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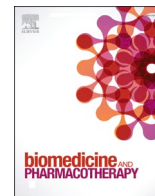
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## CDX2 controls genes involved in the metabolism of 5-fluorouracil and is associated with reduced efficacy of chemotherapy in colorectal cancer

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### ABSTRACT

Most patients affected with colorectal cancers (CRC) are treated with 5-fluorouracil (5-FU)-based chemotherapy but its efficacy is often hampered by resistance mechanisms linked to tumor heterogeneity. A better understanding of the molecular determinants involved in chemoresistance is critical for precision medicine and therapeutic progress. Caudal type homeobox 2 (CDX2) is a master regulator of intestinal identity and acts as tumor suppressor in the colon. Here, using a translational approach, we examined the role of CDX2 in CRC chemoresistance. Unexpectedly, we discovered that the prognosis value of CDX2 for disease-free survival of patients affected with CRC is lost upon chemotherapy and that CDX2 expression enhances resistance of colon cancer cells towards 5-FU. At the molecular level, we found that CDX2 expression correlates with higher levels of genes regulating the bioavailability of 5-FU through efflux (ABCC11) and catabolism (DPYD) in patients affected with CRC and CRC cell lines. We further showed that CDX2 directly regulates the expression of ABCC11 and that the inhibition of ABCC11 improves 5-FU-sensitivity of CDX2-expressing colon cancer cells. Thus, this study illustrates how biological functions are hijacked in CRC cells and reveals the therapeutic interest of CDX2/ABCC11/DPYD to improve systemic chemotherapy in CRC.

### 1. Introduction

Systemic or loco-regional 5-FU-based chemotherapy is frequently administered to patients affected with colorectal cancer (CRC) at high risk of recurrence or having metastases. Chemoresistance, whether occurring from the onset or after prolonged exposure of tumor cells to anticancer agents, is a major concern and is inherent to the molecular heterogeneity of tumor cells. Several mechanisms of chemoresistance have been described, among which drug efflux through an increased expression of ATP-Binding Cassette (ABC) family transporters. A better understanding of the molecular mechanisms involved in chemoresistance towards 5-FU is critical as it would allow the identification of new therapeutic targets and biomarkers having predictive value for the treatment of CRC.

Caudal-type homeobox transcription factor 2 (CDX2) plays a crucial role in the determination of the intestinal identity and is a biomarker of

mature colorectal epithelial tissue [1]. The loss of CDX2 expression in CRC is associated with features of aggressiveness such as poor differentiation, BRAF mutation, high tumor grade, MMR deficiency, advanced stages and CIMP phenotype [2,3]. Furthermore, Dalerba et al. and Pilati et al. recently showed that the loss of CDX2 expression was significantly associated with worse survival in stage II and III CRC [4,5]. Paradoxically, patients with CDX2-positive tumors did not benefit from adjuvant chemotherapy compared to patients with CDX2-negative tumors, suggesting a role of CDX2 in chemoresistance [6]. In addition, CDX2 induces the expression of the multidrug resistance protein 1 (MDR1/ABCB1) gene by binding to its promoter [7], suggesting that CDX2 plays a role in CRC chemoresistance through the expression of ABC family transporters.

In the present study, we developed a translational approach to clarify the role of CDX2 in the chemoresistance of CRC and identify the related molecular mechanisms.

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## 2. Materials and methods

### 2.1. Patients clinical and biological data analyses

#### 2.1.1. CRC patients gene expression datasets

The NCBI-GEO meta-cohort, representing 1257 patients affected with CRC, annotated or not with survival and clinical data ([Supplementary Table S1](#)), was formed by pooling publicly available datasets from the Gene Expression Omnibus at NCBI. 30 gene expression datasets were screened and 12 of them ([Supplementary Table S2](#)) were selected for being homogeneously generated through the Affymetrix platform GPL 570 Human Genome U133 Plus 2.0 Array and analyzed with a Robust Multi Array analysis (RMA) algorithm [8,9]. The 20 other datasets were excluded from the pooling as they did not meet the previous criteria. Raw data were extracted and pooled after computation of the z-score for each data to remove biases due to data having been collected in different centers.

#### 2.1.2. Human tissue samples and clinical data

Frozen tissue samples, mostly originating from the General and Digestive Surgery Department (Haute-pierre Hospital, Strasbourg, France), were obtained through the Biological Resources Center (Strasbourg University Hospital, France). They included 61 healthy tissue samples (stomach  $n = 5$ , duodenum  $n = 5$ , jejunum  $n = 1$ , ileum  $n = 5$ , colon  $n = 40$ , rectum  $n = 5$ ) and 38 CRC samples (i.e. the so-called local cohort). All patients gave their written informed consent for the analyzes of their clinical and biological data. The database complies with applicable local regulations and ethical principles (Declaration of Helsinki, Fortaleza 2013) related to data processing, files and personal freedom and privacy. For each patient, the following data were extracted: gender, age, WHO performance status, BMI, TNM stage and histopathological characteristics ([Supplementary Table S3](#)).

#### 2.1.3. Statistical analyses on CRC patients

The patients of the meta-cohort and the local-cohort were classified into 2 groups (Meta/Local-CDX2-High or Meta/Local-CDX2-Low) according to the CDX2 expression level determined by a Ward's method (hierarchical cluster analysis) with XLSTAT (Addinsoft) and SPSS softwares (IBM). Continuous variables are presented as numbers (with percentage), mean (+/- standard deviation) or median (with range). Groups were compared using the Chi-squared and Fisher's exact tests when appropriate for categorical variables, whereas Mann-Whitney tests were used for continuous variables. Variables with  $p$ -values  $< 0.1$  were defined as potentially confounding variables and were included in a multivariable Cox model. When available, disease free survival (DFS) and overall survival (OS) analyzes were performed on the pooled population using the Kaplan-Meier method and compared using the log-rank test with SPSS software. A  $p$ -value  $< 0.05$  was considered significant.

### 2.2. Cellular and molecular biology analyses

#### 2.2.1. Cell lines

The following cell lines were used and grown as recommended: human colon adenocarcinoma Caco-2/TC7 [10,11] HCT116, SW480 and embryonic kidney HEK293 (ATCC). HT29/CDX2 (formerly TW6) inducible cells and control HT29/CT (formerly TG8) cells were generated in our laboratory from human colon adenocarcinoma HT29 cells modified via the TET-ON system to express CDX2 and/or GFP upon addition of doxycycline (1  $\mu\text{g}/\text{mL}$ , Sigma-Aldrich) to the culture medium [12]. These cell lines were chosen according to their endogenous CDX2 expression ([Supplementary Fig. S1A](#)), transfection rates and/or endpoint. Almost all CDX2 gain of function experiments were performed on stable HT29/CT and HT29/CDX2 inducible cells and repeated in other cell lines (Caco-2/TC7, HCT116, SW480 and/or HEK293) for confirmation. The CDX2 loss of function experiment was performed in SW480 cells which display endogenous expression of CDX2 and good

transfection rates.

#### 2.2.2. Tumor formation

For xenografts, HT29/CDX2 cells were subcutaneously injected ( $2.10^6$  cells in 100  $\mu\text{l}$ /injection point) on 2 injection points in 6 nu/nu mice (Elevage Janvier) as previously described [11]. Induction of CDX2 was performed by adding doxycycline (400  $\mu\text{g}/\text{mL}$ ) in the drinking water of half the mice. After 6 weeks, mice were sacrificed, tumors were measured (mean volumes: 31.7  $\text{mm}^3$  without CDX2 vs 24.8  $\text{mm}^3$  with CDX2 induction;  $n = 5-6$ ,  $p = 0.41$ ) and processed for immunofluorescence analyses. Mice were handled according to the protocol approved by the Committee on the Ethics of Animal Experiments of the University of Strasbourg (CREMEAS, C2EA-35) under the permit number AL/43/50/02/13.

#### 2.2.3. Cytotoxicity assays

HT29/CT, HT29/CDX2 and Caco-2/TC7 cells were treated during 48 h with ranges of 5-FU (Sigma-Aldrich), oxaliplatin (Teva Santé), irinotecan (Sigma-Aldrich) or vehicle, and survival was evaluated after 48 h by MTT assay (Sigma-Aldrich) as previously described [13]. ABC11 activity was reduced by either adding MK571 (50  $\mu\text{M}$ , Sigma-Aldrich) to the culture medium or transfecting the cells with a pool of siRNA before treatment (see transfection procedure below).

#### 2.2.4. Plasmids and small interfering RNA transfections

Transfections were performed according to the recommendations of the suppliers.

pFLAG-hCDX2 (pCDX2) [11] or parental plasmids were transfected for 48 h using the JetPrime transfection reagent (Polyplus transfection).

Stealth siRNA duplexes (CDX2HSS141546 or low GC Duplex #2, Invitrogen, 1 nM) were transfected for 48 h with Lipofectamine RNAi-MAX (Invitrogen).

#### 2.2.5. Chromatin immunoprecipitation (ChIP)

ChIP experiments were performed as previously described [11] using the Magna ChIP G Chromatin Immunoprecipitation kit. qPCR analyzes were performed with the Power SYBR Green PCR Master Mix (Applied Biosystems) as recommended. Enrichments relative to inputs were calculated according to the formula  $\% \text{ input} = 2^{(\text{Ct}_{\text{input}} - 6.64 - \text{Ct}_{\text{ChIP}})} \times 100$ .

#### 2.2.6. RNA extraction and RT-qPCR

Total RNA was extracted using TRI Reagent (Molecular Research Center). For tissues, the Precellys24 homogenizer (Bertin Technologies) was used according to the manufacturer protocol before extraction. cDNA synthesis (RT) was performed with either the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, cell samples) or the Cloned AMV Reverse Transcriptase kit (Invitrogen, tissue samples) according to the suppliers' recommendations. Quantitative PCR (qPCR) reactions were performed with FastStart universal Probe Master-Rox (Roche) and gene-specific TaqMan probe and primers sets (TaqMan Gene Expression Assays, Applied Biosystems, [Supplementary Table S4](#)) on a 7500 real-time PCR System (Applied Biosystems). Data were analyzed using the  $\Delta\Delta\text{Ct}$  method.

#### 2.2.7. Immunofluorescence

Immunofluorescence was performed on fixed frozen sections with anti-ABCC11 (1/250, H-215 or S-19 from Santa Cruz Biotech) and anti-CDX2 (1/5000, Biogenex) as primary antibodies. Imaging was based on optical sectioning using the ApoTome system (Zeiss) and the ImageJ software (NIH).

Additional information and methods are provided in [Supplementary Data](#).

### 3. Results

#### 3.1. The prognostic value of CDX2 in CRC is challenged by chemotherapy

To investigate the role of CDX2 as a biomarker in CRC, we screened thirty-two publicly-available CRC gene expression datasets and pooled the raw data from twelve of them (Supplementary Table S2) to set up the NCBI-GEO meta-cohort ( $n = 1237$ ). Two groups were created according to the expression level of CDX2 in the tumors (Meta-CDX2-High group,  $n = 797$  and Meta-CDX2-Low group,  $n = 440$ ). Clinical data are summarized in the Supplementary Table S1. DFS was significantly higher for patients of the Meta-CDX2-High group compared to the patients of the Meta-CDX2-Low group (Fig. 1A). Furthermore, the Meta-CDX2-High

status was associated with a better DFS in the multivariate analysis (Table 1). However, this DFS difference between the Meta-CDX2-High and Meta-CDX2-Low groups was statistically not significant between the Meta-CDX2-High and Meta-CDX2-Low groups when patients received systemic chemotherapy (Supplementary Fig. S2). In addition, DFS tended to be lower upon chemotherapy in patients with stage II, III and IV CRC in the Meta-CDX2-High group (Fig. 1B).

These results confirm the prognostic role of CDX2 expression for patients affected with CRC, as low level of CDX2 expression negatively impacts DFS. However, in the subgroups of patients treated with chemotherapy, the beneficial effect of CDX2 expression on DFS seems lost and reciprocally.

#### 3.2. CDX2 increases the chemoresistance of CRC cells

To confirm the hypothesis that CDX2 expression bestows tumors with increased chemoresistance, we first performed MTT survival assays on colon cancer HT29/CDX2-inducible cells [12] and showed that cells ectopically expressing CDX2 upon addition of doxycycline in the culture medium were significantly more resistant to ranges of 5-FU doses (Fig. 2A, Supplementary Fig. S1B). No change of the 5-FU sensitivity was observed in the control HT29/CT cells [12] (Supplementary Fig. S3A). HT29/CDX2-expressing cells also tended to be more resistant towards protocols combining 5-FU with oxaliplatin but not with irinotecan (Supplementary Fig. S3B-C). Similar results were obtained with another colon cancer cell line as CDX2 expression [11] also correlated with reduced sensibility towards 5-FU (alone or combined with either oxaliplatin or irinotecan) in Caco-2/TC7 cells (Fig. 2B, Supplementary Fig. S1C).

Thus, we showed here that CDX2 expression correlates with 5-FU chemoresistance in CRC cells.

#### 3.3. CDX2 increases the expression of several genes involved in chemoresistance

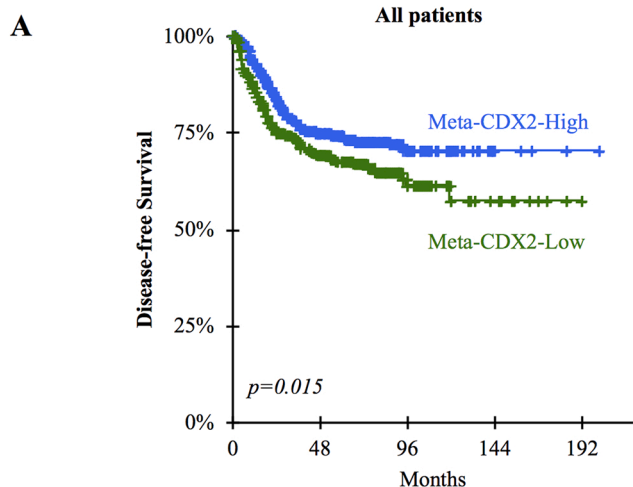
To identify genes that contribute to the CDX2-related chemoresistance, we examined by RT-qPCR the expression of various genes implicated in previously described CRC chemoresistance mechanisms such as drug efflux (ABC family transporters), 5-FU metabolism, apoptosis or the MMR DNA repair mechanism.

RT-qPCR profiling of genes encoding members of the ABC family transporters showed that the expression of both ABCB1 and ABCC11 was increased in the presence of CDX2 in HT29/CDX2 cells (Fig. 3A), whether the cells were treated or not by 5-FU (Supplementary Fig. S4A).

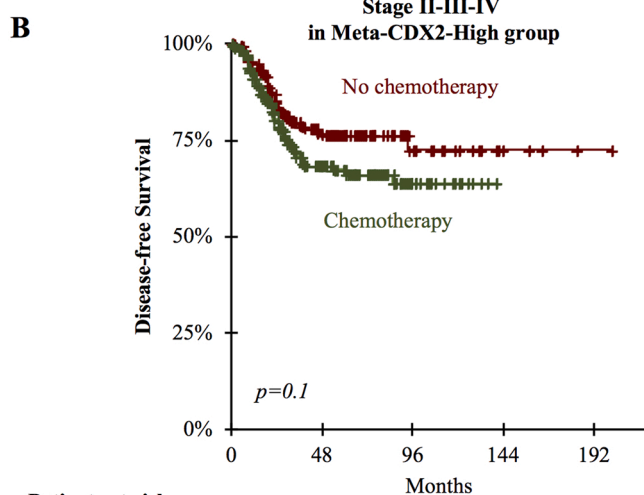
**Table 1**  
Prognostic factors analysis for disease-free survival.

	Multivariate analysis		Univariate analysis	
	HR (95% CI)	p value	HR (95% CI)	p value
All patients with available survival data ( $n = 767$ )				
Meta-CDX2 High (vs Meta-CDX2 Low)	0.69 (0.49–0.97)	0.035	0.71 (0.54–0.94)	0.016
UICC tumor stage (per increase in stage)	2.38 (1.32–4.75)	0.001	2.70 (1.53–4.39)	0.001
Age (modeled as continuous variable)	1.01 (0.99–1.02)	0.12	1.00 (0.99–1.01)	0.23
Female sex (vs male)	0.81 (0.58–1.13)	0.22	0.84 (0.64–1.11)	0.23
Proximal location of CCR (vs distal)	0.76 (0.52–1.11)	0.15	0.82 (0.61–1.09)	0.18
MSS phenotype (vs MSI)	2.03 (1.08–3.8)	0.029	2.26 (1.28–3.98)	0.005
KRAS mutation	0.84 (0.59–1.18)	0.32	0.78 (0.57–1.07)	0.13

CI: confidence interval, n: number, vs: versus, MSS: microsatellite stable, MSI: microsatellite instable, HR: hazard ratio.

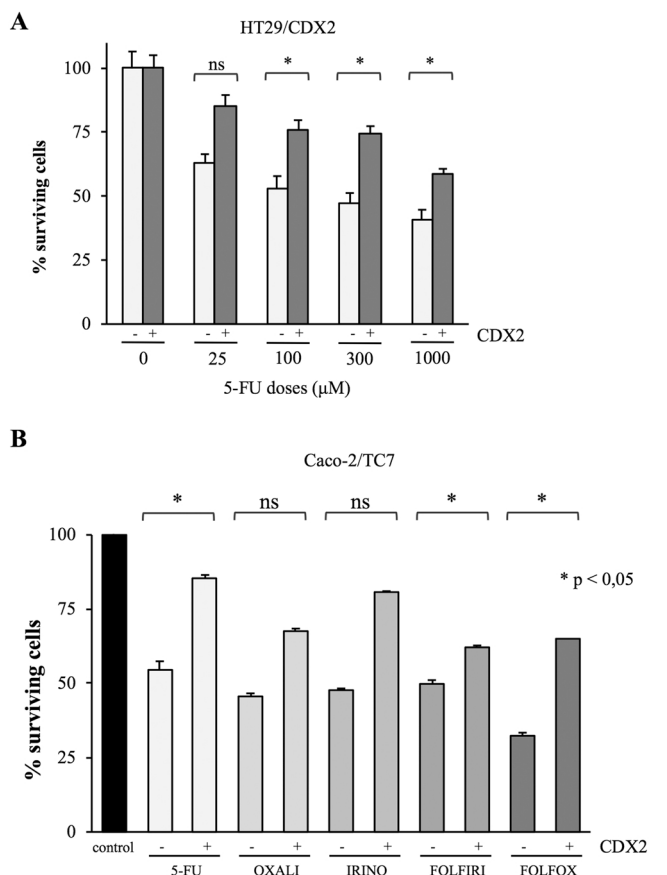


Patients at risk					
Meta-CDX2-High	438	224	49	5	2
Meta-CDX2-Low	329	143	33	11	1



Patients at risk					
Chemotherapy	153	82	21	5	2
No chemotherapy	149	72	18	1	

**Fig. 1.** Impact of CDX2 on survival. Disease-free survival curves in the NCBI-GEO meta-cohort comparing (A) all patients for whom disease-free survival data were available ( $n = 767$ ) according to CDX2 expression and (B) patients of the Meta-CDX2-High group with stage II, III and IV CRC ( $n = 302$ ) according to the administration of systemic chemotherapy,  $p$ -value for (A) and (B) indicates result of the log-rank test.



**Fig. 2.** CDX2 increases the chemoresistance of CRC cells. Survival of (A) HT29/CDX2 cells cultured 24 h in the presence or absence of CDX2 (± doxycycline) and treated during 48 h with ranges of 5-FU doses and (B) Caco-2/TC7 cells cultured for 1 (-) or 11 (+) days and treated during 48 h with different chemotherapy regimens. Data (means ± SD) of a representative experiment are shown and statistics were calculated on 3 experiments \* *p*-value < 0.05, ns not significant.

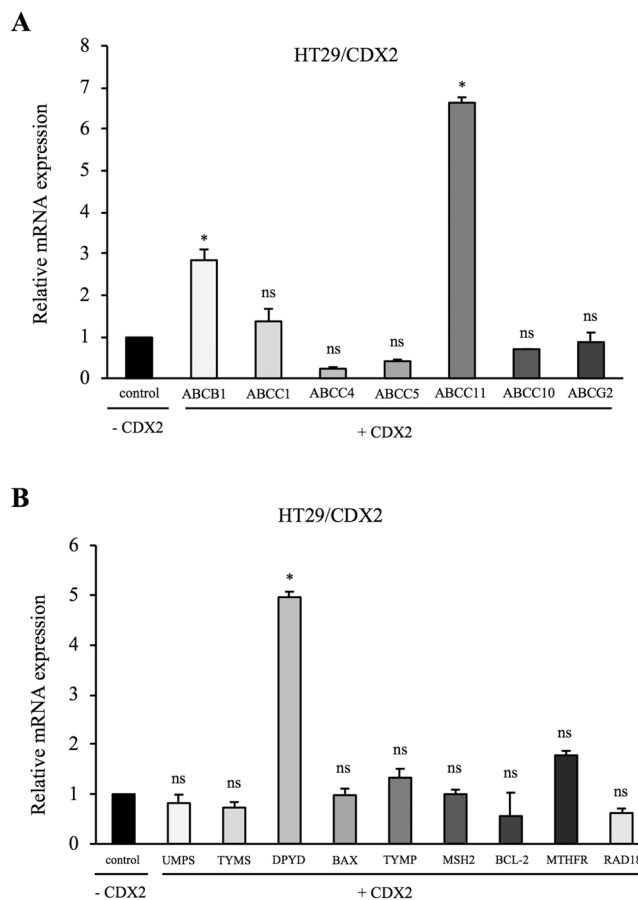
No change was observed in the control HT29/CT cells (Supplementary Fig. S4B). Among the other genes tested, DPYD, which encodes the dihydropyrimidine dehydrogenase responsible for the 5-FU breakdown, was the only gene whose expression was up-regulated in CDX2-expressing HT29 cells (Fig. 3B).

Thus, we identified two genes up-regulated by CDX2, namely ABCC11 and DYDP, that may contribute to 5-FU chemoresistance. Hereafter, we focused on ABCC11 as it was never associated with chemoresistance in CRC but is able to transport 5-FU. ABCB1, which is a CDX2 target [7] but does not transport 5-FU, was used as a positive control.

### 3.4. ABCC11 transcription is directly regulated by CDX2

First, we confirmed the upregulation of ABCC11 upon CDX2 expression in two other colon cancer cell models grown in vitro, namely Caco-2/TC7 cells and HCT116 cells (Supplementary Fig. S5A-B, Supplementary Fig. S1C-D). We also established colon spheroids (Fig. 4A left and Supplementary Fig. S5C) and tumors in nude mice (Fig. 4A right and Supplementary Fig. S5D) using HT29/CDX2 cells to confirm that ABCC11 expression was also increased in the presence of CDX2 in experimental set-ups that mimic more closely tumor growth.

Second, we performed transient gain and loss of function experiments that further confirmed that ABCC11 expression depends on CDX2 levels: the transfection of a CDX2-expressing plasmid led to increased levels of ABCC11 in HCT116, SW480 and HEK293 cell lines (Fig. 4B,



**Fig. 3.** Impact of CDX2 on genes involved in chemoresistance. (A) RT-qPCR detection of the indicated ABC transporter in HT29/CDX2 cells cultured in the presence or absence of CDX2. (B) RT-qPCR analyses of genes implicated in the 5-FU metabolism, apoptosis or the MMR DNA repair mechanism in HT29/CDX2 cells upon CDX2 expression. Data (means ± SD) of a representative experiment are shown and statistics were calculated on 3 experiments \* *p*-value < 0.05, ns not significant.

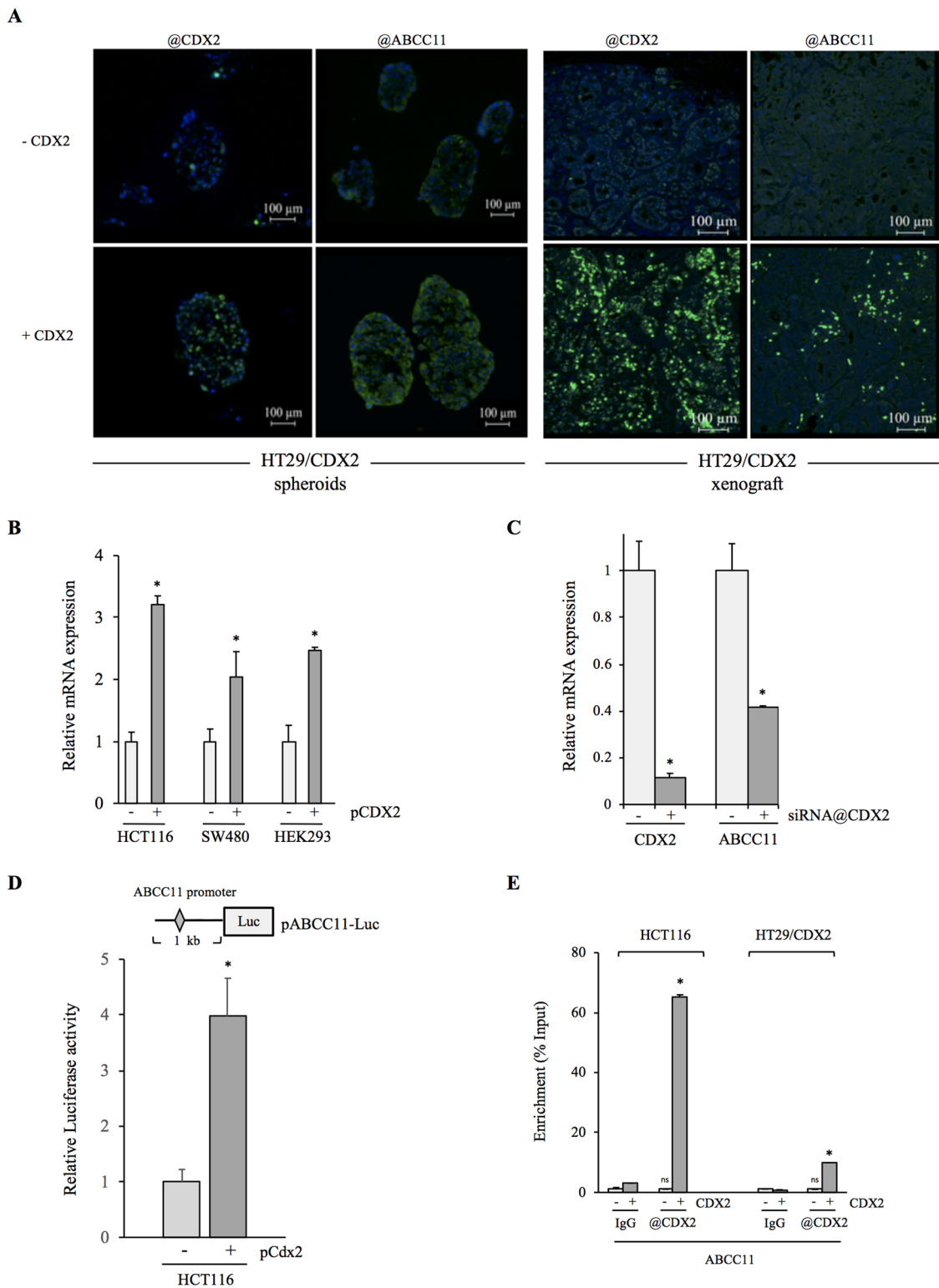
Supplementary Fig. S1E); on the contrary, silencing CDX2 with siRNA significantly decreased ABCC11 expression in SW480 colon cancer cells (Fig. 4C, Supplementary Fig. S1F).

Finally, we investigated if ABCC11 is a direct transcriptional target of CDX2. Manual examination of the genomic sequence in the vicinity of the ABCC11 locus revealed the presence of several consensus binding sites for CDX2 (A/CTTTATATA/G, [14]). Luciferase reporter assays using 1 kb of the proximal promoter region of ABCC11 indicated that CDX2 stimulates the transcription of ABCC11 (Fig. 4D). Furthermore, Chromatin immunoprecipitation (ChIP) experiments were performed in two experimental set-ups to verify the binding of CDX2 to the ABCC11 promoter. As shown in Fig. 4E, a DNA fragment from the ABCC11 promoter region overlapping the consensus CDX2 DNA-binding site was specifically enriched in CDX2-immunoprecipitated chromatin in colon cancer HCT116 cells expressing CDX2. Similar results were obtained in HT29/CDX2 cells (Fig. 4E) and with already known CDX2 target genes (Supplementary Fig. S5E).

Altogether, these data strongly indicate that CDX2 regulates the transcription of ABCC11 by direct interaction with its promoter.

### 3.5. CDX2 and ABCC11 expressions correlate in human healthy tissues and CRC

Given that ABCC11 has been poorly studied so far, we collected samples from human healthy digestive tissues and analyzed them by RT-



**Fig. 4.** Direct regulation of ABCC11 by CDX2. (A) Immunofluorescence detection of CDX2 and ABCC11 on sections of HT29/CDX2-based spheroids (left) or in HT29/CDX2-grafted subcutaneous tumors of nude mice (right) grown in the presence and absence of CDX2. @ antibody. Scale bar, 100  $\mu$ m. (B) Evaluation of ABCC11 expression by RT-qPCR in three cell lines transiently transfected with a CDX2-encoding plasmid (pCDX2: +) or control plasmid (-). (C) Evaluation of ABCC11 and CDX2 expression by RT-qPCR in SW480 cells transiently transfected with control (-) or CDX2-silencing siRNA (+). (D) Up: Schematic representation of the Luciferase reporter plasmid containing the proximal ABCC11 promoter with one consensus CDX2-binding site (A/CTTTATATA/G); Down: Luciferase activity of the pABCC11-Luc plasmid after co-transfection with control (-) or pCDX2 (+) plasmids in HCT116 cells. (E) Chromatin immunoprecipitation with control (IgG) or anti-CDX2 (@CDX2) antibodies of either HCT116 cells transfected with pCDX2 (left side) or HT29/CDX2 cells ( $\pm$  CDX2 via doxycycline, right side), followed by PCR amplification of the ABCC11 promoter. p plasmid, kb kilobase, Ig Immunoglobulin, @ antibody, si small interfering RNA. Data (means  $\pm$  SD) of a representative experiment are shown and statistics were calculated on 3 experiments \* *p*-value < 0.05, ns not significant.

qPCR to determine the expression pattern of ABCC11. Although weak, ABCC11 expression was detected all along the digestive tract and peaked in the colon. Of note, the ABCC11 and CDX2 expression profiles along the digestive tract were similar enough to be compatible with a transcriptional regulation of ABCC11 by CDX2 (Supplementary Fig. S6).

Next, we collected samples from a local cohort of patients suffering from CRC and having undergone elective colorectal cancer surgery. We computed clinical data and analyzed the samples by RT-qPCR and immunofluorescence. Two groups of patients with significant different CDX2 expression levels according to RT-qPCR data (Local-CDX2-High group,  $n = 12$  and Local-CDX2-Low group,  $n = 26$ ) were created. Both groups were comparable for baseline clinical and pathological characteristics (Supplementary Table S3), except for MSI status which tended to be more frequent in the Local-CDX2-Low group. The expression of ABCC11 was significantly higher in the Local-CDX2-High group compared to the Local-CDX2-Low group ( $p < 0.05$ ) (Fig. 5A). A similar trend was observed at the protein level using immunofluorescence on tissue sections (Fig. 5B). Despite the unusually high proportion of CDX2-Low tumors in the local cohort, we confirmed the correlation between CDX2 and ABCC11 expressions in CRC samples from the NCBI-GEO meta-cohort of 1237 patients as well as a correlation between CDX2

and DPYD expressions (Fig. 5C).

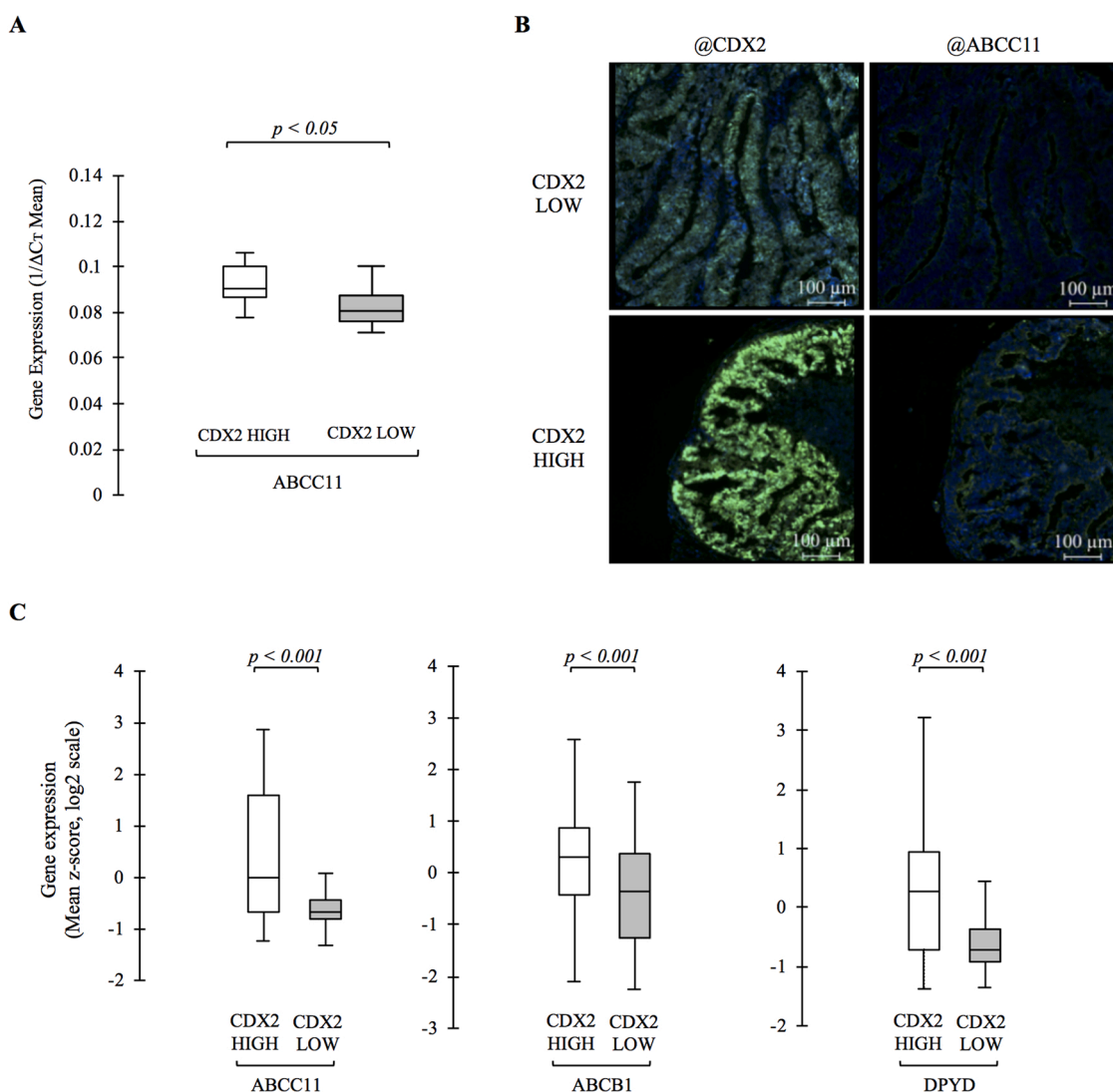
Taken altogether, these data strengthen the proposal of ABCC11 being a new CDX2 target gene in the gut that persists upon CRC.

### 3.6. ABCC11 contributes to CDX2-related chemoresistance

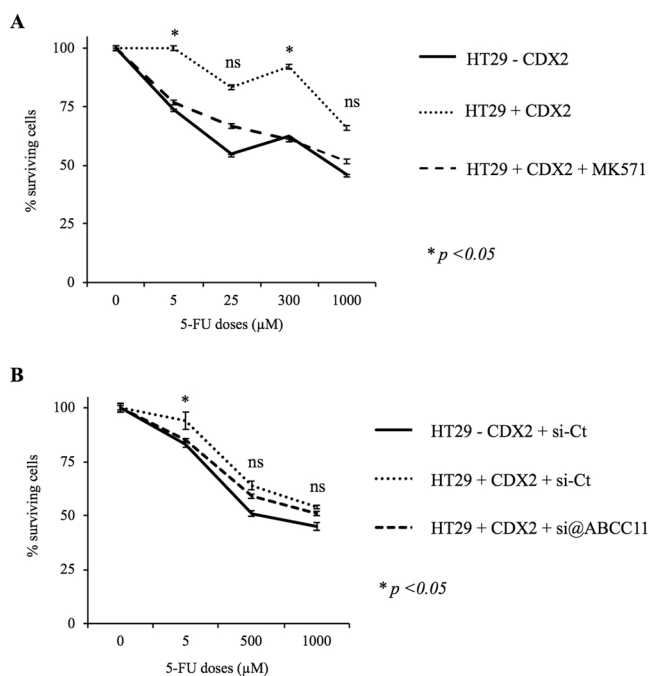
To determine the involvement of ABCC11 in the 5-FU resistance induced by CDX2, we performed survival assays using ABCC11-loss of function approaches.

MTT survival assays were performed on HT29/CDX2-inducible cells treated with 5-FU, in the presence or absence of a pharmacological inhibitor of ABCC11 (MK-571). 5-FU-sensitivity of HT29/CDX2 cells was partially restored upon MK-571 treatment compared to cells only treated with 5-FU (Fig. 6A).

A similar trend was observed upon siRNA silencing of ABCC11 expression in HT29/CDX2 cells, especially with the low doses of 5-FU (Fig. 6B). Thus, enhanced expression of the ABCC11 transporter, secondary to the presence of CDX2, contributes to 5-FU resistance in CRC cells.



**Fig. 5.** Correlation between CDX2 and ABCC11 expressions in human CRC. (A) mRNA expression of ABCC11 in 38 frozen samples of CRC patients according to CDX2 expression level (Local-CDX2-high group,  $n = 12$  and Local-CDX2-low group,  $n = 26$ ). (B) Immunofluorescence detection of CDX2 and ABCC11 proteins in representative CDX2-Low and CDX2-High tumors. (C) Expression of ABCC11, ABCB1 and DPYD in the NCBI-GEO meta-cohort of 1237 patients classified according to CDX2 expression level.  $p$   $p$ -value, @ antibody. Scale bar, 100  $\mu$ m.



**Fig. 6.** Contribution of ABCC11 to CDX2-related chemoresistance. (A) MTT survival assays on HT29/CDX2 cells cultured 24 h in the presence and absence of CDX2 ( $\pm$  doxycycline) and treated during 48 h with ranges of 5-FU and the ABCC11 inhibitor MK-571. Statistics compared the survival of the CDX2-expressing cells treated with the amount of 5-FU, with and without MK571. (B) HT29/CDX2 cells cultured 24 h in the presence or absence of CDX2 ( $\pm$  doxycycline) and transfected with a control (si-Ct) or ABCC11-silencing (si@ABCC11) siRNA before treatment during 48 h with ranges of 5-FU. Data of CDX2-expressing cells (si-Ct vs si-ABCC11) were statistically compared. *p* value, si small interfering RNA.

#### 4. Discussion

Despite the development of more recent targeted- and immunobased therapies, most patients affected with CRC still undergo systemic 5-FU-based chemotherapy but its efficacy is often hampered by toxicity (leading to dose limitation and/or treatment discontinuation) and resistance mechanisms in tumor cells. To overcome these clinical issues, deciphering the molecular determinants involved in the inter-individual variability in drug response is necessary. Here, using a comprehensive translational approach, we provide evidence that the intestinal master gene CDX2 is associated with a reduced efficacy of chemotherapy in CRC and controls the expression of genes involved in the efflux and catabolism of 5-FU metabolites.

The role of CDX2 expression as an independent survival prognostic factor in CRC is exponentially emerging through the publication of several studies based on transcriptomic analyses. There is nowadays no doubt that the loss of CDX2 expression in CRC worsens patients' prognosis and may be a determinant to propose systemic chemotherapy for stage II and III CRC [4,5,15]. The loss of CDX2 correlates with colon cancer cells having a highly immature progenitor-cell phenotype and has been associated with several features of aggressiveness [3,16]. However, this subgroup corresponds to a minority of patients (mean CDX2-Low tumors rate is 16% in Yuan et al. meta-analysis, range 5–50%) [17] and most of CRC tumors express some level of CDX2 [18, 19].

Paradoxically, we found that even if CDX2 expression correlates with better DFS rates, it is associated with lower response rates to systemic chemotherapy in CRC. Similarly, Yuan et al. showed, by pooling 26 retrospective human studies, that a high CDX2 level reflects a favorable 5-year OS for patients with gastric cancer, CRC and other cancer types

such as ovarian and bilio-pancreatic tract cancers [17]. However, CDX2 expression was not associated with the chemotherapy effects in solid malignancies, CRC included. Very recently, Bruun et al. also found that CDX2 expression does not correlate with benefit from systemic chemotherapy in CRC, as well as sensitivity to conventional chemotherapeutics in colon cancer cell lines [6]. On the contrary, a panel of CDX2-negative cell lines was significantly more sensitive to irinotecan and to a weaker extent (i.e. without statistical significance) to 5-FU and oxaliplatin. Using an inducible colon cancer cell model that prevents possible clonal bias and has been validated in vivo [12], we directly provided evidence here that the presence of CDX2 confers resistance towards 5-FU.

At the molecular level, we identified two new genes whose expression in CRC patients and cell lines is up-regulated in the presence of CDX2 and that may specifically contribute to 5-FU chemoresistance. The first gene, ABCC11, encodes a member of the multidrug resistance-associated protein (MRP) family and suggests a mechanism of increased drug efflux. Other genes encoding drug efflux pumps (ABCB1, ABCC7) had been previously identified as transcriptional targets of CDX2 [6]. However, only ABCC11 is able to transport the 5-FU active metabolite [20], which in the cornerstone of actual CRC chemotherapy, and was shown to mediate 5-FU chemoresistance in lung [21] and breast [22] cancer. The role of ABCC11 in CRC has poorly been studied so far but Yabuuchi et al. reported that ABCC11 expression is increased in colon adenocarcinoma [23]. Using gain/loss of function, ChIP and reporter gene assays, we uncovered that ABCC11 is a direct transcriptional target of CDX2. Furthermore, we showed that inhibition of ABCC11 activity clearly reduces the 5-FU resistance conferred by CDX2 expression in colon cancer cells. However, as we were not able to correlate ABCC11 expression and response to chemotherapy in our meta-cohort of CRC patients (data not shown), it is likely that other CDX2 target genes are involved in this phenomenon.

The second mechanism potentially implicated in the 5-FU chemoresistance associated with CDX2 was an overexpression of the DPYD gene, coding for the dihydropyrimidine deshydrogenase (DPD), which is the rate-limiting enzyme in the 5-FU catabolism. The mechanism of DPYD regulation by CDX2 is unknown and should be investigated. However, one may postulate a direct transcriptional regulation (as new target gene) and/or an indirect regulation through REG IV as proposed by Hu et al. [24]. Over 30 genetic polymorphisms have been described for the DPYD gene and the DPD activity is highly heterogeneous among individuals. Interestingly, the reduction or loss (i.e. 0.3% of the population) of DPD activity has been widely correlated with 5-FU cytotoxicity [25,26]. Even if DPD is mostly active in the liver, positive expression has been identified in colon cancer cells [27] and in 47.5% of CRC [28]. In addition, several studies indicate that DPD is a negative prognostic factor for survival and efficacy of 5-FU based regimens [29–31]. Thus, the enhanced DPD activity upon CDX2 expression in colon cancer cells may contribute to their 5-FU resistance and should be demonstrated in future studies.

On a more conceptual level, it seems counterintuitive that CDX2, a gene antagonizing tumorigenesis, would be implicated in chemoresistance. One may argue that CDX2 has dual functions and acts either as a tumor suppressor gene or as an oncogene [32]. Indeed, we and others have clearly established that CDX2 suppresses intestinal tumorigenesis [12,33,34] but CDX2 acts as an oncogene in several others malignancies (esophagus, gastric, ovarian and leukemia) [3,17,35]. This versatility is still not clearly understood and might depend on the molecular context of the tumor cell and its microenvironment [36]. One may also hypothesize that the biological function of CDX2 is hijacked by colon cancer cells. Indeed, CDX2 is driving differentiation and stimulates the expression of multiples genes involved in the physiological function of mature intestinal cells. As a barrier facing the content of the lumen, the colon epithelium plays a protective role, namely against xenobiotics. Therefore, stimulation of genes encoding ABC transporters (i.e. ABCB1, ABCC7 and ABCC11) by CDX2 may reflect its role in intestinal differentiation [7,37]. Similarly, as differentiated cells do not



proliferate, pyrimidine bases may no longer be required for DNA synthesis and need to be catabolized through increased DPD expression.

From a clinical point of view, our data suggest several new avenues of research. First, based on our results and the ones from other recent studies, CDX2 may be a useful marker for predicting drug resistance in CRC and guide therapeutic regimen toward the best drug's sensitivity, avoiding side effects of potentially inefficient chemotherapy. Of note, CDX2 expression profile seems concordant between primary CRC and corresponding liver metastases suggesting the same chemosensitivity between both sites [18,38]. Furthermore, pre-therapeutic screening of DPD activity, using liquid chromatography measures of the dihydrouracil/uracil ratio (UH<sub>2</sub>/U) in plasma [39], will provide valuable information concerning both toxicity and efficacy of a 5-FU based treatment, and allow to adjust the dosage for each patient. Of note, different pharmacological DPD inhibitors such as gimeracil and eniluracil have been added to oral 5-FU formulations to improve 5-FU bioavailability [40–42] and evaluation of individual DPD activity will be informative for the use of these inhibitors. Finally, even if ABC inhibitors have proven inefficient so far [43,44], targeting ABC transporters may still be rational to bypass this type of chemoresistance mechanism. For instance, ABCC11 inhibitors may be developed and evaluated in combination with 5-FU based regimens in randomized clinical trials.

Our study may present some limitations. Since the high number of missing clinical data in the NCBI-GEO meta-cohort, the results should be interpreted with cautiousness. OS data were not usable and the impact of CDX2 on OS is still uncertain. The protocol, timing and efficacy of chemotherapy were also not always specified precluding direct comparison. Furthermore, the contribution of ABCC11 and DPD in 5-FU chemoresistance of colon cancer cells needs to be further investigated. However, through this solid translational approach, we reported convincing results that CDX2 is implicated in the chemoresistance of CRC and identified molecular relays of CDX2 whose overexpression may contribute to 5-FU chemoresistance in CRC. Given the exploratory and retrospective design of our study, these results will need to be further validated.

#### CRediT authorship contribution statement

**Jean-Baptiste Delhorme:** Formal analysis, Investigation, Methodology, Writing – original draft, Visualization, Validation. **Emilie Bersuder:** Investigation. **Chloé Terziolo:** Investigation. **Ourania Vlami:** Investigation. **Marie-Pierrette Chenard:** Resources. **Elisabeth Martin:** Investigation, Resources. **Serge Rohr:** Project administration, Writing – review & editing. **Cécile Brigand:** Project administration, Writing – review & editing. **Isabelle Duluc:** Project administration, Investigation. **Jean-Noël Freund:** Project administration, Writing – review & editing. **Isabelle Gross:** Validation, Project administration, Writing – review & editing, Funding acquisition, Supervision.

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#### Conflict of interest statement

The authors have declared no conflicts of interest.

#### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.biopha.2022.112630](https://doi.org/10.1016/j.biopha.2022.112630).

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## **CDX2 controls genes involved in the metabolism of 5-fluorouracil and is associated with reduced efficacy of chemotherapy in colorectal cancer**

### **Supplementary data**

#### ***1. Supplementary Methods and procedures***

All experiments were at least repeated 3 times. Data shown are representative for one experiment unless specified otherwise.

##### *1.1. Cell lines*

All cell lines were routinely tested for mycoplasma by PCR and were within 20 passages when used for the experiments. Human colon adenocarcinoma Caco-2/TC7 cells [1] were maintained in Dulbecco's Modified Eagle's Medium (DMEM, D. Dutscher) supplemented with heat-inactivated Fetal Bovine Serum (FBS, 20%, Gibco), essential amino-acids (1%, Gibco) and antibiotics. Human colon adenocarcinoma HCT116, SW480 cells (ATCC) and embryonic kidney HEK293 (ATCC) were grown in DMEM supplemented with FBS (10%) and antibiotics. HT29-CDX2 inducible cells (HT29/CDX2, clone TW6) and control cells (HT29/CT, clone TG8) generated from human colon adenocarcinoma HT29-16E/TR cells as described elsewhere [2], are stably modified to express CDX2 and/or GFP upon addition of doxycycline (1µg/ml, Sigma-Aldrich) to the culture medium; they were maintained in DMEM supplemented with FBS (10%), blasticidin (5 µg/ml, Gibco) and zeocin (100 µg/ml, Gibco). Control and CDX2-expressing HCT116 stables cell lines were established at the laboratory upon transfection of HCT116 cells with pCB6 (clone HC1) or pCB6-FLAG<sub>2</sub>-mCDX2 WT (clone HW2); they were maintained in DMEM supplemented with FBS (10%), G-418 (1 mg/ml, Gibco) and antibiotics.

### Cell lines description:

- Human colorectal cancer stable HT29/CDX2 cells: parental HT29 cells do not express endogenous CDX2 and show very poor plasmid transfection efficiency. Hence, they were stably modified in our lab via the TET-ON system to express CDX2 upon addition of doxycycline (Gross et al. 2008). This allows homogenous / long term expression and is ideal to evaluate the impact of CDX2 on chemotherapy. This cell line can also be used to study the impact of CDX2 on direct target genes.
- Human colorectal cancer HCT116 and human embryonic kidney HEK293 cells: these cell lines do not express endogenous CDX2 and have very good transfection efficiency. This allows transient / overexpression and is ideal to study the impact of CDX2 on direct target genes.
- Human colorectal cancer SW480 cells: this cell line expresses some levels of endogenous CDX2 and has good transfection efficiency. This allows both transient overexpression and silencing to study the gain or loss of function of CDX2 on direct target genes.
- Human colorectal cancer Caco-2/TC7 cells: this cell line expresses CDX2, especially upon differentiation, but has low transfection rates.

### *1.2. Colonosphere formation*

For colonospheres, HT29/CDX2 cells were seeded at 1000 cells/well in 6-well Ultra Low Attachment plates (Corning) and grown as 3D-spheroids during at least 15 days in DMEM/F12 medium (Invitrogen) supplemented with B-27 supplement (2%, Gibco), Epidermal Growth Factor (20 ng/ml, Sigma-Aldrich), Fibroblast Growth Factor (10 ng/ml, Peprotech), L-glutamine (1%, Gibco) and antibiotics in the absence or presence of doxycycline (1µg/ml).

### *1.3. Cytotoxicity assays*

HT29/CDX2 cells and HT29/CT cells were seeded at 10000 cells/well in 96-well plates on day 1 and addition or not of doxycycline to the culture medium occurred on day 2. Treatment with ranges of doses of 5-FU (Sigma-Aldrich), oxaliplatin (Teva Santé) and irinotecan (Sigma-Aldrich) or vehicle was started on day 3 and amounts of live cells were evaluated 48 h later by performing MTT assays (Sigma-Aldrich). ABCC11 activity was reduced by either adding MK571 (50  $\mu$ M, Sigma-Aldrich) to the culture medium or transfecting the cells with a pool of siRNA at day 2 (see transfection procedure below).

Caco2/TC7 cells were seeded at  $5 \cdot 10^6$  cells/well in 6-well plates and grown either 1 day (weak expression of CDX2) or 11 days (high expression of CDX2) before treatment with IC<sub>50</sub> doses of 5-FU, oxaliplatin, irinotecan alone or combinations. Cells were harvested 72h after treatment, and manually counted using a hemacytometer and trypan blue staining.

### *1.4. Plasmids and small interfering RNA transfections*

HCT116, HEK293 and SW480 cells were seeded at day 1 with 500000 cells/well in 6-well plates. Cells were transfected at day 2 with 1  $\mu$ g of pFLAG-hCDX2[3] using the JetPrime transfection reagent (Polyplus Transfection) according to the recommendations of the supplier. After 48h of transfection, cells were harvested and analyzed by RT-qPCR (see procedures below).

All siRNA duplexes were transfected with Lipofectamine RNAiMAX (Invitrogen).

For siRNA@CDX2 experiments, SW480 cells were seeded at day 1 with 500000 cells/well in 6-well plates and transfected at day 2 with 1 nM of stealth siRNA duplex oligoribonucleotides CDX2HSS141546 (siRNA@CDX2 546, Invitrogen) or low GC Duplex #2 as negative control (Invitrogen). After 48h of transfection, cells were harvested and analyzed by RT-qPCR (see procedures below).

For siRNA@ABCC11 experiments, CDX2-expressing HT-29/CDX2 cells were seeded at day 1 with 10000 cells/well in 96-well plates. On day 2, cells were transfected with 10 nM of ABCC11 (ON-TARGETplus ABCC11 siRNA, Dharmacon) or control siRNA (ON-TARGETplus Non-targeting Pool, Dharmacon) during 24h and CDX2 was induced by adding doxycycline to the culture medium. Cells were treated with ranges of 5-FU doses at day 3 during 48h and MTT assays were performed at day 5.

### *1.5. Luciferase reporter gene assays*

To obtain the pABCC11-Luc reporter plasmid, a 1kb DNA fragment was generated by PCR using HT29 cells genomic DNA, the PCRx Enhancer System (Invitrogen) and ABCC11-promoter specific forward (F) and reverse (R) primers (F : TTT CTC TAT CGA TAG GTA CCT GCA TGC CAT CAT ACC CAG CCT CA; R: GAT CGCAGA TCT CGA TGT CGA TGC CAC GAT TCA CGA). The PCR product was inserted into KpnI and XhoI of pGL3-basic (Promega) with the « In Fusion HD » cloning kit (Clontech). Each new construct was checked by sequencing (GATC Biotech).

HCT116 cells ( $0.15 \times 10^6$  in 24-well plates) were co-transfected with 700 ng of pABCC11-Luc, 400 ng of pFLAG-hCDX2 [3] and 35 ng of pRL-null (Promega) using JetPEI (Polyplus Transfection). Luciferase activity was performed with the Dual-Luciferase Reporter Assay (Promega) as described[2]. Data presented correspond to a representative experiment performed in triplicate.

### *1.6. Chromatin immunoprecipitation (ChIP)*

ChIP experiments were performed as previously described[3] using either transfected HCT116 cells (pFlag-CMV2 vs pFlag-hCDX2 plasmid) or HT29-CDX2 inducible cells (+/- doxycycline). After 48h, transfected cells were fixed with 1% (v/v) formaldehyde for 10 min at

room temperature and quenched with 0.125 M Glycine for 5 min. ChIP experiments were carried out using the Magna ChIP™ G Chromatin Immunoprecipitation kit as advised by the supplier (Millipore). Sheared crosslinked chromatin from ~ 10<sup>6</sup> cells was incubated overnight at 4°C with 1 µg of normal mouse IgG (Santa Cruz Biotech), or anti-CDX2 (clone 88, Biogenex) antibody. Inputs were used as references and correspond to non-immunoprecipitated sheared crosslinked chromatin from ~ 10<sup>5</sup> cells (1%). qPCR analyses were performed with Power SYBR Green PCR Master Mix (Applied Biosystems) as recommended, using 1/100<sup>th</sup> of purified DNA as template and primers located in the promoter region of either *ABCC11* (F: CAT TCT TCA TTT ATT GAG CAC CTG; R: TGC TTT CCT CGC TGT TGA AT, Invitrogen), *CDH17* (F: TGT CTT TCC TCA TGT CTT CTG AGT; R: GCG CCA TGT CTG AGC AAT, Invitrogen) or *SI* (F: GGC TGG TAA GGG TGC AAT AA ; R : GCC TGT TCT CTT TGC TAT GTT G, Invitrogen). Enrichments relative to inputs were calculated according to the formula % input =  $2^{(Ct_{input} - 6.64 - Ct_{ChIP})} \times 100$ .

### 1.7. RNA extraction and RT-qPCR

Total RNA was extracted using TRI Reagent (Molecular Research Center). For tissues, the Precellys24 homogenizer (Bertin Technologies) was used according to the manufacturer protocol before extraction. cDNA synthesis (RT) was performed with 2 µg of RNA with either the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, cell samples) or the Cloned AMV Reverse Transcriptase kit (Invitrogen, tissue samples) according to the suppliers' recommendations. Quantitative PCR (qPCR) reactions were performed in technical duplicates with diluted cDNAs (5x), FastStart universal Probe Master-Rox (Roche) and gene-specific TaqMan probe and primers sets (TaqMan Gene Expression Assays, Applied Biosystems, supplementary Table S4) on a 7500 real-time PCR System (Applied Biosystems). Analyses of the results were performed with the 7500 software v2.0.1 (Applied Biosystems) using a  $\Delta Ct$  or

a relative  $\Delta\Delta\text{Ct}$  quantification method. B2M (tissue samples) or TBP (cell samples) were used as reference genes.

### *1.8. Immunofluorescence*

Immunofluorescence was performed on dried and fixed (4% PFA, 15 min) frozen sections. After antigen-retrieval (10 min in 10mM Sodium citrate pH6), sections were blocked (5% NGS-0.1% Triton-PBS, 1h at room temperature), incubated overnight at 4°C with diluted primary antibodies (1/250<sup>e</sup> anti-ABCC11 H-215 or S-19 from Santa Cruz Biotech and 1/5000<sup>e</sup> anti-CDX2 from Biogenex), washed 3 times for 5 min with 0.1% Triton-PBS, incubated 1h at room temperature with diluted secondary antibodies (1/500<sup>e</sup> Alexa Fluor 488 goat anti-mouse IgG, Invitrogen), stained for DAPI (1/20000<sup>e</sup> for 15 min at room temperature, Sigma-Aldrich) and mounted with FluorSave Reagent (Millipore). Imaging was based on optical sectioning using the ApoTome system (Zeiss) and the ImageJ software.

### *1.9. Western Blot*

Cells were rinsed with cold PBS, scraped in NP-40 lysis buffer (50 mM Tris pH 8, 150 mM NaCl, 2 % NP-40) containing Complete<sup>TM</sup> protease inhibitors (Roche) and extracts were precleared by centrifugation after 10 min. Protein concentrations were measured using Bio-Rad Protein Assay. 20  $\mu\text{g}$  of proteins were separated by SDS-PAGE and transferred to 0.2  $\mu\text{m}$  Hybond P PVDF membranes (Amersham). Membranes were saturated at room temperature for 45 min in PBS containing 1 % BSA and incubated at 4 °C overnight with one of the following primary mouse monoclonal antibodies: CDX2 (Biogenex; 1/5000) or Actin (clone C4, Chemicon; 1/15000). For the detection, peroxidase-conjugated sheep anti-mouse IgG antiserum (Amersham; 1/5000) were used at room temperature for 45 min. Finally, the membranes were



developed using ECL (Amersham). ReBlot Plus Strong Antibody Stripping Solution (Chemicon) was used as recommended to strip the membranes.

## 2. References

- [1] I. Chantret, A. Rodolosse, A. Barbat, E. Dussaulx, E. Brot-Laroche, A. Zweibaum, M. Rousset, Differential expression of sucrase-isomaltase in clones isolated from early and late passages of the cell line Caco-2: evidence for glucose-dependent negative regulation, *J. Cell. Sci.* 107 ( Pt 1) (1994) 213–225.
- [2] I. Gross, I. Duluc, T. Benameur, A. Calon, E. Martin, T. Brabletz, M. Kedinger, C. Domon-Dell, J.-N. Freund, The intestine-specific homeobox gene *Cdx2* decreases mobility and antagonizes dissemination of colon cancer cells, *Oncogene*. 27 (2008) 107–115. <https://doi.org/10.1038/sj.onc.1210601>.
- [3] I. Hinkel, I. Duluc, E. Martin, D. Guenot, J.-N. Freund, I. Gross, *Cdx2* controls expression of the protocadherin *Mucdhl*, an inhibitor of growth and  $\beta$ -catenin activity in colon cancer cells, *Gastroenterology*. 142 (2012) 875-885.e3. <https://doi.org/10.1053/j.gastro.2011.12.037>.

**Supplementary Table S1. Available clinical data in the NCBI-GEO meta-cohort**

	Total n=1237	Meta-CDX2 Low n=440	Meta- CDX2 High n=797	<i>p-value</i>
<u>Gender</u>				<i>0.69</i>
Male	497 (53)	214 (54)	283 (53)	
Female	433 (47)	181 (46)	252 (47)	
unknown	307	45	262	
Mean Age (years)	65.8 (22-97)	66.6 (22-93)	65.1 (29-96)	<i>0.21</i>
<u>Side</u>				<i>&lt;0.001</i>
Proximal	317 (41)	189 (53)	128 (31)	
Distal	451 (59)	167 (47)	284 (69)	
Unknown	469	84	385	
<u>UICC Tumor stage</u>				<i>0.006</i>
0	1	0	1	
1	87 (9)	25 (6)	66 (11)	
2	383 (39)	158 (40)	225 (38)	
3	324 (33)	146 (37)	176 (29.5)	
4	193 (20)	66 (17)	127 (21.5)	
unknown	251	49	202	
<u>Differentiation</u>				<i>0.001</i>
Well	18 (8)	2 (13)	15 (8)	
Moderate	166 (78)	12 (52)	154 (81)	
Poor	30 (14)	9 (35)	21 (11)	
unknown	1024	417	607	
<u>Molecular features</u>				
<u>KRAS mutation</u>				<i>0.04</i>
Yes	276 (41)	159 (44)	117 (37)	
No	402 (59)	199 (56)	202 (63)	
Unknown	560	82	478	
<u>BRAF mutation</u>				<i>0.001</i>
Yes	65 (10)	59 (18)	6 (2)	
No	579 (90)	277 (82)	302 (98)	
Unknown	593	104	489	
<u>Phenotype</u>				<i>0.001</i>
MSS	554 (85)	267 (79)	287 (92)	
MSI	96 (15)	70 (21)	26 (8)	
Unknown	587	103	484	

Results are presented as numbers (unless specified) and proportion are into brackets.

UICC: Union for International Cancer Control, MSS : Microsatellite Stable, MSI : microsatellite instability

**Supplementary Table S2. List of the datasets included into the NCBI-GEO meta-cohort**

NCBI-GEO dataset	N	Pubmed ID	Reference
GSE35896	62	PMID: 23272949	Schlincker et al., BMC Med. Genomics, 2012
GSE17536	177	PMID:19914252	Smith et al., Gastroenterology, 2010
GSE17537	55	PMID:19914252	Smith et al., Gastroenterology, 2010
GSE39582	566	PMID: 23700391	Marisa et al., PLoS Med., 2013
GSE71222	152	PMID: 26499327	Takahashi et al., BMC cancer, 2015
GSE39084	70	PMID: 25083765	Kirzin et al., PLoS One, 2014
GSE54483	11	PMID: 25375918	Martinez-Garcia et al., Nat Med., 2014
GSE5275	37	PMID: 25730906	Estevez-Garcia, Oncotarget, 2015
GSE62080	21	PMID: 17327601	Del Rio, J Clin Oncol., 2007
GSE3232	17	PMID: 22399497	Khamas et al., Cancer Genomics Proteomics, 2012
GSE62932	64	PMID: 27176004	Chen et al. PloS One 2016
GSE23194	5	No ID	No reference

**Supplementary Table S3. Clinical and pathological characteristics of patients in the local cohort**

	Local-CDX2 Low n=12	Local-CDX2 High n=26	<i>p-value</i>
<u>Gender</u>			<i>0.63</i>
Male	7 (58)	13 (50)	
Female	5 (42)	13 (50)	
Mean Age (years)	69.6 (55-81)	63.5 (32-91)	<i>0.10</i>
<u>WHO performance status</u>			<i>1</i>
0-1	11 (92)	23 (88)	
≥ 2	1 (8)	2 (8)	
Unknown	0	1	
Mean BMI (kg/m <sup>2</sup> )	27.1	29.9	<i>0.25</i>
<u>Site</u>			<i>0.25</i>
Right	5 (42)	16 (62)	
Left	7 (58)	10 (31)	
<u>Tumor stage (T)</u>			<i>0.74</i>
T1	0	0	
T2	2 (17)	3 (12)	
T3	9 (75)	18 (69)	
T4	1 (8)	5 (19)	
<u>Node stage (N)</u>			<i>0.11</i>
N0	9 (75)	11	
N1	2 (17)	5	
N2	1 (8)	10	
<u>Synchronous metastasis</u>			<i>1</i>
Liver	2	3	
Peritoneum	2	3	
<u>Differentiation</u>			<i>0.73</i>
Well	1 (8)	4 (15)	
Moderate	8 (67)	12 (46)	
Poor	0	1	
Mucinous	2 (17)	8 (31)	
unknown	1	1	
<u>Phenotype</u>			<i>0.07</i>
MSS	9 (75)	11 (42)	
MSI	2 (16)	11 (42)	
Unknown	1	4	

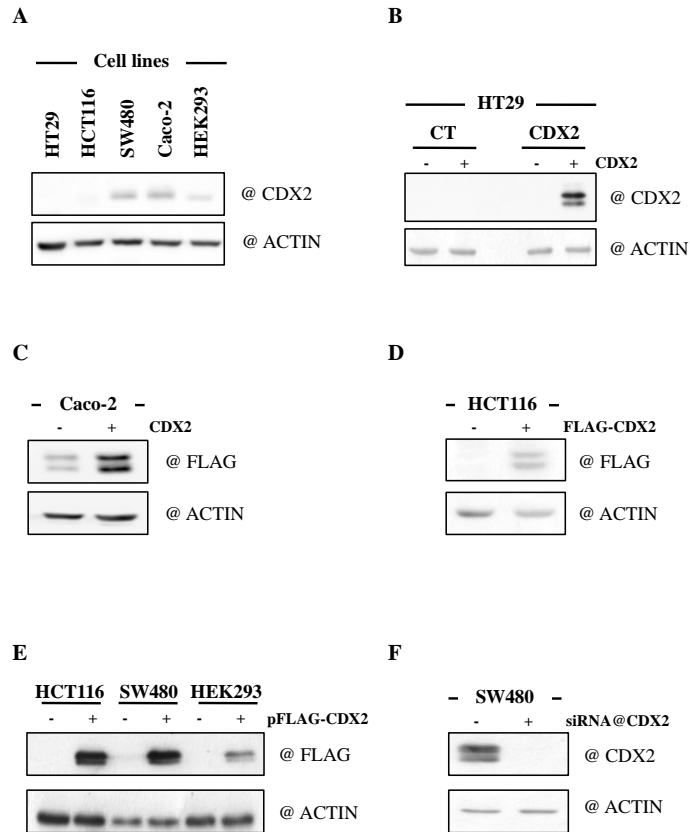
*Results are presented as numbers (unless specified) and proportion are into brackets.*

*BMI: Body mass index, WHO: World health organization, MSS : Microsatellite Stable, MSI : microsatellite instability*

**Supplementary Table S4. List of the Taqman probes used for RT-qPCR experiments.**

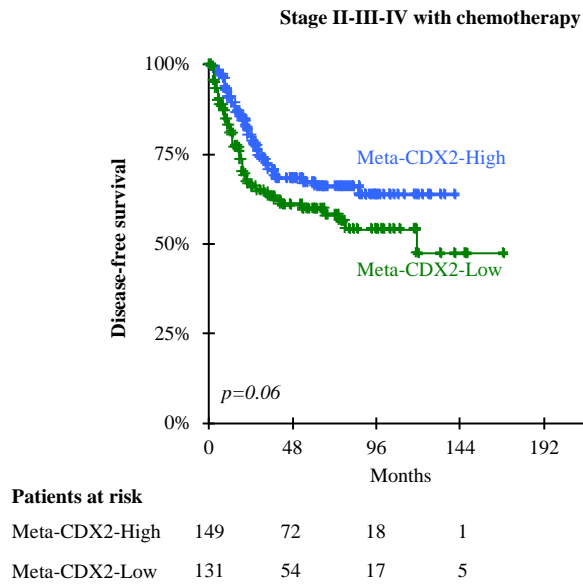
<b>Gene</b>	<b>Catalogue number</b>
ABCB1	Hs00184500_m1
ABCC1	Hs00219905_m1
ABCC4	Hs00988717_m1
ABCC5	Hs00981087_m1
ABCC10	Hs00375701_m1
ABCC11	Hs01090768_m1
ABCG2	Hs01053790_m1
CDX2 (Caudal type homeobox 2)	Hs00230919_m1
CDX2 (Caudal type homeobox 2)	Mm00432449_m1
BCL2	Hs00608023_m1
B2M	Hs00187842_m1
TBP (TATA Binding Protein)	Hs00427620_m1
UMPS	Hs00923517_m1
RAD18	Hs00892551_m1
BAX	Hs00180269_m1
MTHFR	Hs01114487_m1
MSH2	Hs00953527_m1
DPYD	Hs01115750_m1
TYMS	Hs00426586_m1
TYMP	Hs00157317_m1
SI (Sucrase-Isomaltase)	Hs00356112_m1
CDH17 (LI-Cadherin)	Hs00184865_m1

**SUPPLEMENTARY FIGURE 1**



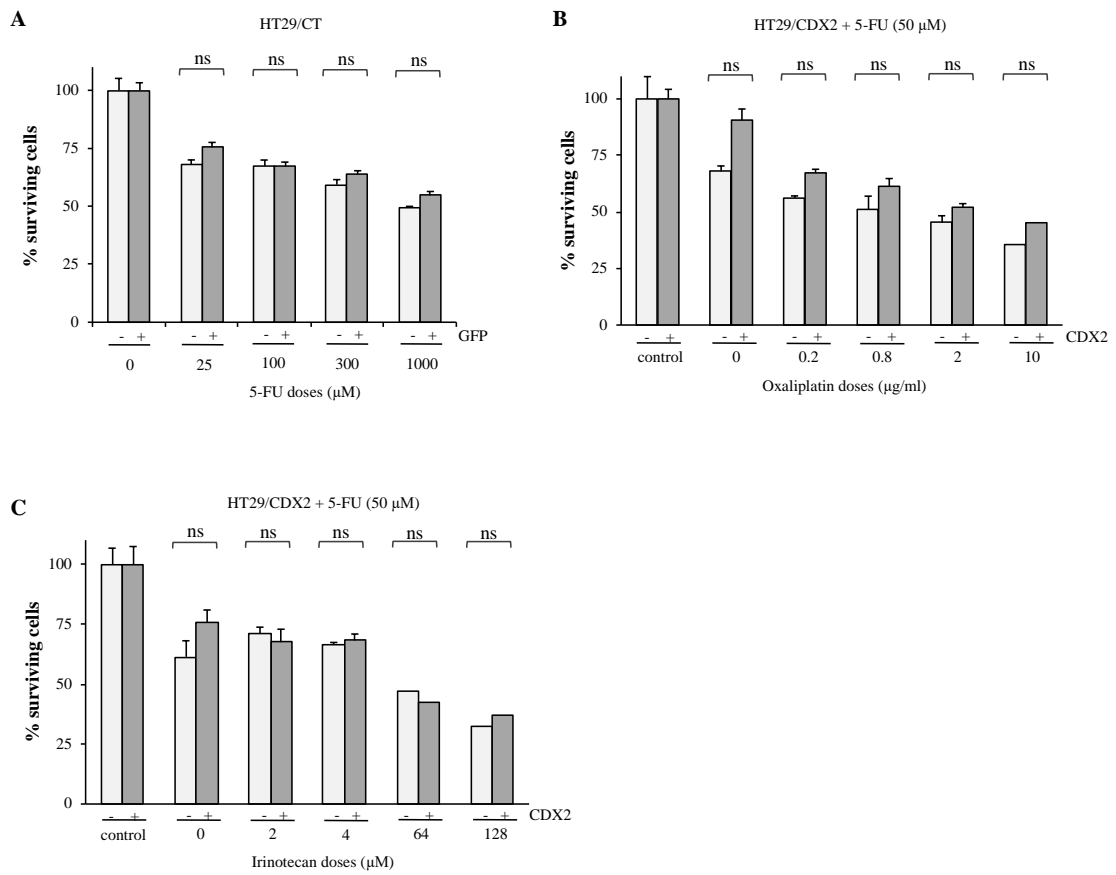
**Supplementary Figure S1.** (A) Western blot detection of CDX2 protein in the different cell lines used throughout the study. Actin was used as lane control. (B), (C) and (D) same as (A) for HT29/CT or HT29/CDX2 stable cell lines ( $\pm$  doxycycline), Caco-2/TC7 cells ( $\pm$  differentiated), HCT116/CT or HCT116/CDX2 stable cell lines, respectively. (E) Western blot detection of ectopically expressed CDX2 in HCT116, SW480 or HEK293 cells transiently transfected with a plasmid encoding FLAG-tagged CDX2 (+). (F) Western blot detection of endogenous CDX2 protein in SW480 cells transiently transfected with control (-) or @CDX2 (+) siRNA.

## SUPPLEMENTARY FIGURE 2



**Supplementary Figure S2.** Disease-free survival curves comparing patients with stage II, III and IV CRC in the NCBI-GEO meta-cohort for whom the administration of a systemic chemotherapy was provided, according to the expression level of CDX2 (Meta-CDX2-High vs meta-CDX2 Low groups). *p-value* indicates result of the log-rank test.

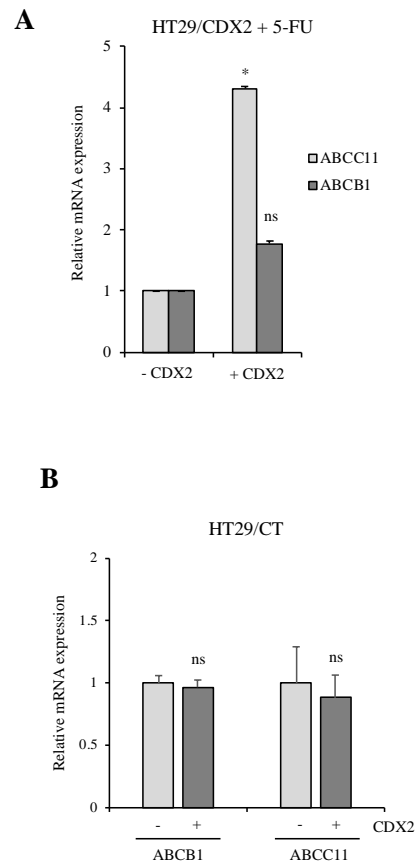
**SUPPLEMENTARY FIGURE 3**



**Supplementary Figure S3.** Survival assays of (A) HT29/CT control cells cultured 24h ( $\pm$  doxycycline) and treated 48h with ranges of 5-FU doses, (B) HT29/CDX2 cells cultured 24h in the presence or absence of CDX2 ( $\pm$  doxycycline) and treated during 48h with FOLFOX-type regimen or (C) FOLFIRI-type regimen. \* *p*-value < 0.05, ns not significant.

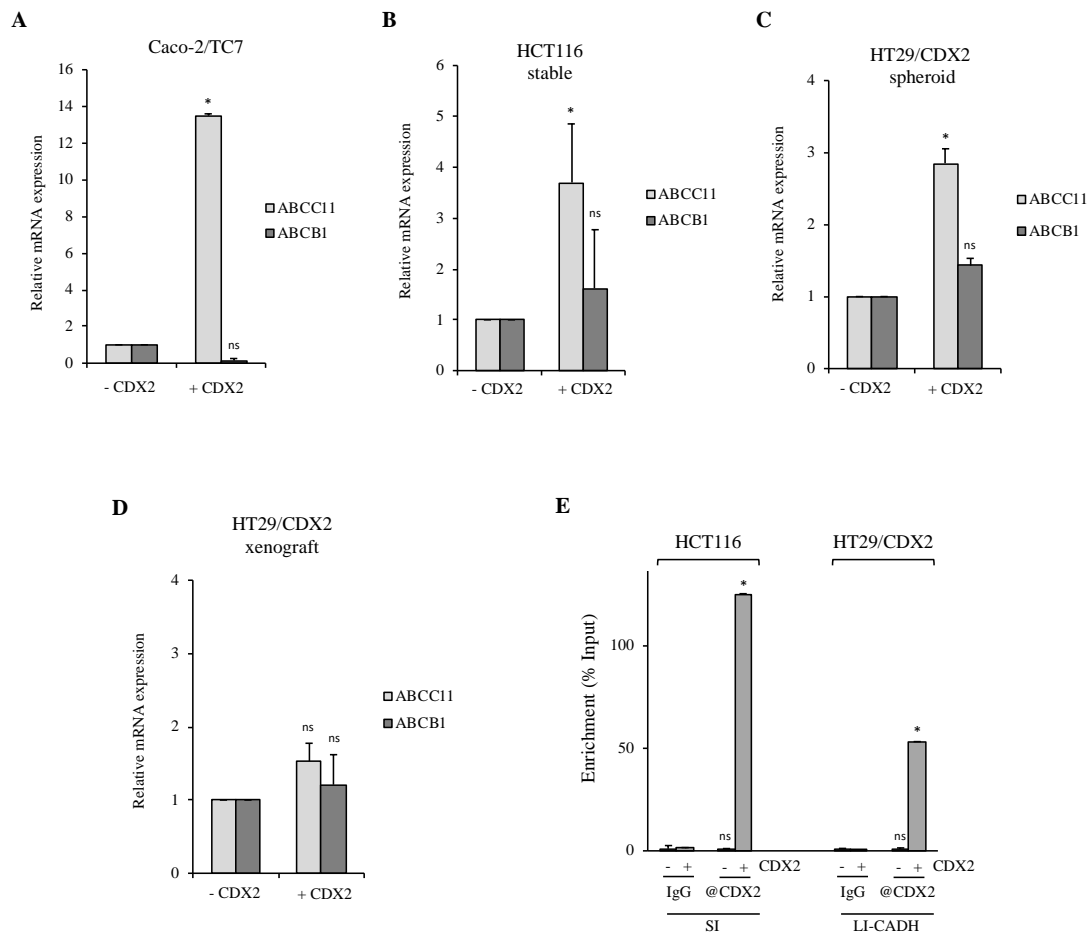


#### SUPPLEMENTARY FIGURE 4



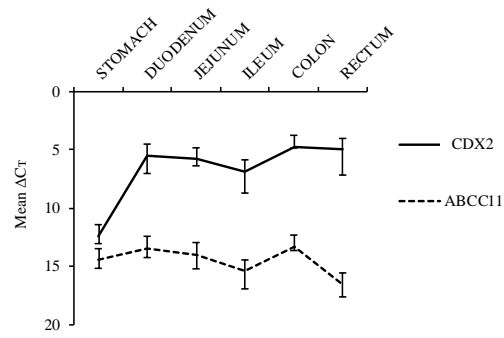
**Supplementary Figure S4.** RT-qPCR detection of ABCB1 and ABCC11 expression in (A) HT29/CDX2 cells ( $\pm$  doxycyclin) after 48h treatment of 5-FU (300  $\mu$ M), (B) HT29/CT cells ( $\pm$  doxycyclin). Data (means  $\pm$  SD) of a representative experiment are shown and statistics were calculated on 3 experiments \* *p*-value < 0.05, ns not significant

**SUPPLEMENTARY FIGURE 5**



**Supplementary Figure S5.** RT-qPCR detection of ABCB1 and ABCC11 expression (A) Caco-2/TC7 cells cultured during 1 (-) and 11 (+) days, (B) control (-) and CDX2-expressing (+) HCT116 stables cell lines, (C) HT29/CDX2 cells-based 3D-grown colonospheres ( $\pm$  doxycyclin), (D) HT29/CDX2 cells-based subcutaneous tumors in nude mice ( $\pm$  doxycyclin). (E) Chromatin immunoprecipitation with control (IgG) or anti-CDX2 (@CDX2) antibodies of either HCT116 cells transfected with pCDX2 (left side) or HT29/CDX2 cells ( $\pm$  CDX2 via doxycyclin, right side), followed by PCR amplification of the SI or LI-CADH promoters as positive controls. Ig Immunoglobulin, @ antibody. Data (means  $\pm$  SD) of a representative experiment are shown and statistics were calculated on 3 experiments \* *p*-value < 0.05, ns not significant

**SUPPLEMENTARY FIGURE 6**



**Supplementary Figure S6. (A)** RT-qPCR expression of CDX2 and ABCC11 in 61 frozen samples from healthy tissues on different organs of the digestive tract (results presented as mean  $\Delta C_t$ ).