Aberrant expression and subcellular localization of ECT2 drives colorectal cancer progression and growth

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SUPPLEMENTARY TABLES

Supplementary Table S1. *APC* and *KRAS* mutation status of CRC cell lines

CRC cell line	APC status	KRAS status
COLO-320-HSR	S811*	WT
LS-1034	E1309fs*4	A146T
SNU-C1	APC-TBST1 fusion	WT
SW48	R2714C	WT
T84	L1488fs*19	G13D
HCT-116	WT	G13D
LOVO	- R1114* - R2816Q - M1431Cfs*42	G13D
LS-174T	WT	G12D
SW480	Q1338*	G12V
SW620	Q1338*	G12V

Data are compiled from cBioportal.org (1) and (2).

Supplementary Table S2. Patient characteristics

Characteristic	**N = 146**
Age, years, mean (range)	66.0 (32-92)
Gender	
Male, n (%)	80 (55)
Female, n (%)	66 (45)
Race	
White, n (%)	113 (79)
Black, n (%)	30 (21)
Tumor stage[a]	
1, n (%)	13 (11)
2, n (%)	40 (35)
3, n (%)	41 (36)
4, n (%)	20 (18)
Tumor differentiation	
Well, n (%)	6 (5)
Moderate, n (%)	86 (77)
Poor, n (%)	19 (17)
Tumor location[b]	
Proximal colon, n (%)	48 (44)
Distal colon, n (%)	60 (56)
Overall survival, days	
Mean (SE)	1344 (56)
Alive, n (%)	84 (58)
Dead, n (%)	62 (42)

[a]American Joint Committee on Cancer/Union, Internationale Contre le Cancer staging. [b]Proximal colon includes cecum, ascending colon, hepatic flexure, transverse colon. Distal colon includes splenic flexure, descending colon, sigmoid colon, and rectum.

Supplementary Table S3. Cox proportional hazard ratios

Variable	Univariate		Multivariate[a]	
	HR (95% CI)	*P* value	HR (95% CI)	P value
Nuclear (10 unit increment)	0.97 (0.92, 1.03)	0.32	0.98 (0.90, 1.06)	0.56
Cytoplasm (10 unit increment)	0.97 (0.92, 1.03)	0.32	0.98 (0.90, 1.06)	0.56
C:N ratio (0.5 unit increment)	0.60 (0.47, 0.77)	<0.01	0.61 (0.38, 0.95)	0.03
Stage (3-4 vs 1-2)	2.14 (1.10, 4.15)	0.02		
Differentiation (Poor vs. Not Poor)	2.52 (1.26. 5.04)	0.01		
Age (10 year increment)	1.38 (1.12, 1.70)	<0.01		
Location (Prox vs Dist)	1.04 (0.58, 1.85)	0.90		
Chemo (Yes vs No)	0.75 (0.34, 1.64)	0.47		

[a]Adjusted for stage, differentiation, age, location and chemotherapy

SUPPLEMENTARY MATERIALS AND METHODS

Immunohistochemistry (IHC) for ECT2 in tissue microarrays (TMAs) and full tissue slides

Cancer Care Outcomes Research and Surveillance Consortium (CanCORS) was a population-based cohort stud, as described previously (3). The North Carolina CanCORS site enrolled newly diagnosed cases of CRC in North Carolina, encompassing rural and urban areas. In addition to information collected by patient surveys and medical record review, the University of North Carolina at Chapel Hill site collected tissue blocks on consenting subjects. Written informed consent was obtained from all patients and the study was approved by the human subjects committees at the University of North Carolina at Chapel Hill. TMAs were constructed from formalin-fixed, paraffin-embedded colorectal tissues. Each microarray block included duplicate or triplicate cores of CRC and adjacent normal tissues from each patient. Twenty-nine TMAs from 441 patients were available for staining. Paired colon and rectum adenoma/carcinoma samples were obtained during pathological routine inspection. The samples are termed “matched” as they co-exist in the same polyp or resection sample. All samples displayed histological evidence of neoplastic progression upon microscopic investigation. For the IHC study with the independent cohort of 16 samples, all patients provided signed consent as part of the clinical documentation protocol of the Charité Universitätsmedizin Berlin (4). To validate the anti-ECT2 polyclonal rabbit antibody (Millipore #07-1364, RRID:AB_10805932) for IHC, we utilized CRC cell lines and tissue. Positive controls included a colon cancer tissue and HT-29 cells. HT-29

cells with *ECT2* gene knockdown were used as negative controls. TMAs, positive and negative control slides were stained using anti-ECT2 antibody at 1:350 dilution using Bond Primary Antibody Diluent (Leica Microsystems). Antigen was heat retrieved in citrate buffer, pH 6, for 30 min. The remainder of the staining was carried out using the Bond Polymer Refine Detection Kit with the Bond Autostainer (Leica Microsystems) for the following times: primary antibody 6 h, post-primary 8 min, polymer 8 min, peroxide block 5 min, 3,3-diaminobenzidine 10 min, hematoxylin 7 min, and bluing 5 min.

Scoring of TMAs : Prior to scoring, crypts and colorectal epithelium in each core on the TMAs were manually annotated to remove the lamina propria and non-epithelial cells. Only the annotated areas were scored. Scoring for ECT2 expression was carried out using Cytoplasm algorithm in ImageScope (Aperio Inc., RRID:SCR_020993). Aperio ImageScope algorithms were used for tumor tissue slide and TMA scoring and demonstrated good concordance with visual scoring in previous studies (5-7). Scoring was confirmed on a random sample of cores by visual inspection. Any cores with less than 300 epithelial cells, missing from falling off in the staining process, exhausted from prior sectioning, or cores with staining artifact were excluded from analysis. ECT2 protein expression was measured by H-scores for nuclear or cytoplasmic staining using the Aperio Cytoplasm Algorithm User's Guide, Aperio. Briefly, H-score is an intensity score derived from the average intensity of the staining of the corresponding area (cytoplasm or nucleus, cellular average). For example, there are three intensity thresholds (1+, weak, 2+, moderate and 3+, intense), and H-score equals sum of (% of cells with 1+

staining)+2*(% of cells with 2+ staining)+3*(% of cells with 3+ staining). This would yield a range of scores from minimum of 0 and maximum score of 300, where 300 would represent 100% of the cells are 3+ in intensity. In this study, H-score and ECT2 scores represent the same, and these words are used interchangeably.

Scoring of the slides: All full slides were also annotated manually to exclude tissue parts that were not included in the scoring (non-epithelial cells). Scoring was then carried out using the Cognition Master Professional Suite (VMscope GmbH Berlin). For nuclear staining, a ki67 method was used to highlight nuclear ECT2 expression. For the IHC study with the independent cohort of 16 samples, nuclear expression levels as well as cytoplasmic expression levels were scored afterwards by an advanced pathologist using the previously mentioned H-score method.

Statistical analysis for TMAs: ECT2 protein expressions were compared between tumor and normal tissues or between nuclear and cytoplasmic areas using a paired t-test. ECT2 tumor scores were divided into tertiles, with cutoffs determined by ECT2 scores in normal tissues. To determine the association between patient characteristics and ECT2 tumor score tertiles, Kruskal-Wallis test was used for 2 category variables, Spearman correlation for variables with more than 2 categories tumor grades, and Wilcoxon rank test for continuous variable (overall survival). Cox proportional hazards models were used to calculate hazard ratios (HRs) and 95% confidence intervals (CIs) for mortality associated with continuous ECT2 scores. Multivariate models were fit to adjust for age (in 10 year increments), stage (American Joint Committee on Cancer/ Union Internationale Contre le Cancer stages 1, 2, 3, or 4), grade (well, moderate or poorly

differentiated), location of CRC (proximal or distal), and chemotherapy (yes or no). Analyses were performed using SAS/STAT 9.2 (SAS Institute, RRID:SCR_008567).

Statistical analysis: Mean ECT2 expression scores as well as ratios for cytoplasmic to nuclear expression in normal, adenoma and carcinoma tissue were tested for significant differences using the Mann-Whitney U test. Statistical analyses were performed using RStudio version 1.1.463 based on the statistical language R version 3.5.1 (RStudio Team (2015), RRID:SCR_000432).

ECT2 constructs

HT-29, HCT-116 and LS-174T cells stably expressing human ECT2 or control shRNAs were described previously (8,9). Lentiviral expression vectors encoding shRNA targeting human *ECT2* (targeting sequence: CGGAATGAACAGGATTTCTAT) were obtained from the Mission shRNA Library (Sigma-Aldrich). A vector encoding a shRNA that does not recognize any human or mouse genes (NS-shRNA) was used a negative control. For lentiviral infections, cells were seeded in 100 mm plates and grown to 70–80% confluency. Culture medium was replaced with 4 ml of fresh medium containing polybrene (5 μg/ml). After 5 min incubation at 37°C, 400 μl of viral supernatant (multiplicity of infection ~3) was added. After 24 h, virus containing medium was replaced with fresh medium. Forty-eight hours after infection, populations of stably transduced cells were selected in 2.5 μg/ml puromycin.

We generated the shRNA-resistant cDNA expression vector of full-length WT ECT2 by introducing silent mutations that disrupt the ECT2 shRNA target site (targeting sequence: CGGAATGAACAGGATTTCTAT, which is not in the 5' or 3' UTR (8,10)). The shRNA-resistant *ECT2* cDNA sequence was used as a template for introduction of missense mutations or generation of truncations by the QuikChange Site-directed Mutagenesis Kit (Stratagene). WT or mutant *ECT2* cDNA sequences were subcloned into the pCDH-EF1α-MCS-IRES-Puro Cloning and Expression Lentivector (Systems Biosciences) to encode an amino-terminal hemagglutinin (HA) epitope tag.

β-catenin overexpression and MYC/KRAS knockdown studies

Virus particles were produced for the pCW317 empty vector (EV) and activated β-catenin (4A) constructs as described previously (11). $5x10^5$ HCT-116 cells were plated in a T25 flask and infected the next day by combining 0.5 ml of EV or 4A virus with 8 mg/ml polybrene (Millipore, #TR-1003-G) in 3 ml of medium. Medium was replaced with DMEM + 10% FBS the next day. After 24 h, cells were seeded into T75 flasks with 2 μg/ml Puromycin (Sigma, #P7255). After 72 h, $3x10^5$ EV- or 4A-infected cells were seeded into individual wells of a 6-well plate. The next day, medium was replaced. Cells were transfected the next day with control nonspecific siRNA (Ambion, #4390844) or validated siMYC (M0: Thermo Fisher Scientific, #3s9129 (4392421), M1: Thermo Fisher Scientific, #s9130 (4392421)). Thirty pmol siRNA were added to 200 μl of Opti-MEM medium followed by 9 μl of Lipofectamine RNAiMax (Thermo Scientific, #13778150). Mixtures were incubated

for 8-10 min before adding to cells. After 72 h, cells were collected for immunoblotting.

For siRNA-mediated depletion of MYC or KRAS, SW480 cells were seeded in a 6-well plate (300,000 cells per well) and transfected with 10 nM nonspecific siRNA (see above) or validated siMYC (M0 or M1, see above) or siKRAS (K1: Thermo Fisher Scientific, # s7939 (4390815), K2: Thermo Fisher Scientific, #s7940 (4390815)), using Optim-MEM medium and Lipofectamine RNAiMax as described above. After 72 h, cell lysates were collected for immunoblotting.

REFERENCES

1. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA*, et al.* The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012;2:401-4.
2. Yeh JJ, Routh ED, Rubinas T, Peacock J, Martin TD, Shen XJ*, et al.* KRAS/BRAF mutation status and ERK1/2 activation as biomarkers for MEK1/2 inhibitor therapy in colorectal cancer. Mol Cancer Ther 2009;8:834-43.
3. Ayanian JZ, Chrischilles EA, Fletcher RH, Fouad MN, Harrington DP, Kahn KL*, et al.* Understanding cancer treatment and outcomes: the Cancer Care Outcomes Research and Surveillance Consortium. J Clin Oncol 2004;22:2992-6.
4. Mamlouk S, Simon T, Tomas L, Wedge DC, Arnold A, Menne A*, et al.* Malignant transformation and genetic alterations are uncoupled in early colorectal cancer progression. BMC Biol 2020;18:116.
5. Chabot-Richards DS, Martin DR, Myers OB, Czuchlewski DR, Hunt KE. Quantitative image analysis in the assessment of diffuse large B-cell lymphoma. Mod Pathol 2011;24:1598-605.

6. Laurinaviciene A, Dasevicius D, Ostapenko V, Jarmalaite S, Lazutka J, Laurinavicius A. Membrane connectivity estimated by digital image analysis of HER2 immunohistochemistry is concordant with visual scoring and fluorescence in situ hybridization results: algorithm evaluation on breast cancer tissue microarrays. Diagn Pathol 2011;6:87.
7. Rexhepaj E, Brennan DJ, Holloway P, Kay EW, McCann AH, Landberg G, *et al.* Novel image analysis approach for quantifying expression of nuclear proteins assessed by immunohistochemistry: application to measurement of oestrogen and progesterone receptor levels in breast cancer. Breast Cancer Res 2008;10:R89.
8. Frederick LA, Matthews JA, Jamieson L, Justilien V, Thompson EA, Radisky DC, *et al.* Matrix metalloproteinase-10 is a critical effector of protein kinase Ciota-Par6alpha-mediated lung cancer. Oncogene 2008;27:4841-53.
9. Justilien V, Ali SA, Jamieson L, Yin N, Cox AD, Der CJ, *et al.* Ect2-dependent rRNA synthesis Is required for KRAS-TRP53-driven lung adenocarcinoma. Cancer Cell 2017;31:256-69.
10. Justilien V, Fields AP. Ect2 links the PKCiota-Par6alpha complex to Rac1 activation and cellular transformation. Oncogene 2009;28:3597-607.
11. Waters AM, Khatib TO, Papke B, Goodwin CM, Hobbs GA, Diehl JN, *et al.* Targeting p130Cas- and microtubule-dependent MYC regulation sensitizes pancreatic cancer to ERK MAPK inhibition. Cell Rep 2021;35:109291.