adenomas. The proteomic profiles of the colon tissues were compared with those of six colon epithelial cell lines (five colon cancer cell lines plus HCEC cells, to our knowledge, the only well-characterized line established from normal colorectal epithelium (23)). Changes in expression levels observed in adenomas (i.e., upregulation or downregulation with respect to normal mucosal levels) were presumed to be epithelial-cell-specific if similar changes were found in the colon cancer cell lines (relative to HCEC cells). After OFFGEL fractionation, duplicate MS analysis of iTRAQ-labeled peptides (24 fractions) from the six cell lines was performed in an LTQ-Orbitrap Velos mass spectrometer, and 11,266 peptides (FDR = 0.5%) were confidently identified and quantified from 27,922 unique PSMs (FDR = 0.4%) (Table 2, Supplementary Table 3). A total of 2017 non-redundant protein families (FDR = 1.1%) were identified and relatively quantified in cell lines; 1957 (97%) were present in all six cell lines (Table 2). In the iTRAQ experiment with cell lines, peptide labeling efficiency was 95% (Supplementary Table 2).

Relative quantification of the proteomes of colorectal tissues and cell lines.

The concentration range for proteins expressed in human tissues spans ten orders of magnitude. We chose not to deplete our protein samples of high-abundance proteins (e.g., albumin, IgG), because with the number of tissue samples being analyzed, additional sample preparation steps were considered potential sources of confounding variability (30). As an alternative, each of the ten pooled iTRAQ-labeled samples (ten experiments) were separated into 12 fractions based on the isoelectric point of peptides,
reducing the complexity of our protein matrix and limiting the risk of a bias toward the more abundant proteins.

The expression levels of the 4325 non-redundant protein families we were able to relatively quantify in colorectal tissues spanned four orders of magnitude, as deduced from the protein Mascot emPAI value (used as a proxy for the emPAI value (31) to estimate protein concentrations) (Figure 2A). Thirty percent (1304/4325) of these families were relatively quantified on the basis of more than one unique peptide. At the top of this list were the large proteins AHNAK, DYNC1H1, DSP, and FCGBP (Figure 2B). In colon epithelial cell lines, 1174 of the 2017 protein families were relatively quantified with more than one unique peptide.

Gene Ontology Annotation in Scaffold was used to identify the subcellular localizations of these protein families and the biological processes they were involved in. The GO categories represented in the tissue and cell line proteomes were fairly similar. In the cell-line proteome, however, the categories generally contained fewer proteins since the total number of proteins detected in these cells was lower than that of the tissues (Figure 2C). Cytoplasmic and organelle- or membrane-associated proteins were the most highly represented categories in our extracts, but nuclear proteins were also readily identified, which indicates that our protein extraction procedure was not strongly biased toward a few cell compartments. The most highly represented biological processes in the tissue proteome were metabolic or biosynthetic processes, whereas cell component organization and developmental processes predominated in the cell line proteome (Figure 2D). Stromal contamination is probably responsible for the increased representation of immune system processes in the tissue proteome (compared with that of the cell lines).

Log₂-expression levels of the protein families identified in all tissues (n=1072) and cell lines (n=1957) (Table 2) were subjected to principal component analysis (PCA), which
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easily distinguished the adenomas from the normal mucosa samples (Figure 3A) and the five colon cancer cell lines from the immortalized normal colon epithelial cell line HCEC (Figure 3B). The cancer cell lines were also segregated into three distinct groups reflecting their patient origins (Figure 3B). When PCA was performed on the expression intensity values of the 1496 non-redundant proteins expressed and quantified in all tissues and cell lines (i.e., those representing the intersection of the tissue [n=10,452] and cell-line [n=5056] protein sets reported in Table 2), colon cancer cell lines clustered with adenomas, while HCEC cells were closer to the normal mucosa samples (Figure 3C).

As a quality control measure, data for the 60 tissue samples (1072 protein families) were subjected to hierarchical clustering analysis. As shown in Supplementary Figure 1, three main clusters emerged: one consisting almost exclusively of normal mucosa samples, a second containing mainly adenomas, and a third that included both tissue types. The 18 samples in the third cluster (nine adenoma/normal mucosa pairs) formed three sub-clusters, which corresponded to three of the 10 experiments for which trypsin digestion, iTRAQ labeling, and LC-MS/MS were performed on the same day. These findings were suggestive of an experimental bias. Indeed, when these 18 potentially sub-standard samples were included in subsequent statistical analyses, they diminished the stringency of our threshold and increased the error margin for false identification. We therefore excluded these samples from the analyses described in the following section.

Proteins displaying dysregulated expression in colorectal adenomas and colon cell lines

To identify proteins whose expression was significantly altered in adenomas (relative to normal mucosa), we analyzed data on the proteins quantified in the remaining 21 tissue pairs. The experimentally derived protein fold-change threshold defining differential expression was based on comparison of the distributions of average intensity log2 ratios in the reference standard (113 vs. 114, seven experiments) and in patient
samples (adenoma vs. normal, seven experiments). The average ratios in the reference sample were centered around 1 (i.e., log₂ 0). Average fold-change ratios for the tissue samples displayed wider variance (Supplementary Figure 2). Seventeen percent of the log₂ ratios for the tissue samples exceeded ± 0.5 log₂ scale (indicating a linear fold change of ≥ ± 1.4) as opposed to only 5% of those for the reference samples. For each protein, a paired t-test was used to compare the intensity ratios in normal and adenomatous samples (i.e., normal/114; adenoma/114). After adjustment for multiple comparison (Benjamini-Hochberg method), we selected a stringent q value cutoff of ≤ 0.02.

The 212 proteins that satisfied this criterion and presented a mean expression fold change of ± 1.4 (log₂ 0.5) or more were classified as significantly dysregulated in adenomas. They included 76 with upregulated expression and 136 with downregulated expression in the tumor samples (Table 3). When protein abundance iTRAQ ratios for these 212 proteins were plotted on a heat map, adenomas and normal mucosa samples formed two distinct clusters (Figure 4A). As shown in Figure 4B, tissue expression levels for the 212 dysregulated proteins showed good correlation (r = 0.74, P < 0.001, 95% CI: 0.67 - 0.79) with those of mRNAs for the same genes (measured by our group in another set of colorectal adenomas) (26).

Table 4 lists the biological processes that were over-represented in this set of proteins. At the top of this list was xenobiotic metabolism, a process already linked with adenoma formation on the basis of enrichment studies of transcriptomic datasets conducted by our group (32). Three of the dysregulated proteins involved in this process (CYP2S1, NQO1, and GSTP1) displayed upregulated expression in adenomas, but most were characterized by tumor-related downregulation (ADH1B, ADH1C/ADH1A, UGT1A9/UGT1A6, UGT1A1/UGT1A4/UGT1A3/UGT1A5, UGT1A7/UGT1A8, UGDH, MAOA, SULT1A3/SULT1A1, PAPSS1/PAPSS2, UGP2). Network-building analysis
revealed that all these proteins were linked by sub-networks controlled by cancer-associated transcription factors, such as SP1 or, less frequently, MYC, HIF1A, or TP53 (Supplementary Figure 3). As noted in Table 4, a very similar picture emerged when gene ontology enrichment was also analyzed in a larger set of 621 dysregulated proteins selected with less stringent criteria (q value cut-off \( \leq 0.2 \); average log₂ fold change \( \geq \pm 0.5 \)).

The expression levels of 111 (52%) of the 212 proteins that were differentially expressed in adenomas were also quantified in cell lines (those shown in boldface in Table 3 and referred to hereafter as the "epithelial cell signature" proteins). Almost half (n=51, 46%) showed directionally similar tumor-related dysregulation in both analyses. Since cell-line studies were conducted with only one non-cancerous line, these findings obviously require further validation. They suggest, however, that these 51 proteins are indeed expressed in the epithelial cells of normal colorectal tissues and that their expression is dysregulated in the epithelial cells of adenomas.

**Upregulation of SORD expression and activity in colorectal adenomas and cancer cell lines**

Sorbitol dehydrogenase, a key enzyme in the polyol pathway, was one of the most significantly upregulated proteins in our colorectal adenomas (based on q values) (Table 3). Because its increased expression could have metabolic consequences with potential impact on tumorigenesis, we performed Western blotting and immunostaining studies to validate this finding. The reliability of the anti-SORD antibody we had chosen was first tested on protein extracts from the six colorectal epithelial cell lines (Figure 5A). The tumor-related log₂ fold changes detected with Western blotting were substantially larger than those documented with iTRAQ (2 to 6 vs. 0.4 to 1, respectively) (Figure 5B), which was not surprising since iTRAQ has been reported to underestimate protein abundance (33). However, the relative quantities of SORD found with the two methods were fully consistent. As for the 21 adenomas, the elevated SORD expression documented in these
tumors by iTRAQ (Figure 5C) showed good correlation with the increased SORD mRNA levels we had previously found in other 42 lesions of this type (26) (Figure 5D). Western blot analysis of four randomly selected adenoma/normal mucosa pairs from the present series revealed obvious upregulation of SORD expression in all four tumors although the magnitude of the increase varied (Figure 5E).

SORD activity was then assayed (see Experimental procedures) to see how it corresponded with the enzyme expression levels reported above. As shown in Figure 5F, the results of cell line assays were fully consistent with the Western blotting data: SORD activity was 7 times higher in HT29 than in HCEC cells, and more limited upregulation was found in SW480. High correlation between enzyme activity and protein level was also documented for three randomly selected adenoma/normal mucosa pairs (Figure 5F).

MS and Western blotting findings were further validated with immunostaining studies, as shown in Figure 6. Cytoplasmic SORD staining was evident in the colon cancer cell line HT29 but weaker or even absent in normal epithelial HCEC cells (Figures 6A and 6B). As for colorectal tissues, SORD cytoplasmic expression was limited to the bottom of the normal epithelial crypts (Figures 6C, 6D, and 6E), but its expression was markedly increased in adenomatous and cancerous glands (Figures 6F - 6I). These findings suggest that SORD is likely to be expressed in proliferating cells, although it was largely absent in HCECs, which undergo regular proliferation in vitro. Furthermore, nuclear localization of SORD was noted in some adenomatous crypts (Supplementary Figure 4A and C), and the cells in question were almost always negative for the well-known proliferation marker Ki-67 (Supplementary Figure 4B and D). This mutually-exclusive staining pattern was also observed in normal crypts of the ileum where SORD, interestingly, appeared to be expressed in the nuclei of putative stem cells (Supplementary Figure 4E and F).

Polyol pathway enzyme expression and metabolite levels in cell lines and tissues.
We then examined the state of the polyol pathway (Supplementary Figure 5A) in colorectal cell lines and tissues. As shown in Supplementary Figure 5B, immunoblot studies revealed decreased AKR1B1 expression in HT29 (vs. HCEC cells) and adenomas (vs. corresponding normal mucosal samples), while SORD expression and that of KHK were upregulated in tumor cells and tissues. As for the metabolites (Supplementary Figure 5C), D-glucose levels were significantly decreased in adenomas. Less dramatic changes were observed in the levels of D-sorbitol and D-fructose, which both showed a tendency to decrease in tumor tissues.

**DISCUSSION**

Although a number of proteomic studies have comparatively analyzed different types of colorectal tissues, precancerous lesions have been considered in only three (21, 34, 35), and in two of these (21, 34), the number of adenomas analyzed was very small (≤ 4). The study by Lam et al. (35) is the only one that compared protein expression in a relatively large number (n=20) of paired adenoma and normal mucosa samples. They used 2-DE to resolve over 1000 proteins in the two tissue groups, and those displaying differential expression were then analyzed with MALDI-TOF/TOF MS. MS/MS validation pinpointed four proteins (ANXA3, S100A11, EIF-5A1, S100P) whose expression in adenomas was significantly increased. Using MS with iTRAQ 8-plex peptide labeling and OFFGEL fractionation allowed us to quantitatively compare protein expression in 30 colorectal adenomas and paired samples of normal mucosa, and to investigate low-abundance proteins that cannot be evaluated with 2-DE-based proteomics. All in all, 4325 non-redundant protein families were quantified in our colorectal tissues (25% of which were identified in all 60 samples) (Table 2). And the 212 proteins we flagged as being significantly dysregulated in adenomas included three of the four proteins identified by Lam
et al. (upregulation of the fourth, EIF-5A1, failed to meet our stringent criterion for significance) (Table 3).

The cell types in which these proteomic changes occur is of obvious interest since colorectal cancer arises from the epithelial component of the colorectal mucosa. Although our findings are preliminary and will naturally require validation in future studies, 51 of the 212 proteins listed in Table 3 were "epithelial cell signature" proteins and showed directionally similar expression changes in colon cancer cell lines vs. HCEC. It therefore seems likely that their dysregulated expression in adenomas is a feature of neoplastic transformation of colorectal epithelial cells. However, epithelial-stromal cell interactions can also play important roles in tumorigenesis (20). Our approach also allowed us to identify 101 proteins displaying adenoma-related dysregulation that were probably of stromal-cell origin since they were not expressed in any of the six epithelial cell lines we examined (Table 3). These proteins were mainly involved in immune-related processes (immune response, complement activation, T cell co-stimulation), which are usually not represented in colon epithelial cell lines. Their expression changes are likely to have important effects on the microenvironment of an epithelial-cell tumor.

Our search for potential biomarkers of early-stage colorectal tumorigenesis focused exclusively on the 76 proteins whose expression was significantly upregulated in adenomas. According to the Human Protein Atlas (HPA) database (36), 69 (91%) of these have cancer-related features, and 16 of the 69 are already classified as candidate cancer biomarkers (Table 3). The HPA database contains information on protein expression in normal and cancer tissues, but not in those regarded as precancerous. The overlap between our findings and those of the HPA suggest that most protein expression changes identified thus far in colorectal adenocarcinomas are probably already detectable in the benign precursors of these lesions.
Supplementary Figure 6 shows the expression profiles of the 10 proteins that were most markedly upregulated in adenomas. This group comprised two of the four proteins identified by Lam et al. (35) as significantly overexpressed in adenomas. **Annexin A3 (ANXA3)**, for example, is at the top of our list (based on q values) (Table 3). An angiogenic factor that induces VEGF production via the HIF-1 pathway (37), ANXA3 belongs to a family of calcium-dependent, phospholipid-binding proteins involved in diverse biological processes, including signal transduction, inflammatory responses, membrane organization, and the regulation of cellular growth (38, 39). Dysregulated ANXA expression is also a common feature of colorectal cancer (39) and most other cancers as well (40). **S100A11** expression was also increased in these tumors, which is consistent with earlier reports (41). The cytosolic S100 proteins interact directly with peptides on the N-terminal domain of annexins (38, 42), and like the annexins, they also have diverse intracellular and extracellular functional roles (43).

Among the other top ten proteins displaying adenoma-related upregulation is **LDHA / LDHB**. Their expression levels were not measured separately, but LDHA is presumably responsible for the increased expression observed in our adenomas. **LDHB** expression is in most cases epigenetically silenced in colon cancer cells (44, 45), whereas **LDHA** is overexpressed, and its activity is maintained via the oncogenic tyrosine kinase FGFR1 (46). LDHA is a key player in the reversible conversion of pyruvate to lactate during aerobic glycolysis, a typical feature of cancer cell metabolism first described by Otto Warburg (47). The sodium- and potassium-coupled chloride cotransporter, **SLC12A2**, is expressed on the basolateral membrane of the normal colon epithelium, where its recruitment and activation are regulated by calcium and cAMP. Loss of SLC12A2 leads to impaired chloride secretion in the intestine (48, 49), but to our knowledge, there are no published data linking this protein to colon cancer. The fifth markedly over-expressed protein is **SET**, one of the five
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proteins that make up the inhibitor of acetyltransferases (INHAT) complex. Two other INHAT components, APEX1 and ANP32A/ANP32B, were also upregulated in adenomas (albeit to a lesser extent than SET) (Table 3). These changes are noteworthy because INHAT binds directly to histones, preventing their acetylation by histone acetyl transferases (50-52), and loss of histone acetylation is a crucial step in gene silencing (53, 54). Thus far, INHAT’s role in cancer has not been widely investigated, but overexpression of the complex components has been observed in serous epithelial ovarian cancer (55). The upregulated expression of **PPA1/PPA2** in our adenomas might play various roles in colorectal tumorigenesis since these proteins are key players in the synthesis of fatty acids, nucleotides, amino acids, and other essential molecules (56). The phosphoprotein **nucleolin (NCL)**, an essential protein for proliferating cells (57), appears to regulate several steps in the biogenesis of ribosomes, including transcription, ribosome assembly, and the processing of precursor ribosomal RNA (58-60), all of which might be instrumental to adenoma growth. As for **OCIAD2**, strong immunoreactivity for this protein has been reported in early-stage adenocarcinomas of the lung and in ovarian cancers (61-63), but there are no published data linking it to colorectal tumorigenesis. In contrast, the secreted protein **REG4**, which promotes mitosis and enhances the motility and invasiveness of colon cancer cells, is strongly expressed in these cells and in the serum of patients with colorectal cancer (64-66).

The final protein characterized by marked adenoma-related upregulation was **SORD**, a key enzyme in the polyol metabolic pathway. It was selected for validation studies, because although aberrant polyol pathway activity has been implicated in diabetic complications (67-70) and myocardial ischemia (71), the role of SORD in tumorigenesis was completely unknown. During the execution of this study, however, upregulated SORD expression was reported in prostate cancer (72) and in colorectal adenomas (21), and
these findings strengthened our resolve to characterize this phenomenon in colorectal
tumorigenesis.

Upregulated SORD expression and activity in adenomas (Figure 5) would
enhance the production of fructose (see schematic of Supplementary Figure 5A), thereby
increasing the generation of triose sugars and diacylglycerol (intermediates in the glycolytic
and lipid signaling pathways, respectively). Fructose is also several times more effective
than glucose in promoting intracellular non-enzymatic glycation (73-75), and advanced
glycation end products may contribute to the vascular complications of diabetes and other
pathologic conditions (67, 76-78). Whether these fructose-driven metabolic events play a
role in the development of adenomas is unclear, but the polyol pathway was very active in
the adenomatous cells we examined. This activity was also reflected by the concomitant
increase of the expression of KHK (Supplementary Figure 5B), the enzyme that catalyzes
the transformation of fructose to fructose-1-P, downstream from the polyol pathway.

The effects of these enzymatic changes on sorbitol and fructose concentrations in
adenomas need to be investigated in larger tissue series, but our preliminary data suggest
that the levels of both are slightly decreased in these lesions (Supplementary Figure 5C). In
contrast, our adenomas exhibited dramatically reduced concentrations of glucose, the initial
substrate in the polyol pathway (Supplementary Figure 5C). Adenoma-related dysregulation
was also noted in the expression of AKR1B1, the enzyme that converts glucose to sorbitol
(Supplementary Figure 5B). Exploitation of the polyol pathway to divert carbon from glucose
to other energy intermediates might provide adenomatous cells with a selective advantage
over normal cells. This pathway might prove to be another means of tumor-related glucose
consumption in addition to the well-known glycolytic and pentose phosphate pathways
(Supplementary Figure 5A). Advanced cancer cells consume glucose at a much higher rate
than normal cells, and much of their energy is generated by aerobic glycolysis rather than
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by oxidative phosphorylation of glucose in the mitochondria (i.e., the Warburg effect) (79). The predominantly glycolytic phenotype of cancer cells results in low glucose levels and high concentrations of lactate (47, 80, 81). The relative concentrations of lactate in the three adenomas we tested were significantly higher than those found in matched samples of normal mucosa (Supplementary Figure 5C), indicating that the Warburg effect is already evident in precancerous colorectal lesions. Studies involving metabolic flux analysis to monitor the fate of isotopic tracers in in vitro and in vivo systems would provide further insight into the biological roles of the polyol pathway in tumorigenesis.

Additional information

The MS data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (82) with the dataset identifier PXD000445 and DOI 10.6019/PXD000445. Further information on selected PSMs, peptides and corresponding assembled proteins can be found in Supplementary Tables 1 (tissues) and 3 (cell lines). Supplementary Figures 7 through 17 show spectra for the proteins identified with a single peptide (listed in Supplementary Tables 1 and 3).

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**FIGURE LEGENDS**

**Figure 1. Project design and iTRAQ 8plex-labeling scheme.** Sample preparation for shotgun MS/MS and important steps in the analysis of proteomic data for the detection of dysregulated proteins in adenomas and colon cancer cell lines. For each experiment on tissue samples, iTRAQ tags were assigned to a duplicate reference (two identical pools of normal and adenoma samples: 113 and 114, respectively), normal tissues (115, 117, 119) and corresponding adenomas (116, 118, 121). The same pattern was
Repeated in all 10 experiments. In cell line experiments, two identical pools, each comprising all six cell lines, were used as reference (113 and 114), and each of the remaining six tags was used to label a single cell line. The data analysis flow chart depicted in this figure is described in *Experimental procedures*.

**Figure 2. Protein coverage with iTRAQ shotgun analysis in colorectal tissues.** (A) Analysis of Mascot emPAI values (used as a proxy for emPAI values) revealed a dynamic range of protein abundance in tissues that spanned four orders of magnitude (y-axis) and corresponded with known abundance estimates for various proteins in these tissues. The high/moderate-abundance proteins (e.g., ACTB, FABP5, CHGA) and low-abundance protein (e.g., POLR3A) relatively quantified in our samples are highlighted relative to their mean Mascot emPAI value. (B) Distribution of reported abundance ranges for the proteins with ≥1 unique peptides identified in tissues, and the high-MW proteins with the highest number of unique peptides identified. (C) Subcellular localizations of the proteins identified in colorectal tissues and cell lines and (D) biological processes in which these proteins are involved. This analysis was performed using Scaffold and Gene Ontology annotations (see *Experimental procedures*).

**Figure 3. Principal component analysis of protein expression.** Three-dimensional principal component analysis (PCA) score plot of log2 protein expression intensity values for (A) tissues (normal mucosa, black; adenomas, red); (B) cell lines (HCEC, cyan; colon cancer cell lines, green); and (C) both. The first three principal components (PCs) account for 40%, 82%, and 36% of the total variance in the tissue, cell line, and tissue+cell line sets, respectively. PC1, the main direction of spread in the three groupings, reflects intergroup variance based on tissue or cell-line type (i.e.,
normal/immortalized versus tumorous). Cell lines derived from the same patient: * SW480 and SW620 cells; ^ HT29 and CX1 cells.

**Figure 4. Analysis of the 212 proteins displaying significant tumor-related dysregulation.** (A) Hierarchical clustering of iTRAQ abundance ratios (normal vs. 114, adenoma vs. 114) for the 212 proteins displaying significant adenoma-related dysregulation grouped tissue samples into two discrete clusters: adenoma (A) and normal (N). (B) Pearson’s correlation test comparing average fold changes (≥ ±0.5 log₂) for the 212 proteins (red, upregulated; blue, downregulated) in the tissue series with average log₂ fold changes for the corresponding mRNAs measured in another set of adenoma/normal mucosal samples.

**Figure 5. Significantly upregulated SORD expression and activity in colorectal cell lines and adenomas.** (A) Tumor-related upregulation of sorbitol dehydrogenase (SORD) in colon cancer cell lines was confirmed with Western blotting. The SORD dysregulation trend was identical to that observed with iTRAQ-based MS/MS, although when immunoblot results were quantified (B), the log₂ fold changes were over five times greater than those documented in the iTRAQ study. (C) SORD protein expression (iTRAQ analysis) in 21 normal mucosa/adenoma tissue pairs. (D) SORD mRNA expression in 42 other normal mucosa/adenomas from a previous study by our group (26). Error bars indicate the means and 95% confidence intervals. (E) Western blots showing tumor-related upregulation of SORD expression in four randomly selected adenoma [A] /normal mucosa [N] tissue pairs of the 21 shown in panel C (see Table 1 for sample descriptions). (F) SORD activity also displayed tumor-related upregulation in cell lines (HT29 and SW480 versus HCEC cells) and tissues (adenomas versus normal mucosa). Columns show mean enzyme
activity measured in at least two replicates; error bars indicate standard deviations from means. The Western blot beneath the graph shows SORD levels measured in the extracts used for the enzyme activity assays.

**Figure 6. Anti-SORD immunostaining of colorectal cell lines and tissues.** Consistent with proteomic data, SORD expression was (A) negligible or absent in HCECs, but (B) clearly expressed in the cytoplasm of HT29 cells. (C) In normal colorectal mucosa, SORD expression was limited to the lower portion of the epithelial crypts, where stem cells and highly-proliferating cells are located. Higher magnification views show staining at (D) the base vs. (E) mouth of colonic crypts. (F) and (G): Its expression was markedly increased in adenomatous glands (red arrowheads) compared with normal crypts (green arrowheads). Panels (H) and (I) show abundant expression of SORD in a large adenoma and in a cancer, respectively.
| Patient number | Age | Sex | Colon segment involved | Maximum lesion diameter (mm) | Paris classification # | Pit pattern classification º | Microscopic appearance | Highest degree of dysplasia in the lesion • | No. of lesions at study colonoscopy = | No. of previously excised lesions ‡ |
|---------------|-----|-----|------------------------|-----------------------------|-----------------------|-------------------------------|-----------------------|----------------------------------------|-----------------------------|----------------------------------|
| 1             | 77  | M   | S                      | 25                          | Ila+Iic               | IIs - IIIL                    | TA                   | LGD                                    | 1                          | 0                               |
| 2             | 73  | F   | A                      | 25                          | Ila+Iic               | IIs-III                        | TA                   | LGD                                    | 1                          | 2                               |
| 3             | 59  | M   | T                      | 30                          | Ila+Iic               | IIs + III                     | TA                   | LGD                                    | 1                          | 0                               |
| 4             | 73  | F   | R                      | 50                          | Is                    | IV                            | VA                   | LGD                                    | 1                          | 0                               |
| 5             | 74  | M   | R                      | 40                          | Is                    | IV                            | VA                   | HGD                                    | 2                          | 1                               |
| 6             | 77  | M   | C                      | 25                          | Ila                  | IIIL                           | VA                   | LGD                                    | 1                          | 0                               |
| 7             | 80  | M   | A                      | 40                          | Ila                  | IIIL                           | TVA                  | LGD                                    | 1                          | 1                               |
| 8             | 82  | M   | A                      | 15                          | Ila                  | IIIL                           | VA                   | LGD                                    | 2                          | 0                               |
| 9             | 73  | F   | S                      | 20                          | Ip                    | IV                            | TVA                  | LGD                                    | 1                          | 0                               |
| 10            | 70  | F   | C                      | 25                          | Ila                  | IIIL                           | TVA                  | LGD                                    | 2                          | 0                               |
| 11            | 63  | M   | A                      | 45                          | Is                   | III-I/IV                        | TVA                  | LGD                                    | 0                          | 0                               |
| 12            | 68  | M   | A                      | 30                          | Ila+Is               | IIIL - IV                      | TVA                  | HGD                                    | 0                          | 0                               |
| 13            | 60  | M   | D                      | 30                          | Is                   | IV - VI                        | TVA                  | HGD                                    | 0                          | 0                               |
| 14            | 55  | M   | C                      | 25                          | Ila+Is               | IIIL-IV                        | SSA                  | LGD                                    | 0                          | 0                               |
| 15            | 70  | M   | A                      | 15                          | Is                   | IV                            | TVA                  | LGD                                    | 7                          | 0                               |
| 16            | 87  | F   | S                      | 25                          | Ila+Ila              | IV                            | TA                   | LGD                                    | 1                          | 1                               |
| 17            | 66  | M   | A                      | 30                          | Ila                  | IIIL                           | TA                   | HGD                                    | 2                          | 0                               |
| 18            | 72  | M   | A                      | 30                          | Is                   | IV                            | TVA                  | HGD                                    | 2                          | 0                               |
| 19            | 71  | M   | S                      | 30                          | Ila                  | IIIL                           | TVA                  | LGD                                    | 2                          | 0                               |
| 20            | 59  | M   | R                      | 60                          | Is                   | IV-Vi                          | TVA                  | LGD                                    | 1                          | 0                               |
| 21            | 78  | M   | A                      | 50                          | Is                   | IV - VI                        | TA                   | LGD                                    | 1                          | 0                               |
| 22            | 75  | M   | R                      | 25                          | Is                   | IV - Vn                        | TVA                  | HGD                                    | 6                          | 0                               |
| 23            | 73  | F   | D                      | 25                          | Is                   | IV                            | TVA                  | LGD                                    | 1                          | 0                               |
| 24            | 69  | F   | R                      | 90                          | Is+Ila               | IV                            | TVA                  | LGD                                    | 1                          | 0                               |
| 25            | 75  | M   | T                      | 18                          | Ila                  | IIIL                           | TA                   | LGD                                    | 1                          | 0                               |
| 26            | 61  | M   | A                      | 40                          | Is+Ila               | IV                            | TVA                  | LGD                                    | 20                         | 0                               |
| 27            | 76  | M   | S                      | 30                          | Is                   | IV - VI                        | TA                   | HGD                                    | 1                          | 0                               |
| 28            | 78  | F   | R                      | 60                          | Ila+Is               | IV                            | TVA                  | LGD                                    | 1                          | 1                               |
| 29            | 89  | M   | R                      | 30                          | Is                   | IV                            | TA                   | LGD                                    | 3                          | 0                               |
| 30            | 75  | M   | A                      | 50                          | Is                   | IV-Vn                          | TVA                  | HGD/cancer                              | 7                          | 0                               |

Abbreviations: M, male; F, female; C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum; TA, tubular adenoma; TVA, tubulovillous adenoma; VA, villous adenoma; SSA, sessile serrated adenoma; LGD, low-grade dysplasia; HGD, high-grade dysplasia.

# Macroscopic appearance of neoplastic lesions was classified according to Paris Endoscopic Classification. The Paris Endoscopic Classification of Superficial Neoplastic Lesions. Gastrointest Endosc 2003;58(suppl.):S3-S27

º Morphological analysis of colon crypt patterns according to the Kudo classification. Kudo S, Rubio CA, Teixeira CR, et al. Pit pattern in colorectal neoplasia: endoscopic magnifying view. Endoscopy 2001;33:367-7

• Low-grade versus high-grade dysplasia as defined by the WHO classification of tumors of the digestive system, editorial and consensus conference in Lyon, France, November 6-9, 1999.IARC

This number includes the lesion included in our proteomic study.
Table 2. Summary of proteomics data

|                               | Tissues (n = 60) | Cell lines (n = 6) |
|-------------------------------|------------------|-------------------|
|                               | Total            | FDR (%)           | Total           | FDR (%)           |
| Peptide spectra matches       | 285,929          | 0.2               | 27,922          | 0.4               |
| Peptides                      | 37,184           | 0.9               | 11,266          | 0.5               |
| Proteins *                    | 10,452           | 1.5               | 5,056           | 1.1               |
| Proteins #                    | 4,325            | -                 | 2,017           | -                 |
| Proteins ^                    | 1,072            | -                 | 1,957           | -                 |

* Total number of proteins quantified in the 10 tissue experiments and the single experiment with cell lines.

# Non-redundant protein families quantified in our dataset.

^ Non-redundant protein families quantified in all 60 tissues or in all 6 cell lines.
Table 3. Proteins displaying differential expression in adenomas vs. normal mucosa

| UniProt accession no.* | Gene name | q value  | Average fold change (log2) |
|------------------------|-----------|----------|----------------------------|
| P12429                 | ANXA3     | 0.00000001 | 1.44                       |
| Q9UN36                 | NDRG2     | 0.00000001 | -0.79                      |
| P00918                 | CA2       | 0.00000003 | -2.26                      |
| P23946                 | CMA1      | 0.00000005 | -1.38                      |
| P00488                 | F13A1     | 0.00000005 | -1.30                      |
| Q9UBR2                 | CTSZ      | 0.00000016 | -1.23                      |
| P10645                 | CHGA      | 0.00000016 | -1.82                      |
| O60844                 | ZG16      | 0.00000024 | -2.02                      |
| P17174                 | GOT1      | 0.00000045 | -0.61                      |
| P31949                 | S100A11   | 0.00000062 | 1.49                       |
| P00338 ^ P07195        | LDHA; LDHB | 0.00000068 | 0.62                       |
| O60701                 | UGDH      | 0.00000104 | -0.70                      |
| P55011                 | SLC12A2   | 0.00000115 | 0.85                       |
| O95571                 | ETHE1     | 0.00000120 | -0.74                      |
| Q01105                 | SET       | 0.00000230 | 0.72                       |
| Q16851                 | UGP2      | 0.00000275 | -0.53                      |
| Q00796                 | SORD      | 0.00000275 | 0.62                       |
| P20231; Q15661         | TPSB2; TPSAB1 | 0.00000275 | -1.51                      |
| Q15181; Q9H2U2 ^       | PPA1; PPA2 | 0.00000421 | 0.64                       |
| Q9H3G5                 | CPVL      | 0.00000467 | -0.69                      |
| P01282                 | VIP       | 0.00000722 | -1.10                      |
| P07339                 | CTSK      | 0.00000856 | -0.71                      |
| P19338                 | NCL       | 0.00000934 | 0.50                       |
| Q6UWP2                 | DHR511    | 0.00001070 | -1.20                      |
| P04066                 | FUC1A     | 0.00001240 | -1.32                      |
| Q53EL6                 | PDCD4     | 0.00001270 | -0.51                      |
| P07585                 | DCN       | 0.00001580 | -1.36                      |
| P02511                 | CRYAB     | 0.00002720 | -1.19                      |
| Q96CX2                 | KCTD12    | 0.00003590 | -0.70                      |
| Q05707; P08123         | COL14A1   | 0.00004020 | -1.21                      |
| P51884                 | LUM       | 0.00004160 | -0.96                      |
| Q15063                 | POSTN     | 0.00004240 | -2.10                      |
| P21397                 | MAOA      | 0.00004240 | -0.83                      |
| O00748                 | CES2      | 0.00004610 | -0.88                      |
| Q56VL3                 | OCIAD2    | 0.00004960 | 0.95                       |
| Q9BYZ8                 | REG4      | 0.00004960 | 0.98                       |
| P55008                 | AIF1      | 0.00005650 | -0.75                      |
| P50224; P50225         | SULT1A3; SULT1A1 | 0.00005760 | -0.63                      |
| O14773                 | TPP1      | 0.00006430 | -0.52                      |
| Q16853                 | AOC3      | 0.00006650 | -1.16                      |
| P53634                 | CTSC      | 0.00006940 | -0.58                      |
| O95881                 | TXNDC12   | 0.00006940 | 0.55                       |
| O75795                 | UGT2B17   | 0.00006940 | -1.57                      |
| O00391                 | QSOX1     | 0.00006940 | -0.70                      |
| Q99538                 | LGMN      | 0.00006940 | -0.63                      |
| P12111                 | COL6A3    | 0.00006990 | -0.74                      |
| P80188                 | LCN2      | 0.00006990 | 1.32                       |
| P12956 ^               | X RCC6    | 0.00007190 | 0.53                       |
| Q6NZI2                 | PTRF      | 0.00007860 | -0.76                      |
| P09382                 | LGALS1    | 0.00007880 | -0.84                      |
| P25815                 | S100P     | 0.00008020 | 3.38                       |
| Q15118                 | PDK1      | 0.00008120 | -0.90                      |
| Q75380                 | NDUFS6    | 0.00008650 | -0.63                      |
| Q9HAW8                 | UGT1A10   | 0.00009090 | -0.95                      |
| P01042                 | KNG1      | 0.00009660 | -0.74                      |
| O75356                 | ENTPD5    | 0.00012385 | -0.54                      |
Table 3. (continued).

| Protein ID  | Gene Symbol | GO Term | Fold Change |
|-------------|-------------|---------|-------------|
| Q15293      | RCN1        |         | 0.00012867  | 0.61        |
| P17931      | LGALS3      |         | 0.00013151  | -0.62       |
| P36952      | SERPINB5    |         | 0.00013151  | 1.31        |
| P09211      | GSTP1       |         | 0.00014809  | 0.55        |
| P20774      | OGN         |         | 0.00015467  | -1.52       |
| Q8NB4J5     | GOLM1       |         | 0.00015467  | -0.51       |
| Q16563      | SYPL1       |         | 0.00015568  | 1.16        |
| P04229; P13761; Q30134; Q9TQE; Q9 Giy3; Q29974; P04440; P79483 | HLA-DRB1; HLA-DRB1; HLA-DRB1; HLA-DRB1; HLA-DRB1; HLA-DRB1; HLA-DRB3 |         | 0.00015568  | -0.81       |
| Q9H4M9; Q9NZN4; Q9H223; O00754 | MAN2B1; DYSPL2; S100A6; ABP1; EIF2S2; HDGF; MRE11A; CLIC1; CLIC4 |         | 0.00017278  | -0.56       |
| P20774      | OGN         |         | 0.00018023  | -0.55       |
| Q8NB4J5     | GOLM1       |         | 0.00018025  | 1.18        |
| P19801      | S100A6      |         | 0.00018203  | 1.18        |
| P20042      | EHD2        |         | 0.00022289  | -0.50       |
| P16104      | EHD4        |         | 0.00024174  | 0.64        |
| Q43252; Q95340 | PAPSS1; PAPSS2 |         | 0.00027464  | -0.57       |
| Q12765      | SCRN1       |         | 0.00029349  | 0.67        |
| Q9HB40      | SCPEP1      |         | 0.00030328  | -0.87       |
| P48556      | PSMD8       |         | 0.00032019  | 0.56        |
| Q9Y6R7      | FCGBP       |         | 0.00035167  | -0.73       |
| P61626      | LYZ         |         | 0.00036558  | 0.74        |
| Q9Y224      | C14orf166   |         | 0.00038868  | 0.52        |
| P01765; P01764 | (Ig heavy chain V-III region TIL; Ig heavy chain V-III region VH26) |         | 0.00038868  | -0.69       |
| Q9NVP1      | DDX18       |         | 0.00038996  | 0.67        |
| P30365      | HSD11B2     |         | 0.00039413  | -0.68       |
| P39687; Q92688 | ANP32A; ANP32B |         | 0.00039654  | 0.56        |
| Q86WA6      | BPHL        |         | 0.00046215  | 0.52        |
| P24298      | GPT         |         | 0.00047338  | -0.56       |
| Q12874      | SF3A3       |         | 0.00052166  | 0.50        |
| P04899; Q14344; P63092 | GNAI2; GNA13; GNAS |         | 0.00062192  | -0.51       |
| Q15124      | PGM5        |         | 0.00068593  | -0.68       |
| Q9HAW7; Q9HAW9; O60656 | UGT1A7; UGT1A8; UGT1A9 |         | 0.00071202  | -0.87       |
| P19224      | UGT1A6      |         | 0.00071202  | -0.87       |
| P06748      | NPM1        |         | 0.00071508  | 0.81        |
| Q9NUV9      | GIMAP4      |         | 0.00071508  | -0.89       |
| P18283      | GPX2        |         | 0.00071508  | 0.54        |
| P13688      | CEACAM1     |         | 0.00072482  | -1.06       |
| P01591      | IGJ         |         | 0.00084082  | 0.66        |
| P19823      | ITIH2       |         | 0.00085565  | 0.71        |
| P01774; P01776; P01779 | (Ig heavy chain V-III region POM; Ig heavy chain V-III region WAS; Ig heavy chain V-III region TUR) |         | 0.00085565  | -0.79       |
| P27695      | APEX1       |         | 0.00086971  | 0.54        |
| Q9C002      | NMES1       |         | 0.00087016  | 0.91        |
| Q96F85      | CNRIP1      |         | 0.00088956  | 1.27        |
| Q9BPX5      | ARPC5L      |         | 0.00095098  | 0.67        |
| P62263      | RPS14       |         | 0.00095182  | 0.52        |
| Q9BY32      | ITPA        |         | 0.00095634  | 0.51        |
| P01625      | (Ig kappa chain V-IV region Len) |         | 0.00097041  | -0.61       |
Table 3. (continued).

| Accession | Gene      | Log2 Fold Change | p-value |
|-----------|-----------|-----------------|---------|
| Q15582    | TGFBI     | 0.00097041      | 0.90    |
| Q07021    | C1QBP     |                 |         |
| P00738    | HP        | 0.00102030      | -0.61   |
| O15143    | ARPC1B    | 0.00103757      | -0.50   |
| Q03154    | ACY1      | 0.00105871      | 0.60    |
| Q9HC86    | SPON1     | 0.00115609      | -0.89   |
| Q96HE7    | ERO1L     | 0.00119027      | 0.50    |
| P08575    | PTPRC     | 0.00119950      | -0.52   |
| Q9Y266    | NUDC      | 0.00152358      | 0.56    |
| P63313; P62328 | TMSB10; TMSB4X | 0.00159602 | 0.98 |
| Q96SQ9    | CYP2S1    | 0.00162405      | 0.84    |
| P07136    | TUBA1A    | 0.00162405      | 0.54    |
| P00915    | CA1       | 0.00163258      | -1.18   |
| P04844    | RPN2      | 0.00165748      | 0.55    |
| P09669    | COX6C     | 0.00171230      | -0.61   |
| P21980    | TGM2      | 0.00174251      | -0.55   |
| P00325    | ADH1B     | 0.00175658      | -1.18   |
| O14745    | SLC9A3R1  | 0.00175658      | -0.51   |
| Q9H8H3    | METTL7A   | 0.00179938      | -0.50   |
| P61009    | SPSC3     | 0.00186488      | -0.69   |
| Q15746; O15264; Q16539 | MYLK; MAPK13; MAPK14 | 0.00186488 | -0.53 |
| P01876; P01877; Q92973 | IGHA1; IGHA2; TNPO1 | 0.00191760 | -1.01 |
| P12109    | COL6A1    | 0.00194792      | -0.67   |
| Q9BX66    | SORBS1    | 0.00205902      | -0.64   |
| E9PGJ9    | CC2D1A    | 0.00213982      | -0.53   |
| P49006    | MARCKSL1  | 0.00235323      | 0.51    |
| Q01524    | DEFA6     | 0.00235323      | 1.60    |
| P01620    | (lg kappa chain V-III region SIE) | 0.00238585 | -0.56 |
| P36873    | PPP1CC    | 0.00240171      | 0.58    |
| Q07507    | DPT       | 0.00240576      | -1.28   |
| P37840    | SNCA      | 0.00261720      | -0.57   |
| P00326; P07327 | ADH1C; ADH1A | 0.00271753 | -1.17 |
| P22105    | TNXB      | 0.00271753      | -0.51   |
| Q95299    | NDUFA10   | 0.00272901      | -0.84   |
| Q9NR00    | OSTC      | 0.00275806      | 0.76    |
| P10082    | PYY       | 0.00282902      | -1.59   |
| P21810    | BGN       | 0.00283401      | -0.66   |
| Q8IV08    | PLD3      | 0.00295758      | -0.74   |
| P01857; P01859; P01860; P01861 | IGHG1; IGHG2; IGHG3; IGHG4 | 0.00343621 | -0.57 |
| P62330    | ARF6      | 0.00343645      | 0.84    |
| Q93135    | CAV1      | 0.00346186      | -0.71   |
| P22309; P35503; P22310; P35504 | UGT1A1; UGT1A3; UGT1A4; UGT1A5 | 0.00349148 | -0.87 |
| Q9NSU2    | TREX1     | 0.00349148      | -0.76   |
| Q9UKY7    | CDV3      | 0.00355684      | 0.79    |
| Q7Z4V5    | HDGFRP2   | 0.00360855      | 0.65    |
| P07357    | C8A       | 0.00372327      | -0.62   |
| Q99757    | TXN2      | 0.00377179      | -0.73   |
| P13686    | ACP5      | 0.00385765      | -0.80   |
| Q8WVA0    | ITLN1     | 0.00392858      | -1.30   |
| P62861    | FAU       | 0.00395014      | 0.51    |
| P57737    | CORO7     | 0.00401004      | -0.83   |
| P10606    | COX5B     | 0.00412793      | -0.71   |
| Q9Y259    | CHK5      | 0.00420774      | -0.59   |
| Q9Y2J8    | PADI2     | 0.00435864      | -0.50   |
| Q94919    | ENODD1    | 0.00451988      | -0.80   |
| B9A064; P0CG05; A0M8Q6 | IGLL5; IGLC2; IGLC7 | 0.00451988 | -0.56 |
| P20039    | HLA-DRB1  | 0.00459324      | -0.82   |
Table 3. (continued).

| Accession | Protein      | Expression | Significance |
|-----------|--------------|------------|--------------|
| P63167; Q96FJ2 | DYNLL1; DYNLL2 | 0.00468484 | 0.63 |
| Q9UNN8 | PROCR | 0.00480932 | -0.78 |
| P07099 | EPHX1 | 0.00486543 | -0.54 |
| P32322 | PYCR1 | 0.00495977 | 0.55 |
| Q9POJ0 | NDUFA13 | 0.00534323 | -0.60 |
| E7EUF8; E9PFN5 | EPB41L3; GSTK1 | 0.00539222 | -0.95 |
| O75531 | BANF1 | 0.00560405 | 0.73 |
| P26447 | S100A4 | 0.00562754 | -0.53 |
| Q9UNN8 | PROCR | 0.00562754 | 0.50 |
| Q8N752 | CSNK1A1 | 0.00562754 | 0.52 |
| P40616 | ARL1 | 0.00583778 | 0.60 |
| Q96GA7 | SDSL | 0.00583778 | -0.82 |
| P01275 | GCG | 0.00607808 | -1.33 |
| P15289 | ARSA | 0.00633336 | -0.57 |
| O75521 | ECI2 | 0.00635218 | -0.60 |
| P62158 | CALM1; CALM2; CALM3 | 0.00657472 | 0.67 |
| P49821 | NDUVF1 | 0.00669319 | -0.66 |
| Q15746-5 | MYLK | 0.00678109 | -0.51 |
| Q96BM9 | ARL8A | 0.00686655 | 0.54 |
| Q6UX06 | OLFM4 | 0.00696505 | 1.14 |
| P10153 | RNASE2 | 0.00724902 | -0.50 |
| P19075; P32322 | TSPAN8 | 0.00837908 | 0.59 |
| Q8WU39 | PACAP | 0.00837978 | -0.56 |
| P21953 | BCKDHB | 0.00837978 | 0.54 |
| O76041 | NEBL | 0.00837978 | 0.71 |
| Q9H4G4 | GLIPR2 | 0.00849532 | -1.10 |
| P01766; P01767; P01768 | MBNL1; MBNL2 | 0.00996176 | 0.55 |
| Q9NR56; Q5VZF2 | STOM | 0.01083127 | -0.51 |
| P27105 | RPLP2 | 0.01109030 | 0.62 |
| P05387 | ISOC2 | 0.01164408 | -0.51 |
| Q96BM9 | TGFBI1 | 0.01198321 | -0.57 |
| Q8752 | TSPAN8 | 0.01211602 | 0.55 |
| Q96DG6 | CMBL | 0.01289211 | -0.51 |
| P61619 | SEC61A1 | 0.01375705 | 0.59 |
| P56381; P5VTSU8 | ATP5E; ATP5EP2 | 0.01440856 | -0.52 |
| P14174 | MIF | 0.01488262 | 0.51 |
| P12110 | COL6A2 | 0.01526347 | -0.53 |
| Q14956 | GPMBM | 0.01546825 | -0.63 |
| P46952 | HAAO | 0.01570996 | -0.53 |
| Q86VN1 | VPS36 | 0.01610077 | 0.67 |
| Q96S52 | PIGS | 0.01626862 | -0.61 |
| P15559 | NQO1 | 0.01626862 | 0.56 |
| O60575 | SPINK4 | 0.01810104 | 0.77 |
| P55735 | SEC13 | 0.01827155 | 0.59 |
| P02452 | COL1A1 | 0.01933726 | -1.32 |
| P00403 | MT-CO2 | 0.02012815 | -0.62 |

* Designated candidate cancer biomarkers in the Human Protein Atlas database

* Two or more accession numbers: proteins from the same family or isoforms from the same gene.

Boldface numbers indicate "epithelial cell signature" proteins. (See text.)

(Ig heavy chain V-III region BRO; Ig heavy chain V-III region BUT; Ig heavy chain V-III region CAM)
Table 4. Gene Ontology (GO) biological processes enriched in the set of 212 proteins whose expression displayed adenoma-related dysregulation (see Table 3).

| GO ID       | GO Term                                      | Annotated * | Significant * | Up in adenomas | Down in adenomas | Expected * | elim P value * |
|-------------|----------------------------------------------|-------------|---------------|----------------|------------------|------------|----------------|
| GO:0006805  | xenobiotic metabolic process †               | 64          | 21            | 3              | 18               | 2.8        | 1.70E-09       |
| GO:0006958  | complement activation, classical pathway †   | 74          | 17            | 0              | 17               | 3.24       | 1.10E-08       |
| GO:0051552  | flavone metabolic process †                 | 5           | 5             | 0              | 5                | 0.22       | 1.50E-07       |
| GO:0052696  | flavonoid glucuronidation †                 | 5           | 5             | 0              | 5                | 0.22       | 1.50E-07       |
| GO:0052697  | xenobiotic glucuronidation †                 | 5           | 5             | 0              | 5                | 0.22       | 1.50E-07       |
| GO:0045087  | innate immune response §                     | 249         | 33            | 4              | 29               | 10.91      | 7.80E-07       |
| GO:0031295  | T cell costimulation §                       | 34          | 10            | 0              | 10               | 1.49       | 1.10E-06       |
| GO:0030199  | collagen fibril organization †              | 8           | 5             | 0              | 5                | 0.35       | 7.80E-06       |
| GO:0001501  | skeletal system development                  | 54          | 11            | 1              | 10               | 2.37       | 1.60E-05       |
| GO:0070208  | protein heterotrimerization †                | 5           | 4             | 0              | 4                | 0.22       | 1.70E-05       |
| GO:0050852  | T cell receptor signaling pathway §          | 48          | 10            | 0              | 10               | 2.1        | 3.20E-05       |

* Annotated: proteins in TopGO Background list; Significant: 212 dysregulated proteins of Table 3; Expected: Number of significant proteins expected to map to the GO term if the significant proteins were randomly distributed over all GO terms. elim P value: P value from the elim method (ref. no. 25). Only processes with an elim P value < 1.0E-04 are shown.

† Processes that were also among the top 12 processes displaying enrichment in a larger set of 621 dysregulated proteins selected with less stringent criteria (q value ≤ 0.2; average log₂ fold change ≥ ± 0.5; see Results section for details); § processes that shared a common GO ancestor (immune system process) with the process displaying most significant enrichment in the larger set.
Uzozie A. et al. Figure 1

**Tissue or cells**
- protein extract
- trypsin digestion
- peptides
- iTRAQ 8plex labeling
- OFFGEL electrophoresis (OGE)

**Raw data converted to Mascot generic format (MGF) using Mascot Distiller**
- Peak picking and merging of CID and HCD
- MGF files searched against UniProt/SwissProt database
- .dat files converted to XML
- Mascot XML results parsed to TXT
- Confident peptides filtered

**Median score normalization on log2 protein intensity ratios (e.g., 115/114)**

**Different iTRAQ experiments combined into one matrix**
- Family no. assigned to protein hits

**Proteins assembled from peptides and protein intensity calculated**

**Statistical analysis**
- Selected threshold: q<0.02; fold change = +/- 0.5 log2 scale
- List of dysregulated proteins in adenomas and colon cancer cell lines

**Specific family numbers used to select non-redundant proteins**

**Gaussian distribution histogram for protein ratios**

| 113 | 114 | 115 | 116 | 117 | 118 | 119 | 121 |
|-----|-----|-----|-----|-----|-----|-----|-----|
| 3 x N | 3 x N | 3 x A | 1 x N | 1 x A | 1 x N | 1 x A | 1 x N | 1 x A |

10 experiments for 30 normal mucosa samples (N) and 30 adenomas (A)

**6 cell lines**
- HCEC
- HT29
- Caco2
- CX1
- SW480
- SW620

| 6 cell lines | 3 x N | 6 cell lines | 3 x A | HCEC | HT29 | Caco2 | CX1 | SW480 | SW620 |
|-------------|-------|-------------|-------|------|------|-------|-----|-------|-------|
| 3 x N | 1 x N | 1 x N | 1 x N | 1 x N | 1 x N | 1 x N | 1 x A | 1 x A | 1 x A |

6 cell lines
- HCEC
- HT29
- Caco2
- CX1
- SW480
- SW620
212 proteins (Table 3)

Adenomas Normal mucosa

![Graph showing protein and mRNA levels with Intensity and Average log₂ fold change axes. The Pearson correlation coefficient is r = 0.74.]

Uzozie A. et al. Figure 4
Uzozie A. et al. Figure 5

A

B

C

SORD protein

P < 0.0001
fold change (log2) = 0.62

Expression intensity value (log2)

normal mucosa
adenomas

D

SORD mRNA

P < 0.0001
fold change (log2) = 1.98

Expression intensity value (log2)

normal mucosa
adenomas

E

F

Enzyme activity (mU/mg protein)

β tubulin

SORD

E

F

Enzyme activity (mU/mg protein)

β tubulin

SORD
Uzozie A. et al., Figure 6

A

B

C

D

E

F

G

H

I