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ABSTRACT

Resveratrol belongs to the Bioactive Food Component (BFC) family. It seems admitted that its cytotoxic action impacts tumor cells and spares healthy cells, but the published proofs remain rare. We hypothesized that cells may differentially metabolize resveratrol and lead to different systemic impacts. For this, resveratrol metabolization was evaluated by ultra-high-performance liquid chromatography (UHPLC) coupled with diode array detection (DAD), and correlated with the expression of Uridyl-diphosphate-Glucuronosyl Transferase 1A (*UGT1A*) genes. The expression of *UGT1A* genes in human colorectal tissues was studied with RNAseq databases. Functional validation of UGT1A enzymes implication in resveratrol sensitivity of colorectal cells established by UGT1A expression modulation. As resveratrol impacts the S phase of the cell cycle, nucleotide metabolic balance was assessed. We found that resveratrol was more cytotoxic in cells with downregulation of UGTs, i.e. tumor cells. Conversely, overexpression of the *UGT1A10* gene in an initial resveratrol-sensitive tumor cell line restored the metabolization accompanied by cytotoxicity diminution. Resveratrol affected intestinal sensitive tumor cell homeostasis with a cell growth/proliferation decoupling, cell-cycle modulation, and UXP/AXP nucleotide imbalance resulting in a global reduction of transcription and translation. This impact on global cell activity was restricted to tumor cells. This study improves resveratrol's general knowledge and explains how its antitumor action can spare non-tumor cells. It also paves the way to select colorectal tumors eligible for resveratrol treatment potentiation without additional toxicity to healthy digestive tissues.

1. Introduction

Colorectal cancer (CRC) is the third most frequent cancer in the world, accounting for about 10 % of the total cancer cases (Sung et al., 2021). CRC treatment, combining surgical resection, chemotherapy, and radiotherapy, depending on the tumor stage, shows variable efficiencies and toxicities. Despite the improvement of surgical and therapeutic strategies, numerous patients relapse, with numbers varying from 17 to 25 %, depending on the treatment regimen and the stage (Dekker et al., 2019; Schellenberg et al., 2022). Thus, innovative approaches are still needed to potentiate the chemo and radiotherapy efficiencies in CRC, while keeping low systemic toxicity (Wan et al., 2016; Xiao et al., 2018;

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Habiba et al., 2019). Bioactive food components' antitumor properties seem particularly relevant to CRC (Amintas et al., 2022). According to the American Dietetic Association, BFCs are "physiologically active food compounds, derived from animal or plant sources, including compounds belonging to the food of a basic diet, for which a role has been shown to be healthy and the consumption of which is not harmful to health" (Hasler & Brown, 2009). Preventive and therapeutic benefits of BFCs in cancer have been increasingly reported over the past two decades. Among BFCs, the stilbenoid resveratrol provided promising results in CRC models. On one hand, resveratrol potentiated conventional chemotherapy by increasing Reactive Oxygen Species (ROS) production and promoting cell cycle arrest and apoptosis (Buhrmann et al., 2015; Khaleel et al., 2016; Blanquer-Rosselló et al., 2017). This observation does not belong to CRC only since we and others demonstrated chemotherapy and radiotherapy potentiation in other cancers with or without association with another BFC (C. Lin et al., 2012; Vendrely et al., 2017, 2019; Cocetta et al., 2021; Farghadani & Naidu, 2022). Thus, the anti-cancer effect of resveratrol has been largely described and reviewed (Delmas et al., 2011; Aires & Delmas, 2015; Rauf et al., 2018). On the other hand, numerous studies have described the benefits of resveratrol in physiological processes and non-cancer diseases (Meng et al., 2020).

It is commonly accepted that resveratrol may impact tumor cells while sparing the surrounding healthy tissues. This interesting hypothesis is supported by a few publications that explored tumor cells compared to the corresponding non-tumor cell response to resveratrol. For example, we observed pancreatic tumor-specific toxicity of resveratrol while the pancreatic epithelial non-tumor cells and fibroblasts seemed insensitive (Vendrely et al., 2017), although we did not further describe the molecular basis of the differential response. In the same way, the pro-oxidative cytotoxic action of resveratrol was specific to astrocytoma cancer cells as compared to healthy astrocytes, related to their different glutathione levels and oxidative stress amplitude (Gran et al., 2021). Remarkably, a systematic analysis of more than 20 tumor and non-tumor cells showed a hormetic effect of resveratrol, with beneficial activity at low concentrations turning into adverse impact at high concentrations (Mattson et al., 2010). This biphasic behavior was observed for many other BFCs (Bao et al., 2014; Wätjen et al., 2005) and could contribute to their differential effect on the tumor and non-tumor cells. Multiple downstream targets were reported involved in tumor cell death or healthy cell support, but none of them seemed to be directly regulated by resveratrol, and the first-step mechanisms remain elusive.

Thus, it seems admitted that resveratrol specifically targets tumor cells, but published proofs are actually scarce. To fill up this gap in resveratrol general knowledge, using the CRC model, we investigated how resveratrol antitumor action could be tumor-specific, leading to treatment potentiation without additional toxicity for healthy digestive tissues.

2. Material and methods

2.1. Colorectal cell lines and drugs

HT29, HCT116, SW480, MICC12, and CT26 cells were obtained from the ATCC (Teddington, United Kingdom). NCM460 cells were obtained from Incell (San Antonio, Texas, USA). HT29, HCT116, SW480, and MICC12 cells were maintained in Dulbecco's minimal essential medium (DMEM, Invitrogen, Saint Aubin, France) with 10 % Fetal Calf Serum (FCS, Invitrogen) and 1/100 Penicillin/Streptomycin (Invitrogen). CT26 cells were maintained in Roswell Park Memorial Institute (RPMI, Invitrogen) with 10 % FCS with Penicillin/Streptomycin 1/100 (Invitrogen). NCM460 cells were maintained in M3BaseF medium (Incell) with 10 % FCS and 1/100 Penicillin/Streptomycin. Resveratrol (R), Resveratrol-4′-O-glucuronide (R4′OG) were from Sigma Aldrich.

2.2. Survival assay

Cells were plated at 5.10^3 cells per well in 96-well plates. The day after, different doses of resevratrol or resveratrol-4′-O-glucuronide were applied and cells were kept in culture for 72 h. The medium culture was removed and cells were washed with 1X PBS. Cells were then stained with 100 µL 0.5 % crystal violet and allowed to incubate at room temperature for 15 min. After removing the crystal violet solution, wells were washed three times with 1X PBS to remove unbound dye. Finally, all wells were filled with 100 µL acetic acid (33 %) to release the dye from the cells. The optical density (OD) of stained adherent cells was determined with an absorbance microplate reader at a wavelength of 570 nm. To correct background staining, the OD values of the control wells were averaged and subtracted from the mean OD value obtained for each condition. Results are expressed as cell survival: (OD treated/OD control)*100. Each measure was performed at least in quadruplets. Each experiment was repeated at least three times separately for each condition and the average values were calculated with standard deviation (SD).

2.3. Gene expression analysis

Total RNA was isolated using the Direct-zol® kit (Zymo Research, California) and treated by DNAse according to the manufacturer's instructions. cDNAs were synthesized using a Reverse transcriptase cDNA synthesis kit (Roche Applied Science, Meylan, France). Gene expression levels were determined by reverse transcription/real-time quantitative PCR (qRT-PCR), using the Promega GoTaq® qPCR kit (Promega, Wisconsin) and the comparative MNE (Mean Normalized Expression) method (Lo et al., 1999). The primers used for qRT-PCR analyses are listed in Supplemental Tables 1 and 2. To normalize the qRT-PCR data, we used the primers for the Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. Melting curves showed that primers amplified only the specific fragments.

2.4. Methylation assay

Cells were plated at 15.10^4 cells per well in 12-well plates. The day after, cells were treated with 5-azacytidine (1 mM) for 24 h and with 5-azacytidine (1 mM) + trichostatin A (500 µM) for the next 24 h. Cells were harvested with trypsin 0.25 % and collected in a microtube. RNA extraction and RT-qPCR expression analysis were performed.

2.5. UGT1A expression modulation

UGT1A downregulation was carried out using shRNA technology using the following sequences: shRNA 601 (shRNA *UGT1A10*-1) 5′-CCGGGCAACTGGAAAGATCACTGAA-3′, shRNA 602 (shRNA *UGT1A10*-2) 5′-GCACAGGCAC AAAGTATATTT-3′ and shRNA 603 (shRNA pan-*UGT1A*) 5′-GGATCAATGGTCTCAGAAATT-3′, shRNA scramble (control), cloned in lentivirus expressing puromycin resistance, produced by the Bordeaux University vectorology platform (Vect'UB). Cell lines were transduced at a multiplicity of infection of 5, followed by a 5-days puromycin selection step (1 µM). UGT1A10 overexpression vector was designed using the full coding sequence of the *UGT1A10* gene (Cunningham et al., 2022) cloned in a lentivirus with puromycin resistance gene (Vect'UB). A vector with the same viral backbone and including green fluorescent protein (GFP) and puromycin resistance gene was used as control. Cell lines were transduced with lentivectors at a multiplicity of infection of 10 and puromycin selection.

2.6. Resveratrol metabolites quantification in cell medium

Cells were plated at 15.10^4 cells per well in 12-well plates (2.10^5 cells per well for shRNA modified cells). The day after, cells were treated with resveratrol at 50 µM for 3, 6, 12, and 24 h. At each time point, the

medium was removed, centrifuged for 20 min (20,000 g, 4 °C), and immediately frozen at −80 °C before UHPLC analysis. Quantification of R and resveratrol-glucuronides medium-concentrations were performed by ultra-high-performance liquid chromatography (Agilent 1290 Series) coupled with UV–vis diode array detector (DAD) and mass spectrometer. Two microliters were injected into an Agilent SB-C18 column (1.8 μm, 2.1 × 100 mm). Samples were eluted with solvent A (H_2O 0.1 % formic acid) and solvent B (acetonitrile 0.1 % formic acid) by the following gradient program: 0–1.7 min, 10 % B; 1.7–3.4 min, 10–20 % B; 3.4–5.1 min, 20–30 % B; 5.1–7.8 min, 30 % B; 7.8–8.5 min, 30–35 % B; 8.5–11.9 min, 35–60 % B; 11.9–15.3 min, 60–100 % B; 15.3–17 min, 100 % B; 17–17.3 min, 100–10 % B. The flow rate was set to 0.4 mL/min. The quantifications were carried out by integration of the peaks at the absorbance of 320 nm and using appropriate calibration curves. The cell media alone were also analyzed to eliminate any potential matrix effect.

2.7. Metabolomic assay

Cells were plated at $5x10^5$ cells per well in 6-well plates and were treated the day after with resveratrol at 50 μM for 24 h. Metabolite extraction by ethanol boiling method on subconfluent cells ($<10^5$ cells/cm^2) and metabolites separation by high-pressure ionic chromatography on an ICS 3000 chromatography station (Dionex) were respectively described in (Patel et al., 2014; Ceballos-Picot et al., 2015). Sample normalization was done based on cell number and cell volume both determined with a Multisizer® IV particle counter (Beckman Coulter, CA). AXP and UXP content corresponds to the sum of ATP + ADP + AMP and UTP + UDP + UMP contents, respectively. Adenylate energy charge was defined as AEC = (ATP + ½ ADP)/AXP) (Atkinson & Walton, 1967). AXP, UXP, and AEC were calculated with each nucleotide pure content given in nmol/sample (inferred from standard curves using nucleotides pure compounds). Each experiment was repeated at least three times separately for each condition and the average values were calculated with SD.

2.8. Cell cycle analysis

Non-synchronized subconfluent cells were harvested with trypsin 0.25 % and washed twice with 1X PBS. Cells were fixed with paraformaldehyde (PFA) 3,7% for 15 min and permeabilized with triton 0.5 % for 15 min at room temperature. Cells were washed twice with 1X PBS and incubated with RNAse (100 μg/mL, Sigma-Aldrich) overnight at 37 °C. Cells were washed twice with 1X PBS + 5 % BSA and incubated with propidium iodide (PI) solution (4 μg/mL, Sigma-Aldrich) for 15 min in the dark. The samples were examined on a BD Accuri™ C6 flow cytometer and data were analyzed with BD Accuri™ C6 Plus software (BD Biosciences, Le Pont de Claix, France).

2.9. Transcription rate assay

Cells were plated at 15.10^4 cells per well in 12-well plates. The day after, cells were treated with resveratrol at 50 μM for 24 h. For each condition, cells of 1 well were harvested with trypsin 0.25 % and collected in a microtube. DNA and RNA extractions were performed using respectively QIAamp® DNA isolation kit (Qiagen, Germany) and Direct-zol® RNA isolation kit (Zymo Research, California). DNA and RNA concentrations were measured with Nanodrop® one/one spectrophotometer (ThermoFisher). Transcription quantification was determined using the ratio of RNA concentration to DNA concentration for each condition.

2.10. Translation rate assay

Cells were plated at 1.10^5 cells per well in 6-well plates. The day after, cells were treated with resveratrol at 50 μM for 24, 48, and 72 h. At each time point, 10 μg/mL puromycin was added to the culture medium for 10 min. Immediately after, cells were washed with cold 1X PBS, and frozen at −80 °C in RIPA buffer. Protein extraction was performed using RIPA buffer and protein extracts were processed for western blotting. Membranes were incubated with the anti-puromycin antibody (Clone 12D10, Sigma Aldrich ref MABE343). Rabbit anti-GAPDH antibody (Cell Signaling Technologies, Saint-Quentin-en-Yvelines, France) was used to normalize the loading of the samples. anti-puromycin antibody was detected with specific anti-mouse-IgG-HRP (Cell Signaling Technologies). Proteins were visualized using the ECL detection system (Amersham Pharmacia Biotech, Orsay, France). Quantification by densitometry was performed with the ImageJ software.

2.11. RNAseq databases analysis

RNAseq raw data files from studies GEOD-50760 and GEOD-76987 were obtained with Expression Atlas / EMBL-EBI (https://www.ebi.ac.uk/gxa/home). Raw RNAseq counts for *UGT1A8* and *UGT1A10* genes were normalized using the « median of ratios » normalization method (Anders & Huber, 2010). Log2 fold changes of UGT1A enzyme genes were directly obtained from Expression Atlas / EMBL-EBI analysis.

2.12. Survival analysis

Overall survival Kaplan-Meier curves of COAD (colorectal cancer) and READ (rectal cancer) TCGA databases patients according to the expression of UGT1A1, UGT1A7, UGT1A8, or UGT1A10 enzymes were obtained directly with GEPIA survival analysis (http://gepia.cancer-pku.cn). Group cut-offs used median expression value and high and low cut-offs were equal to 50 %. UGT1A expression correlation analysis in healthy and tumor samples of COAD and READ databases was performed directly using the GEPIA correlation analysis tool.

2.13. Statistical analysis

Statistical tests were performed using the Graph-Pad Prism software (v6.04). Results are expressed as mean ± SD, analyzed by unpaired, bilateral Student's t-tests. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Resveratrol differential toxicity is related to colorectal cell line metabolization capacity

To assess tumor and non-tumor cell line sensitivity to resveratrol, HCT116, HT29, SW480, NCM460, CT26, and MICC12 cells were treated with increasing doses of resveratrol (Fig. 1a). The non-tumor cells NCM460 presented the highest IC_{50} (Inhibitory concentration 50) value (IC_{50} = 125 μM), which was 2.4 and 4.8-fold higher than that of HCT116 (50 μM) and SW480 (25 μM) tumor cells, respectively. HT29 IC50 (IC_{50} = 80 μM) was 1.6 to 3.2-fold higher than that of HCT116 and SW480. Similar results were obtained for the mouse tumor colorectal cell line CT26, with a 5-fold higher sensitivity to resveratrol compared to the non-tumor mouse intestinal cells MICC12 (Supplemental Fig. 1a).

Resveratrol passively crosses the cell plasma membrane to reach the cytosol where it can be metabolized into sulfo- and/or glucuronoconjugates with metabolic enzymes, followed by an efflux of the metabolites in the extracellular compartment (Wenzel & Somoza, 2005; Delmas et al., 2011). To assess resveratrol metabolization in tumor and non-tumor colorectal cells, we performed a time course quantification of resveratrol and its metabolites in the culture medium after resveratrol exposure (Fig. 1b). The resveratrol-sensitive tumor cells HCT116 and SW480 did not present any resveratrol metabolization after 24 h, as no resveratrol glucuroconjugates were detected, and resveratrol medium concentrations remained unchanged. By contrast, the HT29 cells metabolized half of the initial resveratrol into glucuroconjugates after

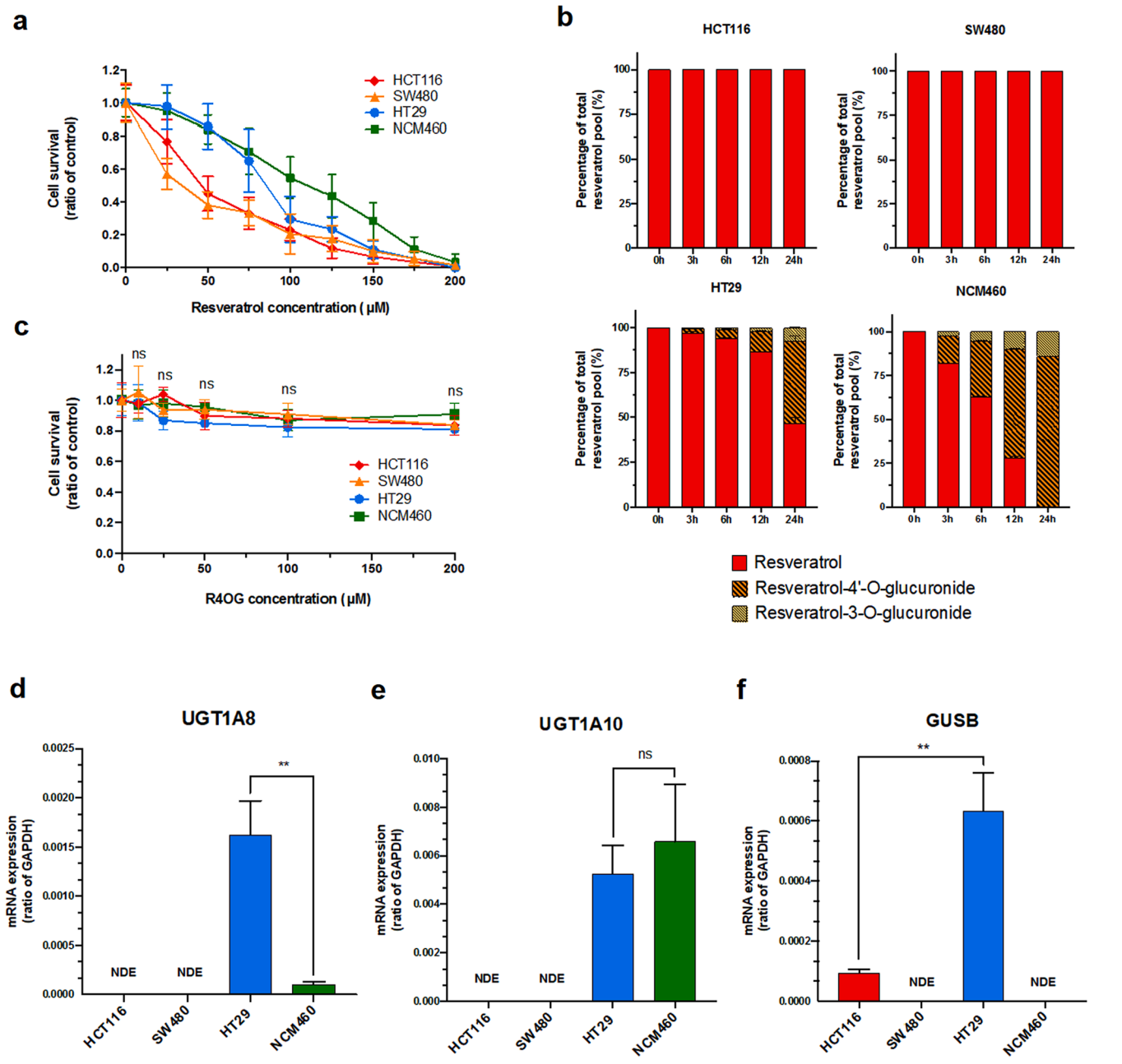

Fig. 1. Human tumor and non-tumor colorectal cell lines resveratrol sensitivity is related to metabolization capacity and expression of UGT1A genes. Dose-response experiments were carried out by treating human colorectal cancer cell lines (HCT116, red diamond, SW480, orange triangle, HT29, blue dots) with increasing doses of resveratrol (a) and resveratrol-4′-O-glucuronide (c). The transformed NCM460 human intestinal cell line (green squares) was used as healthy human non-tumor cells. Resveratrol (red) and its glucuronide derivates resveratrol-4′-O-glucuronide (dashed orange) and resveratrol-3-O-glucuronide (dashed yellow) were quantified in cell culture media after 0, 3, 6, 12 and 24 h incubation with the different cell lines (b). Expression of UGT1A8 (d), UGT1A10 (e) and GUSB (f) transcripts were assessed in the 4 cell lines by RT-qPCR, using GAPDH transcripts as a normalization reference. Results are expressed with mean +/- SD. **: $p < 0.01$; ns: non-significant; NDE: Non-detectable expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

24 h, with a large proportion of the resveratrol-4′-O-glucuronide (R4OG) compared to resveratrol-3-O-glucuronide (R3OG). The non-tumor NCM460 intestinal cells, performed complete metabolization of resveratrol after 24 h, with 85 % R4OG and only 15 % of R3OG. Mouse CT26 tumor cells produced half the amounts of R4OG as compared to the non-tumor MICC12 intestinal cells (Supplemental Fig. 1c).

These results suggest that cell potential to metabolize resveratrol parallels their resistance to resveratrol, so tempted that resveratrol-derived metabolites are non-toxic. To test this hypothesis, we determined whether R4OG was cytotoxic. Dose-response experiments showed no cytotoxic effect for all the tested mouse or human cell lines (Fig. 1c, Supplemental Fig. 1b).

Resveratrol is preferentially metabolized by glucuronidation by the UDP-glucuronosyltransferases (UGTs) 1A (UGT1A) (Wenzel & Somoza, 2005, p.). As the intestinal cell lines displayed distinct capacities to metabolize resveratrol, we determined the mRNA levels of *UGT1A1, 1A6, 1A8, 1A9,* and *1A10* genes. None of the cell lines expressed *UGT1A1, 1A6,* and *1A9* (data not shown). HT29 and NCM460 cells but not HCT116 and SW480 cells exhibited expression of *UGT1A8* and *UGT1A10* mRNAs, (Fig. 1d and 1e). In the same way, the mouse MICC12

cells expressed UGT1A1, 1A6, and 1A10 transcripts while the tumor CT26 cells expressed only the UGT1A6 transcript (Supplemental Fig. 1d). The β-glucuronidase enzyme (encoded by the *GUSB* gene) reverses glucuronidation. HCT116 and HT29 were the only human cells expressing *GUSB* transcripts (Fig. 1f), while both the murine CT26 and MICC12 cells expressed β-glucuronidase enzyme transcripts (Supplemental Fig. 1d).

Taken together, these data suggest that resveratrol resistance was observed in cells armed with UGTs, especially UGT1A8 and UGT1A10, able to resveratrol glucuronidation rather than cells expressing GUSB and able to revert resveratrol metabolization. This is supported by the fact that R4OG was not toxic, even in the GUSB-positive cells.

3.2. The expression of UGT1A genes is downregulated in human colorectal tumors

We found that resveratrol resistance appeared linked to the expression of UGTs in intestinal tumor and non-tumor cell lines. We observed strong expression of UGT1A8 and UGT1A10 in the cell lines resistant to resveratrol. According to protein sequence homology, the UGTs enzyme family is divided into 5 subfamilies sharing expression regulation mechanisms. For instance, UGT1A7, 1A8, 1A9, and 1A10 constitute an independent sub-group, with structural and expression regulation similarities (Meech et al., 2019). To evaluate whether this observation was relevant to colorectal cancers, we looked at the expression of UGT1A genes in RNAseq databases providing gene expression profiles of cancer and matched healthy tissues. Using the GEOD-76987 dataset of colorectal primary tumor tissues and normal mucosa tissues (Kanth et al., 2016), we found a systematic decrease in the expression of *UGT1A1, 1A7, 1A8, and 1A10* (Fig. 2a-c). Focusing on the expression levels of UGT1A8 and UGT1A10 genes, we observed a significant downregulation in colorectal primary tumors compared to healthy digestive tissues (Fig. 2b and 2c). Similar observations were made using the GEOD-50760 dataset obtained from colorectal primary tumor tissues with matching colorectal tumor hepatic metastasis and normal mucosa tissues (Kim et al., 2014) (Fig. 2d-f). In this dataset, we observed that hepatic metastasis had even stronger repression of UGT1A8 and UGT1A10 genes.

Next, we used the GEPIA platform gene correlation analysis tool and found that the expressions of UGT1A8 and UGT1A10 are closely correlated in healthy colorectal samples (Fig. 2g, $r = 0.95$), which agrees with their phylogeny. By contrast, this correlation was lost in tumor samples (Fig. 2h, $r = 0.63$), suggesting a disruption of the expression regulation mechanisms. No correlation was found for UGT1A1 or UGT1A7 with UGT1A8 or UGT1A10 (Supplemental Fig. 2a-h).

Epigenetic marks, like promoter hypermethylation, have been previously involved in UGT1A gene downregulation (Bélanger et al., 2010, p. 1; Hu et al., 2014). We tested if demethylation could switch back on the expression UGT1A8 and UGT1A10 in HCT116 cells. SFRP1 and SFRP2 genes were used as positive controls as both genes are known to be downregulated in colorectal tumors by promoter hypermethylation (Pehlivan et al., 2010). While demethylation did turn on the expression of the SFRP1 and SFRP2 genes, UGT1A8 and 1A10 genes were still off (Fig. 2i). Thus, the downregulation of UGT1A8 and UGT1A10 genes seemed independent of DNA methylation status.

Last, we examined whether UGT1A gene expression was prognostic using the GEPIA platform (*http://gepia.cancer-pku.cn*). Kaplan-Meir overall survival curves according to UGT1A gene expression showed that low levels of UGT1A8 and UGT1A10 were negative prognostic markers for patient survival (Fig. 2l and 2 m). This was not the case for UGT1A1, UGT1A7 (Fig. 2j and 2 k), or other UGT1A genes (data not shown).

These results show that UGT1A8 and UGT1A10 genes might be clinically relevant to colorectal cancer, especially regarding response to polyphenols such as resveratrol but also for prognosis assessment.

3.3. Functional impact of UGT1A expression modulation on non-tumor and tumor cell resveratrol toxicity

There is a known link between UGT1A enzymes and colorectal cell sensitivity to drugs (Landmann et al., 2014). To provide more insight into the relationship between UGT1A expression and resveratrol cell sensitivity, the expression of UGT1A enzymes was modulated. First, we designed 2 different shRNA to downregulate the expression of all the UGT1A enzymes (sh pan-UGT1A) or the UGT1A10 enzyme only (sh UGT1A10). UGT1A10 gene was found expressed both in non-tumor mouse and human cell lines. Moreover, this isoform is strongly present in colorectal healthy tissues and resveratrol is a known target of this enzyme (Iwuchukwu & Nagar, 2008). shRNA impact on the UGT1A8 (sh pan-UGT1A) and UGT1A10 (Sh pan-UGT1A and sh UGT1A10) expression was verified by RT-qPCR as compared to scrambled control (Fig. 3a and 3b). The downregulation of pan-UGT1A or UGT1A10 increased resveratrol sensitivity in HT29 cells, similar to that observed for the sensitive tumor cells HCT116 ($IC_{50} = 50$ μM; Fig. 3C). Moreover, the resveratrol metabolization capacity of HT29 with UGT1A downregulation was decreased by 7 to 8 folds. Interestingly, the downregulation of *UGT1A10* resulted in a diminution of the resveratrol metabolization by about 50 % and up to 35 % with *pan-UGT1A* repression (Fig. 3d). Moreover, while R4OG and R3OG were detected in similar amounts after UGT1A10 repression, R3OG was almost absent with global UGT1A downregulation. These results suggest that the UGT1A10 enzyme may be the major enzyme responsible for resveratrol metabolization, mainly into R4OG. Importantly, UGT1A10 downregulation in the NCM460 non-tumor cells (Supplemental Fig. 3a) led to cell sensitization to resveratrol, lowering the IC_{50} by about 2-fold (Supplemental Fig. 3b).

Next, we wondered whether the overexpression of UGT1A10 could confer resistance to resveratrol. HCT116 cells expressed UGT1A10 when transduced with a lentiviral vector bearing the UGT1A10 gene coding sequence (Fig. 4a). The expression of the UGT1A10 gene in HCT116 diminished about twice their sensitivity to resveratrol (Fig. 4b). Moreover, UGT1A10 expression allowed resveratrol metabolization (Fig. 4c).

These data show that sensitivity to resveratrol depends on the capacity of the cells to perform glucuronidation by UGT1A enzymes. Moreover, we found a significant correlation between resveratrol IC_{50} and metabolization capacity ($r = 0.76$, $p = 0.017$, Supplemental Fig. 3c).

3.4. Resveratrol impacts differentially non-tumor and tumor cells on global cell homeostasis

During the viability assays with resveratrol, we observed that treated HCT116 tumor cells appeared bigger than non-treated cells (Supplemental Fig. 4a and 4b). We determined that the cell volume of resveratrol-treated tumor cells was 1.7-fold bigger as compared to untreated cells (Fig. 5a). Importantly, this observation was not observed for non-tumor treated cells. Cell size is dependent on the balance between cell growth, which leads to an increase in volume, and cell division kinetics, which is defined by total cell cycle length. We hypothesized that resveratrol may disturb the tumor cell cycle, whereas non-tumor cells would not be affected. To test this, we analyzed the distribution of asynchronous cells in the phases of the cell cycle. As expected and already published, resveratrol (50 μM) induced a significant accumulation of HCT116 tumor cells in the S and G2/M phases (Fig. 5b). Resveratrol also affected the cell cycle of the NCM460 cells, although to a lesser extent, which was surprising according to its little impact in the initial cell viability tests and the unchanged cell size (Fig. 1a). Resveratrol IC_{50}s were determined after 72 h of treatment. Resveratrol may induce a cell cycle arrest in normal cells, which then recover from this initial effect, after full glucuronidation. Thus, we compared the initial impact of resveratrol (50 μM) on cell viability of all 4 tumor and non-tumor cell lines treated for 24 h and found a similar moderate decrease, which may account for cell cycle arrest

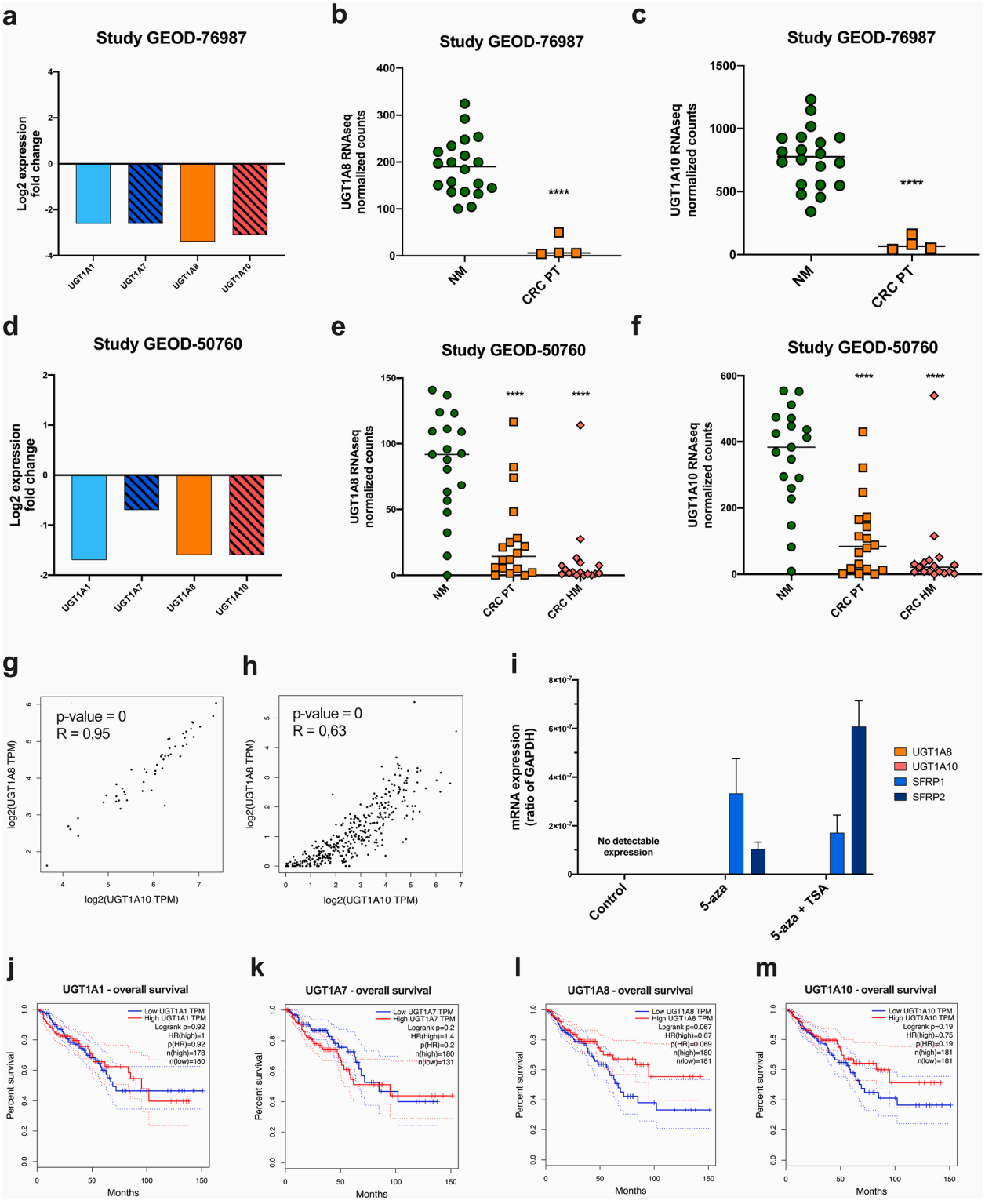

Fig. 2. *UGT1A8* and *UGT1A10* are downregulated in colorectal tumor tissues. Log2 fold change of *UGT1A1/A7/A8/A10* expression (respectively light blue, dashed blue, orange and dashed red) between colorectal tumor and healthy digestive tissues in GEOD-76987 (a) and GEOD-50760 (d) datasets. RNAseq normalized counts for *UGT1A8* (b) and *UGT1A10* (c) in normal colorectal mucosa (green dots) and primary colorectal tumor (orange squares) of the GEOD-50760 dataset. RNAseq normalized counts for *UGT1A8* (e) and *UGT1A10* (f) in normal colorectal mucosa (green dots), primary colorectal tumor (orange squares) and hepatic metastases (red triangles) of the GEOD-50760 dataset. Pearson correlation analysis between *UGT1A8* and *UGT1A10* expressions in healthy colorectal tissues (g) and colorectal cancers (h). Expression of *UGT1A8* (orange), *UGT1A10* (red), *SFRP1* (blue) and *SFRP2* (deep blue) genes in HCT116 cells after demethylation treatment with 5-aza-cytidine and trichostatin A (i). Overall survival of the COAD (colorectal cancer) and the READ (rectal cancer) TCGA databases patients, depending on the tumor expression of *UGT1A1* (j), *UGT1A7* (k), *UGT1A8* (l) and *UGT1A10* (m). Blue lines: low expression, red lines: high expression. Results are expressed with mean +/- SD. **** : $p<0.0001$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

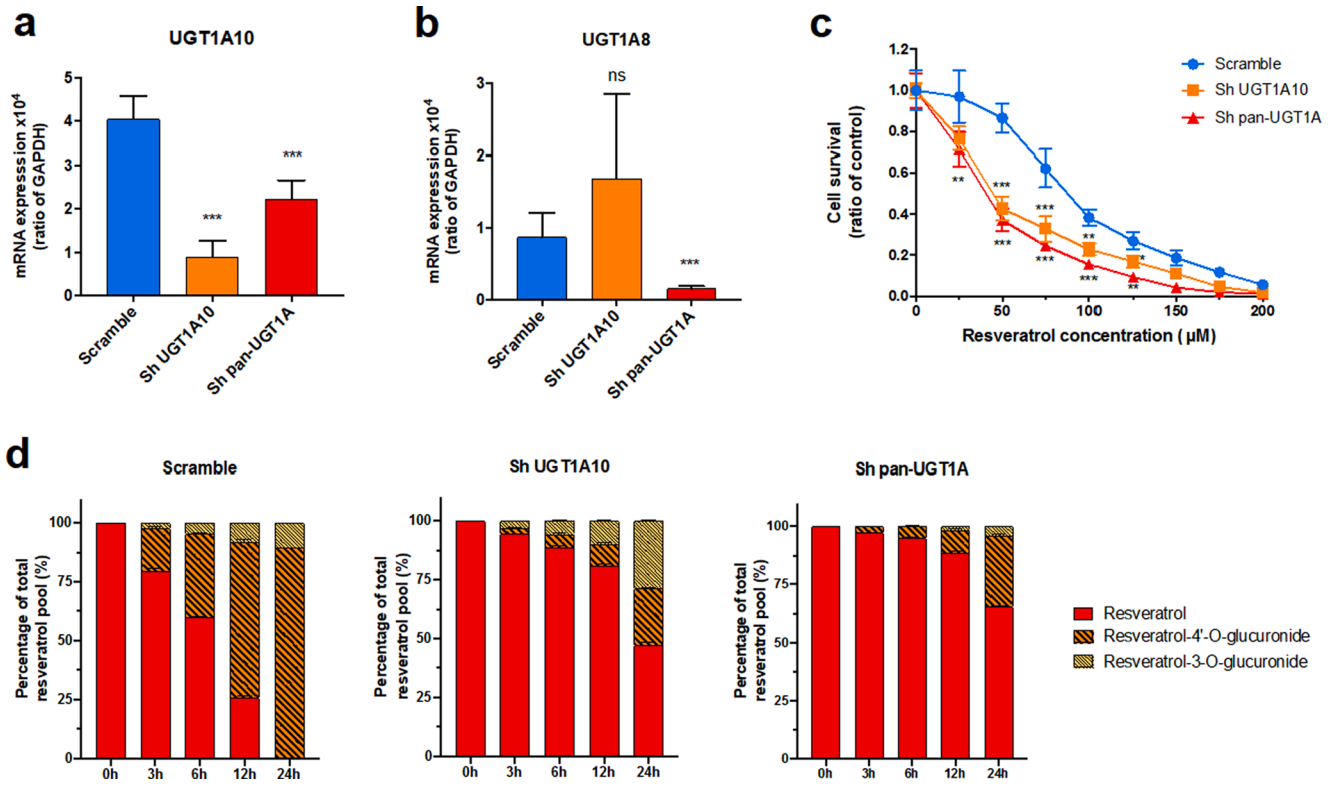

Fig. 3. Downregulation of UGT1A gene expression impacts colorectal cell lines resveratrol metabolization capacities and sensitivity. Expression of the UGT1A10 (a) and UGT1A8 (b) genes was assessed in different shRNA (sh scramble: blue, sh UGT1A10: orange, sh pan-UGT1A: red) transduced HT29 cells by RT-qPCR, using GAPDH transcripts for normalization. Resveratrol dose–response experiments were carried out after shRNA transductions in HT29 cells (c, sh scramble: blue dots, sh UGT1A10: orange squares, sh pan-UGT1A: red triangles). Resveratrol (red) and its glucuronide derivates resveratrol-4′-O-glucuronide (dashed orange) and resveratrol-3-O-glucuronide (dashed yellow) were quantified in cell culture media after 0, 3, 6, 12 and 24 h incubation with the different shRNA-transduced HT29 cells (d). Results are expressed with mean +/- SD. ***: $p < 0.001$; ****: $p < 0.0001$; ns: non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Supplemental Fig. 4c). Nevertheless, resveratrol-induced cell cycle arrest may not be the only global consequence on tumor cells, which by contrast to non-tumor cells, displayed cell volume increase.

Recent data show that cell growth and division can be uncoupled downstream of nucleotide imbalances (Diehl et al., 2021). Moreover, resveratrol inhibits mTORC1 and promotes AMPK activity, both actions leading to indirect pyrimidine synthesis inhibition (J.-N. Lin et al., 2010; Liu et al., 2010; Park et al., 2016). We hypothesized that resveratrol could differentially impact nucleotide concentrations of tumor cells and non-tumor cells, especially pyrimidines. Levels of purine nucleotides (ATP / ADP / AMP / GTP / GDP) and pyrimidine nucleotides (CTP / CDP / UTP / UDP / UMP) were measured in HCT116 and NCM460 cells after 24 h of resveratrol exposure (50 µM). Adenylic nucleotide metabolism was higher in NCM460 compared to HCT116, as shown by the increase in AXP contents (AXP = ATP + ADP + AMP) (Supplemental Fig. 4d and 4g). By contrast, Adenylic Energy Charge remained constant after resveratrol exposure, excluding that an energetic depletion leads to cancer cell proliferation arrest (Supplemental Fig. 4h). GTP, GDP, and CDP nucleotide levels were similar in both cell lines and remained unchanged after resveratrol treatment (Supplemental Fig. 4i-k). By contrast, although basic levels were roughly similar in tumor and non-tumor cells, we observed a significant decrease in all the tested pyrimidine monophosphate and triphosphate nucleotide contents in resveratrol-treated tumor cells but not non-tumor cells. Indeed, UTP, UMP, and CTP levels decreased in resveratrol treated HCT116 cells (Fig. 5c-e, Supplemental Fig. 4k). UDP levels were stable (Fig. 5d), but UDP being the less abundant uridylate nucleotide in HCT116 cells, its contribution to the global uridylate nucleotide pool, UXP, is neglectable (Fig. 5f).

Altogether, these results show that resveratrol treatment creates a pyrimidine nucleotide unbalance specifically in tumor cells.

Pyrimidine nucleotides and especially uridylate nucleotides are major metabolites of transcription. Lower UXP levels may result in transcription inhibition (Huang et al., 2008; Lafita-Navarro et al., 2020). To assess global transcription, we quantified the RNA/DNA ratios in tumor (HCT116) and non-tumor (NCM460) cells, treated or not for 24 h with resveratrol (50 µM). RNA/DNA ratios dropped in HCT116 treated cells but not in NCM460 cells (Fig. 5g).

A general slowdown of transcription may result in diminished global translation (Slobodin et al., 2017). We used a puromycine pulse-chase staining to observe that resveratrol treatment resulted in the decrease of translation in HCT116 cells after 48 and 72 h (Fig. 5h and Supplemental Fig. 4m). Translation in NCM460 cells was not affected even after 72 h of resveratrol exposure (Fig. 5h and Supplemental Fig. 4n). Our results suggest that resveratrol impacts specifically the whole transcription-translation process of colorectal tumor cells while sparing non-tumor cells.

4. Discussion

Numerous studies, including ours, showed resveratrol toxicity in tumor cells and its differential impact on non-tumor cells, but the mechanistic bases of this difference remain unclear. This work highlights the essential role of UGT1As to protect cells from resveratrol toxicity. When UGT1As are downregulated in cancer cells, resveratrol perturbs the nucleotide UXP/AXP balance leading to global diminution of transcription and translation rates.

Dietary resveratrol passively crosses the plasma membrane of

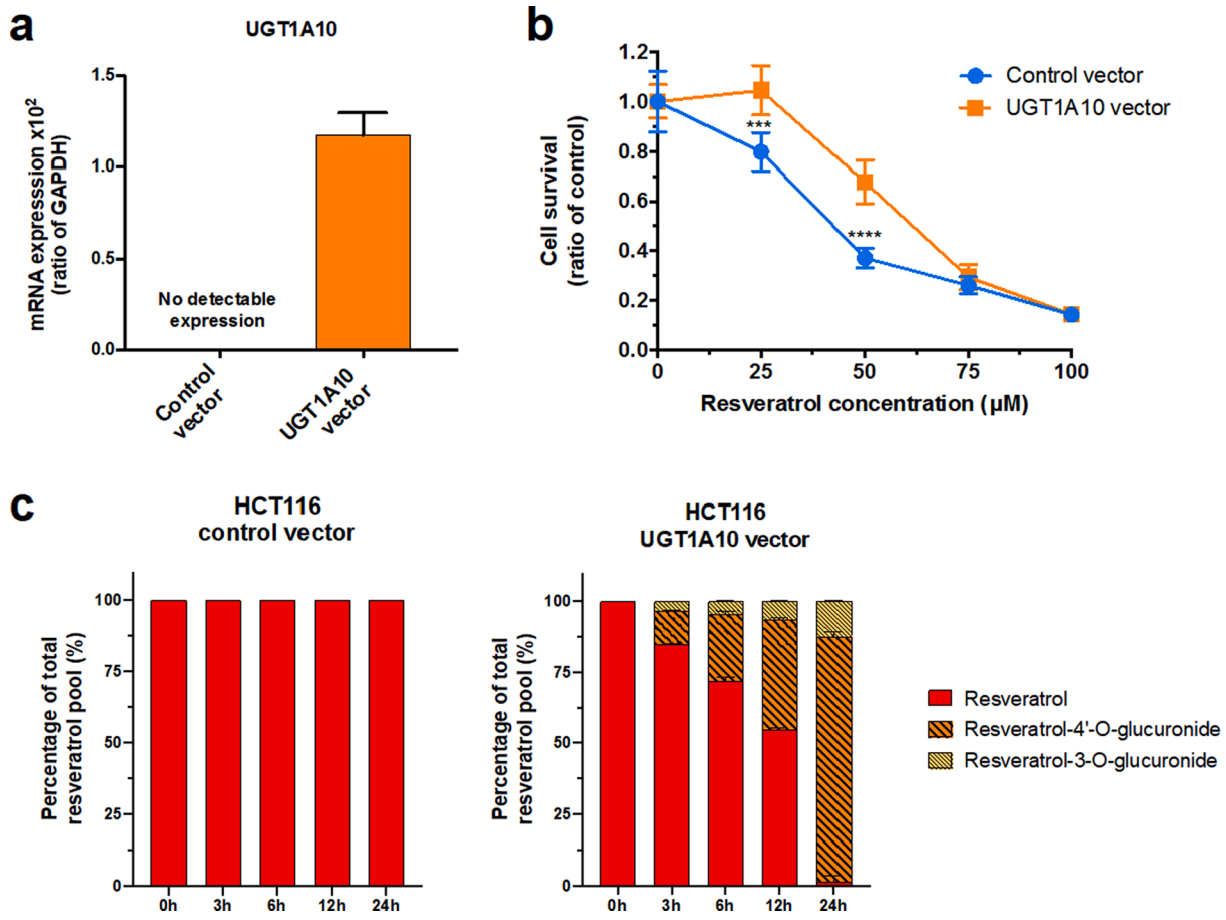

Fig. 4. Upregulation of UGT1A gene expression impacts colorectal cell line sensitivity to resveratrol and resveratrol metabolization. Expression of UGT1A10 was assessed in HCT116 cells transduced with UGT1A10 expression vector (orange) and control vector (blue) by RT-qPCR, using GAPDH transcripts for normalization (a). Resveratrol dose–response experiments were carried out after transductions of HCT116 cells with UGT1A10 expression (orange squares) and control vectors (blue dots) (b). Resveratrol (red) and its glucuronide derivates resveratrol-4′-O-glucuronide (dashed orange) and resveratrol-3-O-glucuronide (dashed yellow) were quantified in cell culture media after 0, 3, 6, 12 and 24 h incubation with HCT116 cells transduced with UGT1A10 expression vector and control vector (c). Results are expressed with mean +/- SD. **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

enterocytes to be metabolized into sulfoconjugates and glucuronoconjugates by metabolic enzymes. These conjugates are released into the bloodstream and the conjugation can be reversed in target tissues. In addition, metabolites may undergo enterohepatic recirculation enabling new absorption in the intestine. In the intestine, metabolized forms can be unconjugated by the microbiota (Marier et al., 2002), and reenter the enterocytes for a new metabolization/circulation cycle. Thus, the intestine is more exposed to the native form of resveratrol, which may optimize its therapeutic impact on colorectal tumors. The enterohepatic cycle of resveratrol is of particular interest in colorectal cancer application since the resveratrol-4′-O-glucuronide metabolite has no cytotoxic activity, as confirmed in this study, which may also be true for the 3-O-glucuronide and 3-O-sulfate. Moreover, their production soon after resveratrol absorption limits its bioavailability to distant tissues.

Our work revealed that *UGT1A* expression status was closely linked to resveratrol sensitivity in cell line models since metabolization activity was correlated to sensitivity. *UGT1A* expression seemed correlated to cell differentiation. Indeed, the HCT116 and SW480 cell lines form undifferentiated tumors and belong to the CRC consensus molecular subtype 4, CMS4, (Berg et al., 2017; Kai et al., 2009), whereas the HT29 cell line forms well-differentiated tumors resembling grade I carcinomas (CMS3, Berg et al., 2017). Of note, the fold difference in the expression of a few genes, including *UGT1A1*, was the highest in colon-like cell lines, such as HT29. This suggests that loss of differentiation during cancer progression is accompanied by loss of *UGT1A* expression. This hypothesis is supported by the *in-silico* analysis presented here, finding a systematic decrease in the expression of *UGT1A1, 1A7, 1A8, and 1A10* in tumor colon tissues. Metastasis showed further downregulation, confirming the relationship between *UGT1A* loss and cancer aggressiveness. This assumption is also in agreement with our finding that low levels of UGT1A8 and UGT1A10 were negative prognostic markers for patient survival. Moreover, downregulation of UGT1A expression in colorectal and other digestive cancer tissues has been described in the literature (Giuliani et al., 2005; Strassburg et al., 1997). UGT1A1 expression is tightly regulated by promoter methylation (Bélanger et al., 2010; Yasar et al., 2013, p. 1) and UGT1A8 and UGT1A10 are expressed from distinct promoters (Hu et al., 2014). We showed here that global demethylation of the HCT116 cell line did not turn on *UGT1A8* and *UGT1A10* genes. Thus, it is probable that UGT1A downregulation is more a consequence of cell dedifferentiation during cancer progression, due to the loss of the transcriptional program, rather than a selective loss of function. This hypothesis agrees with the high correlation of expression of both genes in normal tissues (Fig. 2g), which is lost in cancer tissues (Fig. 2h). This hypothesis is further supported by Delmas et al. who showed that daily resveratrol treatment failed to impact subcutaneous tumor growth of a well-differentiated colorectal rat tumor cell line while a growth delay was observed on a human undifferentiated CRC cell line-derived tumor (Colin et al., 2014). It would be very interesting to assess UGT1A expression in both CRC tumor cell lines. Further exploration is needed, especially regarding tumor response to treatments that are metabolized

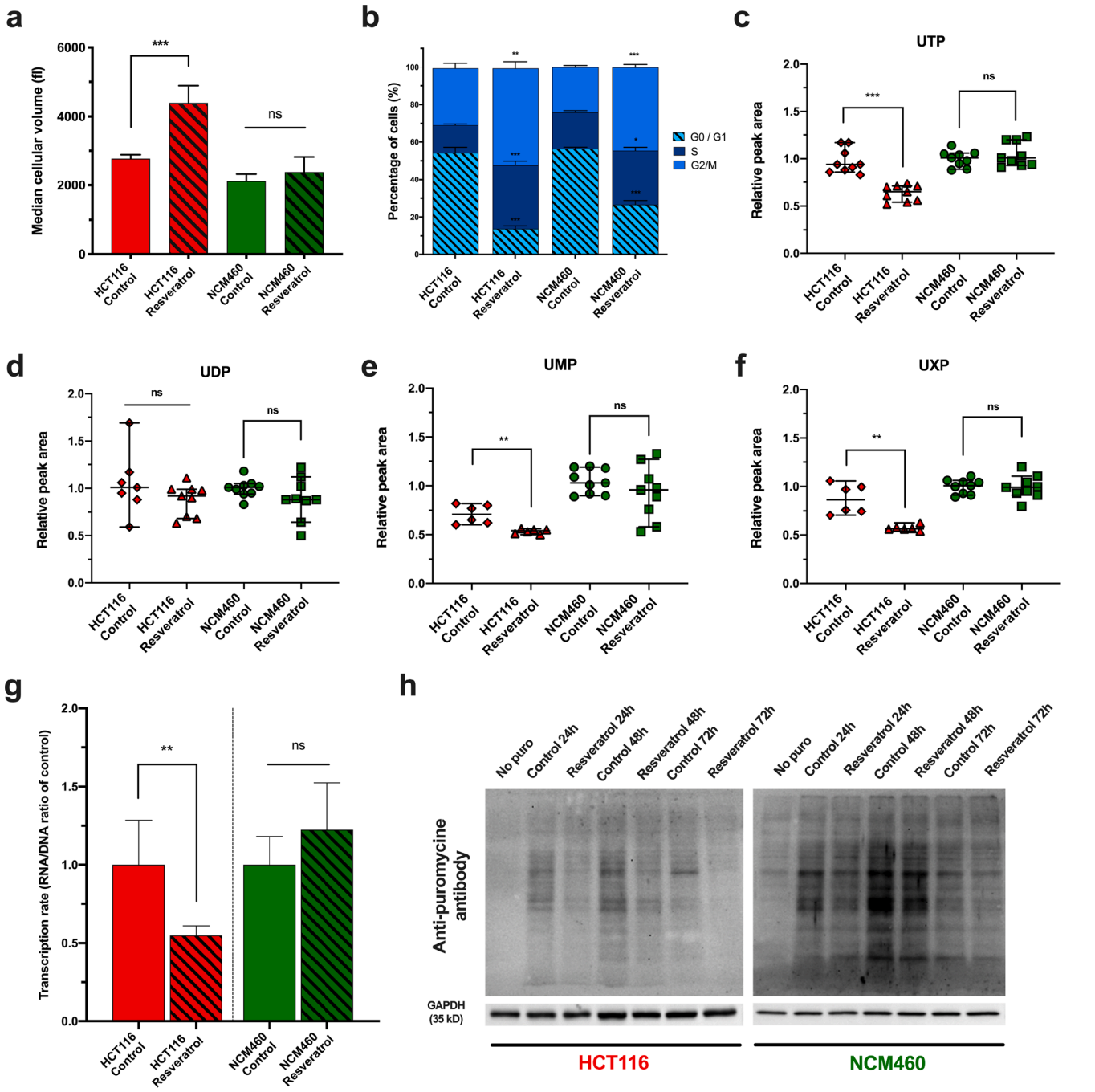

Fig. 5. Resveratrol differentially impacts tumor and non-tumor cell homeostasis. Cell-volume change of HCT116 (red and dashed red) and NCM460 cells (green and dashed green) after 24 h of 50 μM resveratrol exposure (a). Cell-cycle analysis of HCT116 and NCM460 cells after 24 h of 50 μM resveratrol exposure (b, G0/1G1 phase: dashed light blue, S phase: deep blue, G2/M: blue). Relative content (untreated NCM460 value was used as the reference) for UTP (c), UDP (d), UMP (e) and UXP (f) nucleotides in HCT116 (untreated = red diamonds ; treated = red triangles) and NCM460 (untreated = green dots ; treated = green squares) cells after 24 h 50 μM resveratrol exposure. RNA/DNA ratio of HCT116 (red and dashed red) and NCM460 (green and dashed green) cells 24 h 50 μM resveratrol exposure (g). Results are expressed as the ratio of untreated cells for each cell line. Puromycine pulse-chase analysis by western-blot in HCT116 (right side) and NCM460 (left side) after 24 h, 48 h and 72 h of 50 μM resveratrol exposure (h). Results are expressed with mean +/- SD. * : $p<0.05$; ** : $p<0.01$; *** : $p<0.001$; ns : non-significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by UGT1As. Indeed, UGTs may be involved in the intrinsic drug resistance in CRC. Resistance to irinotecan-derived SN38 metabolized by UGT1A1/UGT1A6 and ganetespib conjugated by UGT1A1 (Landmann et al., 2014) might be more active with inhibition of UGTs (Cummings et al., 2003). This agrees with the resistance to resveratrol reversion by *UGT1A* downregulation in HT29 and NCM460 cells observed in the present study. Thus, CRC tumor *UGT1A* expression profiling could be predictive of intrinsic resveratrol sensitivity. In this context, undifferentiated *UGT1A*-negative aggressive CRC tumors could be preferred resveratrol targets. Moreover, as resveratrol could compete with other drugs for UGT1A active sites (Brill et al., 2006; Cummings et al., 2003), it could participate to enhance the efficiency of antineoplastic therapeutics, by competing for inactivation. The treatment combining resveratrol with for example irinotecan may lead to improved CRC chemotherapy response. UGT enzymes are activated by phosphorylation, mainly by the protein kinase C (PKC) (Basu et al.,

2003; Volak & Court, 2010). Thus, combining resveratrol with PKC inhibitors, such as curcumin, another well-studied polyphenol, could enhance the resveratrol CRC anti-tumor effect by decreasing its metabolization.

Our study aimed at understanding the differences in resveratrol responses between non-tumor and tumor cells. Besides the impaired capacity to metabolize the compound, tumor cells displayed a profound decrease in all the tested monophosphate and triphosphate pyrimidine nucleotide contents after resveratrol treatment, while these contents were unchanged in non-tumor cells. We further found that this drop affected tumor cell transcription and translation, probably in close relation with the cytotoxic effect of resveratrol. We did not have access to the deoxyribonucleotide quantification. It is possible that they were depleted too as resveratrol is known to inhibit ribonucleotide reductase (Fontecave et al., 1998) and to induce replicative stress (Benslimane et al., 2020), leading to dNTP pool decrease and cell accumulation in the S phase (Delmas et al., s. d.). Very few studies have focused on resveratrol's impact on NTP metabolism (Schmatz et al., 2009), especially in cancer tissues (Li et al., 2019), even if indirect pyrimidine synthesis negative imbalance can result from resveratrol mTORC inhibition and AMPK activation (J.-N. Lin et al., 2010; Liu et al., 2010; Park et al., 2016). A recent *in silico* docking analysis described resveratrol as a potential inhibitor of multiple enzymes involved in nucleotide metabolism, especially RNA polymerases (Wu et al., 2020). Thus, resveratrol-induced downregulation of pyrimidine nucleotides may not be the unique cause of the observed cancer cell transcription rate inhibition. The disruption of pyrimidine nucleotide balance could impact the translation in tumor cells. The mTOR pathway is a key activator of translation (Nandagopal & Roux, 2015) with oncogenic mutations leading to increased translation rates (Averous & Proud, 2006; Hsieh et al., 2011). Resveratrol has been widely described as an inhibitor of the Akt pathway and a direct inhibitor of mTOR (Jiang et al., 2009; Liu et al., 2010), especially in CRC tumor cells (Park et al., 2016). In addition to the modulation of translation activators, the transcription rate inhibition could enhance translation decrease in HCT116 cells. It is well demonstrated that resveratrol does interfere with the activities of multiple cellular pathways and alters the behavior of many proteins. In this context, the "one-drug-one-target" concept does not apply. However, resveratrol affects the overall homeostasis of tumor cells in multiple ways and many of which are yet to be elucidated. One highlight of our work is the resveratrol-induced decreased UXP and unchanged AXP in tumor cells. This nucleotide imbalance could participate in decoupling cell growth and proliferation, as recently described (Diehl et al., 2021).

This study reinforces the grounds for the use of resveratrol in CRC treatment, in particular as to sensitize cancer cells to conventional treatments. However, resveratrol exhibits low bioavaibility when given orally, because of its high metabolization by the digestive tract and the liver. Conversely, this high metabolization rate prevents resveratrol toxicity (Shaito et al., 2020). Doses up to 1000 mg/d were reported safe in healthy people. However, higher doses could lead to adverse events like the modulation of cytochrome P450 activity, nephrotoxicity and other gastrointestinal problems (Shaito et al., 2020). Resveratrol bioavaibility optimization is being designed to by-pass this bioavaibility issue, for example with nanoformulations (Sharifi-Rad et al., 2021). Nevertheless, rectal tumors are particular good candidates for direct local administration with appropriate galenic forms, to target the tumor while reducing potential local and systemic toxicities.

5. Conclusion

In conclusion, we understand for the first time that colorectal tumor-specific cytotoxicity of resveratrol is directly linked to cell glucuronidation capacity. This strengthens its prospects for use in CRC as a conventional treatment sensitizer agent. From a broader perspective, as for resveratrol, UGT enzymes play a key role in the tumor response to numerous antineoplastic drugs. Thus, tumor UGT expression profiling represents a potential predictive marker for drug sensitivity assessment and *UGT1A* expression modulation could represent a promising therapeutic strategy.

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Ethical Statement

Hereby, I Sandrine Dabernat, consciously assure that for the manuscript "Differential Uridyl-diphosphate-Glucuronosyl Transferase 1A enzymatic arsenal explains the specific cytotoxicity of resveratrol towards tumor colorectal cells", the following is fulfilled:

1) This material is the authors' own original work, which has not been previously published elsewhere.

2) The paper is not currently being considered for publication elsewhere.

3) The paper reflects the authors' own research and analysis in a truthful and complete manner.

4) The paper properly credits the meaningful contributions of co-authors and co-researchers.

5) The results are appropriately placed in the context of prior and existing research.

6) All sources used are properly disclosed (correct citation). Literally copying of text must be indicated as such by using quotation marks and giving proper reference.

7) All authors have been personally and actively involved in substantial work leading to the paper, and will take public responsibility for its content.

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Author contributions

SA wrote the whole manuscript and prepared all figures, design the study, and perform analysis, assembly, and interpretation of all data. PB collected and analyzed data for Fig 1, 3, and 4 and prepared corresponding figures. CD collected and analyzed data for Fig 1, 3, and 4. IM collected and analyzed data for Fig 1, 3, 4, and 5. IL collected and analyzed data for Fig 1, 3, 4, and 5. EP collected and analyzed data for figure 3. BF EP collected and analyzed data for Fig 2. ABi: Designed and produced lentiviral vectors. JB collected data for Fig 2. TR collected and analyzed, data for Fig 1, 3, and 4. SK: PB collected and analyzed data for Fig 1, 3, and 4 and prepared corresponding figures. FMG participated in design, financial support, and final approval of the manuscript. ABe participated in to design, financial support, and final approval of the manuscript. DC designed the methylation experiment and prepared Fig 2. BP collected, analyzed, and interpreted data for Fig 5 and prepared the corresponding figure. VV participated in to design, financial support, writing and final approval of the manuscript. SD wrote the whole manuscript and participated to the conception and design of the project, the analysis, assembly and interpretation of data, and supported financially. All authors reviewed the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105345.

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