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Solange Vivier[1] | Fabrice Bray[2] | Stéphanie Flament[2] | Lucile Guilbert[1,3] | Florence Renaud[4] | Christian Rolando[2] | David Launay[1,5] | Sylvain Dubucquoi[1,3] | Vincent Sobanski[1,5,6]

[1]Univ. Lille, Inserm, CHU Lille, U1286 - INFINITE - Institute for Translational Research in Inflammation, Lille, France | [2]Univ. Lille, CNRS, UAR 3290 - MSAP - Miniaturisation pour la Synthèse, l'Analyse et la Protéomique, Lille, France | [3]Institut d'Immunologie, Centre de Biologie Pathologie, CHU Lille, Lille, Hauts-de-France, France | [4]Sorbonne Université, INSERM, Unité Mixte de Recherche Scientifique 938 and SIRIC CURAMUS, Centre de Recherche Saint-Antoine, Equipe Instabilité des Microsatellites et Cancer, Equipe labellisée par la Ligue Nationale contre le Cancer, Paris, France | [5]CHU Lille, Département de Médecine Interne et Immunologie Clinique, Centre de Référence des Maladies Auto-immunes et Auto-inflammatoires Systémiques Rares du Nord, Nord-Ouest, Méditerranée et Guadeloupe (CeRAINOM), Lille, Hauts-de-France, France | [6]Institut Universitaire de France (IUF), Paris, France

Correspondence: Vincent Sobanski (vincent.sobanski@univ-lille.fr)

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Keywords: colorectal cancer | endoplasmic reticulum stress | epithelial cells | LC-MS/MS | unfolded protein response

ABSTRACT

Purpose: High throughput technologies have identified molecular patterns in colorectal cancer (CRC) cells, aiding in modeling responses to anti-cancer treatments. The different responses observed depend on the type of cancer, the tumour grade and the functional programme of the cancer cells. Recent studies suggest that the unfolded protein response (UPR), autophagy and apoptosis could be involved in treatment resistance mechanisms by interacting with the tumour microenvironment (TME).

Experimental Design: We analysed by LC-MS/MS the proteome of two representative colon adenocarcinoma epithelial cell lines from different tumour grades (CCL-233 and CCL-221) at the basal state or after the UPR induction.

Results: Cell lines expressed a different proteome on about 10% of their total proteins identified, especially on UPR, autophagy and apoptosis pathways proteins at basal state. After UPR induction, the proteome of the cells was modified with a greater adaptive response to cellular stress in CCL-221 cells where the UPR was strongly activated at the basal state.

Conclusions and Clinical Relevance: CRC cell lines at different tumour grades expressed different functional programmes at the proteomic level and were characterised by different responses to the UPR induction. This study suggests that baseline cancer cell stress status could have an impact on the efficiency of cancer therapies.

Abbreviations: BFA, brefeldin A; BP, biological process; BTZ, bortezomib; CC, cellular component; CRC, colorectal cancer; ER, endoplasmic reticulum; FC, fold change; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MF, molecular function; PC, protein class; PCA, principal component analysis; TME, tumour microenvironment; UPR, unfolded protein response.

Sylvain Dubucquoi and Vincent Sobanski contributed equally to this study.

Summary

- This study showed that two colon adenocarcinoma epithelial cell lines associated with early or late tumour grades expressed partially different proteomes and by extension different metabolic profiles, especially on the unfolded protein response (UPR) activation state studied with and without induction of cellular stress.
- The fate of cancer cells treated with therapies based on the homeostasis of proteins is conditioned by their baseline stress status, leading in some cases to treatment failure.
- This in vitro cellular model associated with LC-MS/MS could be used to assess experimentally by the efficiency of drugs on key pathways in the context of colorectal cancer (CRC).

1 | Introduction

Colorectal cancer (CRC) is one of the deadliest cancers worldwide. It is considered the third most frequent cancer, accounting for approximately 10% of all diagnosed cancers and cancer-related deaths in 2020. CRC is therefore a major public health challenge with a higher incidence in high-income countries, which has increased since the last decades [1–3]. CRC is caused by an accumulation of genetic mutations and epigenetic instability in epithelial cells, leading to their proliferation [4].

During tumourigenesis, cells undergo diverse stresses such as hypoxia, oxidative stress or glucose deprivation leading to changes in their homeostasis [5, 6]. Modifications in the metabolism and cellular stress can alter the general process of protein synthesis in the endoplasmic reticulum (ER) (accumulation, aggregation, unfolding or misfolding) thereby activating the unfolded protein response (UPR) [7]. This adaptive mechanism in response to cellular stress has been associated with the survival and proliferation of cancer cells by preventing autophagy and apoptosis [8]. The tumour pathological grades could be associated with different levels of UPR activation, which can influence the response to anti-cancer therapies [9–13]. Tumour grades are defined as a classification of the appearance of cells in tissue under microscopy, ranging from a normal appearance with differentiated cells to abnormal, undifferentiated pro-proliferative cells extending into the tissue. This classification depends on the type of cancer, ranging from grade I to IV, and helps clinicians define the appropriate therapeutic strategy and prognosis. In the case of CRC, multiple tests can be performed to determine tumour grade (genetic mutations and protein expression in blood, stool or biopsies) (National Cancer Institute, https://www.cancer.gov/). By the development of high-throughput sequencing techniques, molecular profiling improved the knowledge of diversity in CRC and enhanced treatment strategies [14–16].

To gain a better understanding of the influence of UPR activation in CRC, we evaluated the proteomic profiles of two colon adenocarcinoma epithelial cell lines of ATCC CCL-233 grade I–II cells (intermediate grade, moderately differentiated cells) and ATCC CCL-221 grade III cells (high grade, poorly differentiated cells) at basal state and after exposition to UPR inducing agents, brefeldin A (BFA) and bortezomib (BTZ).

2 | Materials and Methods

2.1 | Cell Culture

DLD-1 cells (ATCC CCL-221, Manassas, VA, USA) were cultured in DMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) with 10% of foetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% of penicillin and streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in an incubator supplemented with 5% CO_2 and a temperature of 37°C. SW-1116 cells (ATCC CCL-233, Manassas, VA, USA) were cultured in Leibovitz's L-15 medium (Pan Biotech, Germany) with 10% of foetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% of penicillin and streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) in an incubator at a temperature of 37°C without supplementation in CO_2. For the analysis with UPR-inducing agents, 50,000 cells were seeded in 12-well plates with culture medium. After 24 h of starvation, cells were stimulated or not with brefeldin A (BioLegend, United Kingdom) at a concentration of 50 ng/mL or bortezomib (SelleckChem, Germany) at a concentration of 500 nM for 24 or 48 h. For the condition basal state (T0), one million cells were recovered before plating and directly processed for proteomic analysis.

2.2 | Sample Preparation for Proteomic Analysis

For the mass spectrometry analysis, cells were washed with PBS, trypsinised with Trypsin-EDTA (0.05%), phenol red (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and centrifuged at 500 × *g* for 5 min. The supernatant was eliminated, and cells were frozen in a dry pellet at −80°C for the LC-MS/MS analysis.

2.3 | Protein Extraction, Digestion and Analysis in LC-MS/MS

All samples were lysed and digested using the eFASP method—enhanced Filter Aided Sample Preparation as previously described [17, 18]. The concentration of each sample was adjusted to 1 µg/µL by dilution with ultrapure water containing 0.1% formic acid (Sigma-Aldrich, Saint-Louis, Missouri, USA) and 1 µL of each peptide sample by replicate was analysed. LC-MS/MS protein analysis was performed on an Orbitrap Q-Exactive plus Mass Spectrometer hyphenated to a U3000 RSLC Microfluidic HPLC System (Thermo Fisher Scientific, Waltham, MA, USA). Each sample was injected in triplicate.

2.4 | Bioinformatics Quantification in LC-MS/MS

LC-MS/MS analysis was performed as previously described [19]. Briefly, analysis of the raw LC-MS/MS data was performed using MaxQuant (version 1.5.3.30) [20] and the Andromeda search engine [21] was used for database searching against the

UniProtKB human database (UniProtKB version 2021-04). For a more precise label-free quantitation of proteins, MaxQuant uses a dedicated algorithm called MaxLFQ for intensity determination and normalization procedure [22].

2.5 | Graphic Representations and Statistical Analysis in LC-MS/MS

Venn diagrams were generated with Venny 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/). The UpSet plot was generated with the R environment (version 4.2.2) using the 'ComplexHeatmap' package (version 2.15.4). Statistical analysis was as previously described [19]. Briefly, analysis was performed using Perseus (version 1.6.2.3) [23]. One-way ANOVA tests were performed on $\log_2$ transformed data to assess the difference between all conditions considered in the analysis (basal state or after exposition to UPR-inducing agents). The *t*-tests and volcano plots were performed using the Student's *t*-test with standard parameter (S0 = 0 and false discovery rate [FDR] = 0.05). Principal component analysis (PCA) was based on the total proteins quantified and identified, and biological analysis was carried out on data with a $|\log_2(\text{Fold Change})| \geq 1.5$. Heatmaps were generated with GraphPad Prism10.

2.6 | Gene Ontology Terms and Protein Class Enrichment Analysis

The gene ontology (GO) terms and protein class (PC) enrichment were done on differentially expressed protein lists from Perseus analysis with GO slim enrichment analysis and Panther (version 16) [24–26]. Only GO terms and PC significantly overrepresented and underrepresented were analysed (Fisher's exact test, adjusted $p < 0.05$, Benjamini–Hochberg-FDR method).

2.7 | KEGG Genes Pathways Analysis

The gene pathways for the UPR (hsa04141), the autophagy (hsa04210) and the apoptosis (hsa04140) were extracted from the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (2021–08) [27]. The entire data from LC-MS/MS analysis was matched with the three pathways and gene lists of proteins identified were generated. The proteins identified by their gene names were analysed for each comparison of interest.

3 | Results

3.1 | Protein Expression Patterns of CCL-233 and CCL-221 Cells at Basal State

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify and quantify the proteome of the two cell lines.

First, we analysed the global proteome of CCL-233 and CCL-221 cells at basal state. There was good reproducibility with respectively 83.6% (2512 ± 25 proteins) and 84.4% (2610 ± 24 proteins) of proteins common between the triplicates. Keeping only proteins identified and quantified in triplicate in each cell line, 2287 proteins were identified in CCL-233 cells and 2385 proteins in CCL-221 cells (Figure 1A). For complete comparisons of the number of identified proteins between each LC-MS/MS quantification at basal state, see Figure S1. Comparing the two pooled protein lists, CCL-233 and CCL-221 cells shared a common list of 2038 proteins (77% similarity); 249 proteins were exclusive to CCL-233 cells and 347 proteins were exclusive to CCL-221 (Figure 1B).

To study the protein expression patterns, an analysis with Perseus was performed with a filter of three valid values in at least one group, here represented by each cell line. A total of 3302 proteins were identified (Table S1) and 1898 proteins were differentially expressed (ANOVA, $p < 0.05$). PCA was performed and showed a clear separation between the two cell lines with component 1 explaining 68% of variance and good reproducibility between replicates with component 2 explaining only 10% of variance (Figure 1C). Using a volcano plot representation with all proteins identified and a cutoff at $|\log_2(\text{Fold Change})| \geq 1.5$ ($|\log_2(\text{FC})| \geq 1.5$), the Student's *t*-test revealed that 289 proteins were overexpressed in CCL-233 cells and 244 proteins were overexpressed in CCL-221 cells (permutated based-FDR adjusted $p < 0.05$) (Figure 1D). From these latter protein lists extracted from the Student's *t*-test, biological process (BP) GO terms, and PC enrichment analysis were used to identify functional profiles of the cell lines. The enrichment analysis showed that CCL-233 cells were characterised by an overrepresentation of processes involved in the regulation of immunity, metabolic processes (biosynthetic processes), mitochondrial processes, enzymatic activity related to protein modifications and an underrepresentation of transcriptional factors and regulators compared to CCL-221 cells (Figure 2A,B). In CCL-221 cells, the enrichment profile was more oriented toward transcription processes and cell structure than in CCL-233 with an overrepresentation of nucleic acids metabolic processes, ATP synthase and cytoskeletal protein (Figure 2C,D) (for complete GO terms and PC lists, see Figure S2 and Table S2).

3.2 | UPR, Autophagy and Apoptosis Processes at Basal State

The identification of different functional profiles between our two cell lines led us to further analyse certain signalling pathways to better understand the proteome profiles of each cell line. Previous studies have identified UPR, apoptosis and autophagy as pathways that could be altered in cancer cells and modified responses to anti-cancer treatments [7, 28–30]. Thus, we further focused on UPR, autophagy and apoptosis by comparing proteins identified in our analysis with pathways 'UPR', 'Autophagy' and 'Apoptosis' obtained from the KEGG pathway database. On the global LC-MS/MS analysis including all conditions for the two cell lines, we identified 82 proteins involved in the UPR pathway, 29 in the autophagy pathway and 43 in the apoptosis pathway (Table S3). At basal state, the two cell lines expressed proteins involved in the three pathways. CCL-233 cells expressed mainly proteins of the apoptosis pathway as cathepsins (CTSs), Inositol 1,4,5-Triphosphate Receptor type3 (ITPR3), Poly(ADP-Ribose) Polymerase 4 (PARP4) and Diablo IAP-binding mitochondrial protein (DIABLO). In CCL-221 cells,

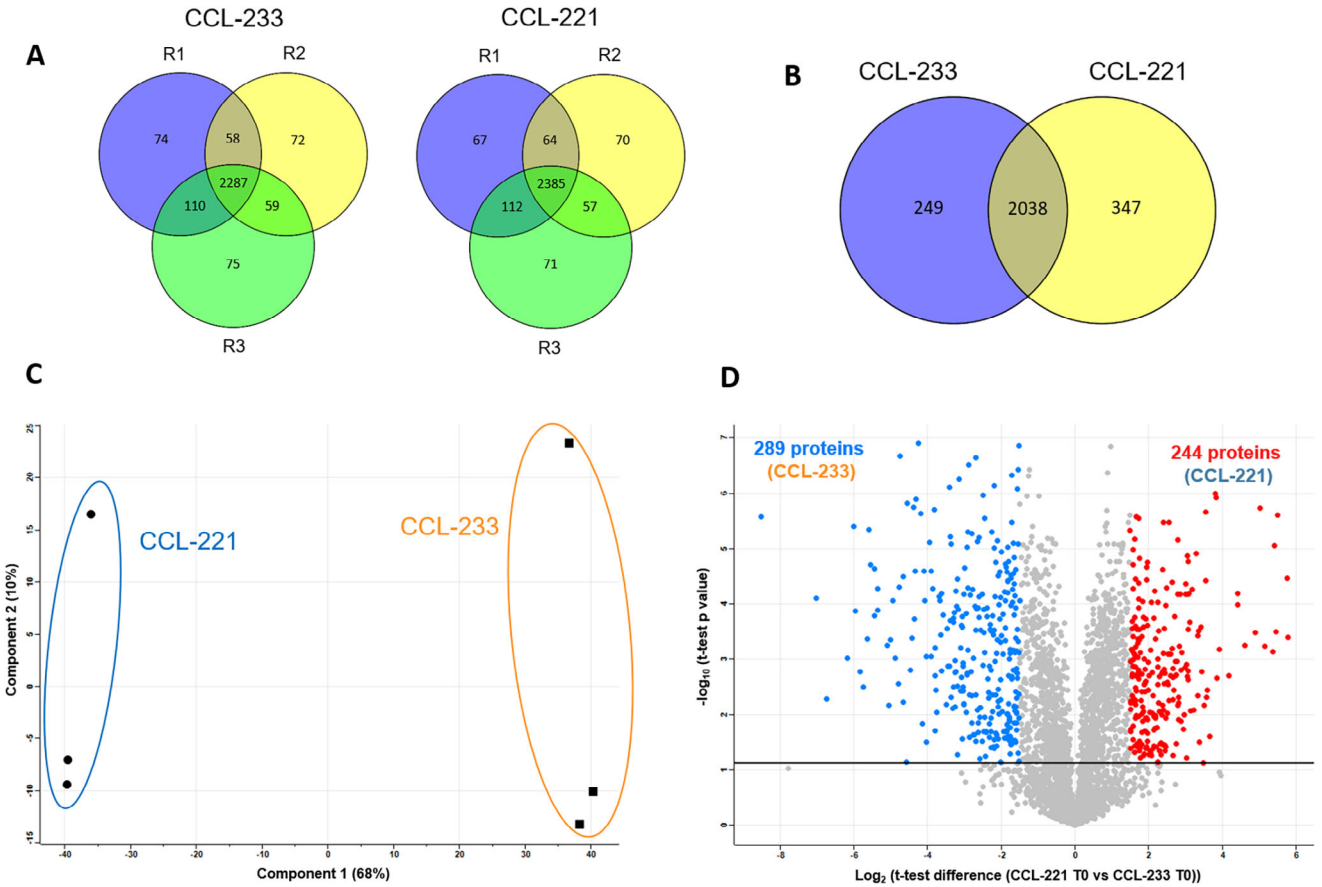

FIGURE 1 | Proteome profiles of CCL-233 and CCL-221 cells at basal state. (A) Venn diagram showing the comparison of the number of identified and quantified proteins between the three replicates (R1, R2 and R3) for CCL-233 and CCL-221 cells. (B) Venn diagram showing the comparison of the number of proteins common and exclusive to both CCL-233 and CCL-221 cells based on the common protein replicate lists for each cell line. (C) Principal component analysis of the CCL-233 and CCL-221 cells. (D) Volcano plot representing proteins differentially expressed in CCL-233 and CCL-221 cells (Student's *t*-test, permutated based-FDR adjusted $p < 0.05$). Proteins significantly overexpressed in CCL-233 cells are represented in blue and in CCL-221 cells are represented in red with a cut-off at $|\log_2(\text{Fold Change})| \geq 1.5$.

the proteins of the UPR pathway were the most represented with chaperones and co-chaperones (Heat Shock Protein HSP 90-beta [HSP90AB1], BAG family molecular chaperone regulator 2 [BAG2]) and proteins of transport and localisation of proteins (protein transport protein SEC23B [SEC23B], Cytoskeleton-associated protein 4 [CKAP4] and Translocon-associated protein subunit alpha [SSR1]) (Table 1).

These results showed that, at basal state, CCL-233 and CCL-221 cells expressed already proteins involved in the ER stress, but CCL-233 cells expressed more proteins involved in the apoptosis pathway whereas CCL-221 cells expressed more proteins involved in the UPR pathway.

3.3 | Impact of Induction of the UPR on Proteomic Profiles in CCL-233 and CCL-221 Cells

Cellular stress is in cancer cells and changes their metabolism to enhance survival and proliferation [29]. One of the mechanisms involved in these processes is the UPR. The study of the expression of UPR proteins in CRC could be used to better determine the stage of cancer and its malignancy, as demonstrated in melanoma, and understand the resistance to some anti-cancer therapies [31]. To explore this hypothesis, we performed stimulation with UPR inductors to assess whether UPR pathways were altered in our cell lines, which could lead to a different response to cellular stress.

Each cell line was then analysed at the proteomic level after induction of the UPR to compare changes under stress conditions (stressed) and basal conditions (unstressed). Among the 3302 proteins identified, 2910 proteins were differentially expressed (ANOVA, $p < 0.05$) between the two cell lines, all conditions considered. PCA was performed and showed again a clear separation in groups of the two cell lines with a 'component 1' explaining 48% of variance which confirms that cell lines have different functional programme at the proteomic level and 'component 2' (8%) which separated the different conditions for each cell line. CCL-233 cell line showed few differences between the conditions unstressed and stressed, whereas in CCL-221 cells differences were observed depending on the time (24 or 48 h) and the condition of stimulation with BFA or BTZ compared to unstimulated cells (Figure 3A). The proteome profiles of each condition were more visible on separate PCA by cell line (Figure S3). Using a volcano plot representation with all proteins identified and a cut off at $|\log_2(FC)| \geq 1.5$, the Student's *t*-test (permutated based-

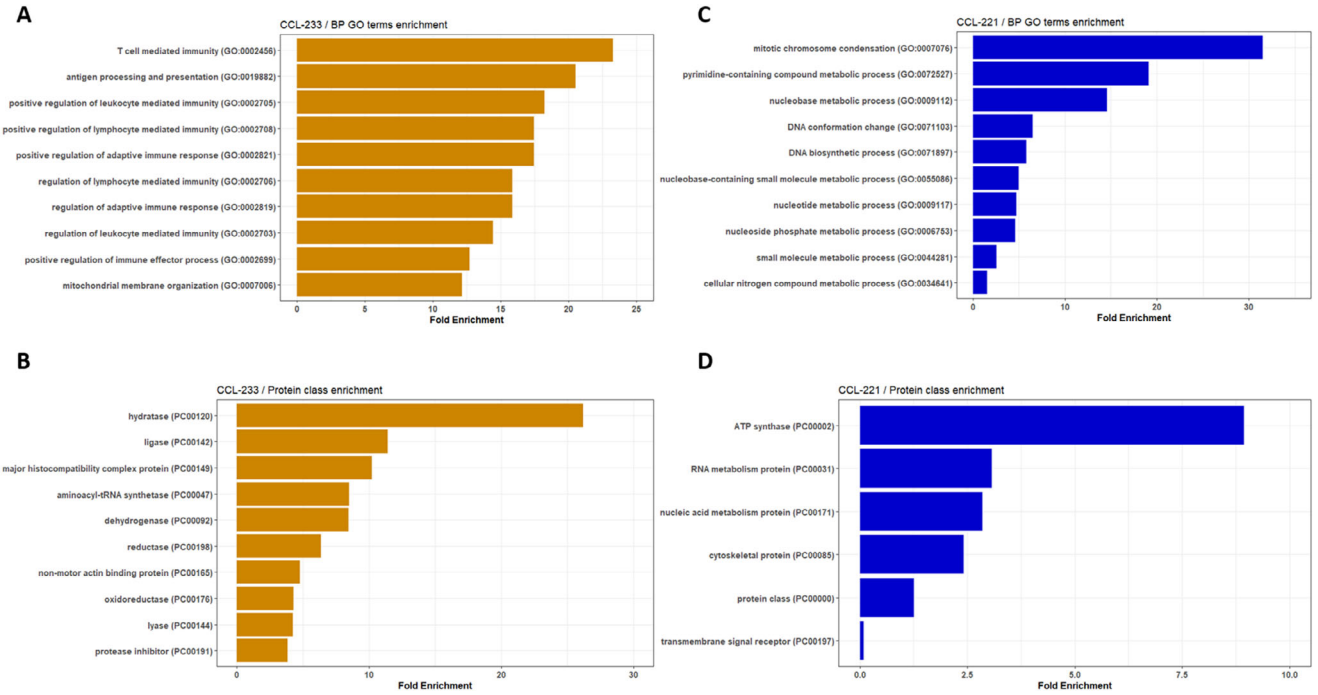

FIGURE 2 | Top 10 of biological process (BP) GO terms and protein class (PC) enriched in CCL-233 cells and CCL-221 cells at basal state generated with significant ANOVA proteins lists from Perseus analysis. (A) BP GO terms enriched in CCL-233 cells. (B) PC enriched in CCL-233 cells. (C) BP GO terms enriched in CCL-221 cells. (D) PC enriched in CCL-221 cells. All BP GO terms and PC represented by fold enrichment had a significant adjusted p value (Fisher's exact test, adjusted $p < 0.05$, Benjamini–Hochberg-FDR method).

TABLE 1 | Proteins of the unfolded protein response (UPR), the autophagy and the apoptosis identified in CCL-233 and CCL-221 cells at basal state (adjusted $p < 0.05$, $\log_2$(Fold Change)| ≥ 1.5).

	CCL-233	**CCL-221**
	Proteins from Perseus analysis ($\lvert\log_2(FC)\rvert \geq 1.5$)	**Proteins from Perseus analysis ($\lvert\log_2(FC)\rvert \geq 1.5$)**
UPR	UGGT1; ERLEC1; SEC31A	BAG2; CKAP4; SSR1; SEC23B; HSP90AB1
Autophagy	VAMP8; PRKCD; LAMP1	SMC2; PRKACB; RAB8A
Apoptosis	DIABLO; CTSS; ITPR3; CTSH; CTSC; CTSZ; PARP4	BAX; CASP3; PARP1

FDR adjusted $p < 0.05$) revealed protein characteristics to each condition unstressed and stressed for each cell line. For example, in CCL-223 cells after 48 h of stimulation with BFA, 18 proteins were overexpressed and 26 were underexpressed in stressed condition compared to unstressed condition, while in CCL-221 cells the same comparison revealed 101 proteins overexpressed and 66 proteins underexpressed (Figure 3B). To illustrate the dynamic of expression level under UPR-inducing agents compared to unstimulated cells, we generated heatmaps considering $\log_2$(FC) with only significant proteins with a $|\log_2(FC)| \geq 1.5$ for each cell line (Figure S4). Taken together, these results showed that fewer proteins were differentially expressed in the CCL-233 cells than in CCL-221 cells after UPR induction. These observations suggested that the CCL-233 cells proteome was less affected by the UPR induction than CCL-221 cells. Yet, considering each cell line independently, the number of proteins differentially expressed increased with the time of stimulation whatever the pharmacological agent.

To better decipher the proteome profiles, BP, molecular function (MF) and cellular component (CC) GO terms enrichment analysis was performed with the differentially expressed proteins for each comparison. As expected, the very low number of differentially expressed proteins in CCL-233 cell conditions did not allow to define enrichments except for the BTZ condition after 48 h of stimulation with an overrepresentation of CC GO terms related to mitochondrial complexes, ribosomes and cytoplasm. For CCL-221 cells, after UPR induction with BFA, enrichment analysis showed an overrepresentation GO terms mainly related to proteins processing (folding and degradation) and transcription activity and regulation. With BTZ, GO terms enriched and overrepresented were mostly related to protein processing (folding and binding), adaptive immunity and RNA splicing. Conversely, other GO terms involved in DNA binding and transcription regulation were underrepresented with both UPR inductors (Tables S4 and S5; and Figure S5). Overall, GO terms enriched in the stressed conditions were related to the consequences of the activation of

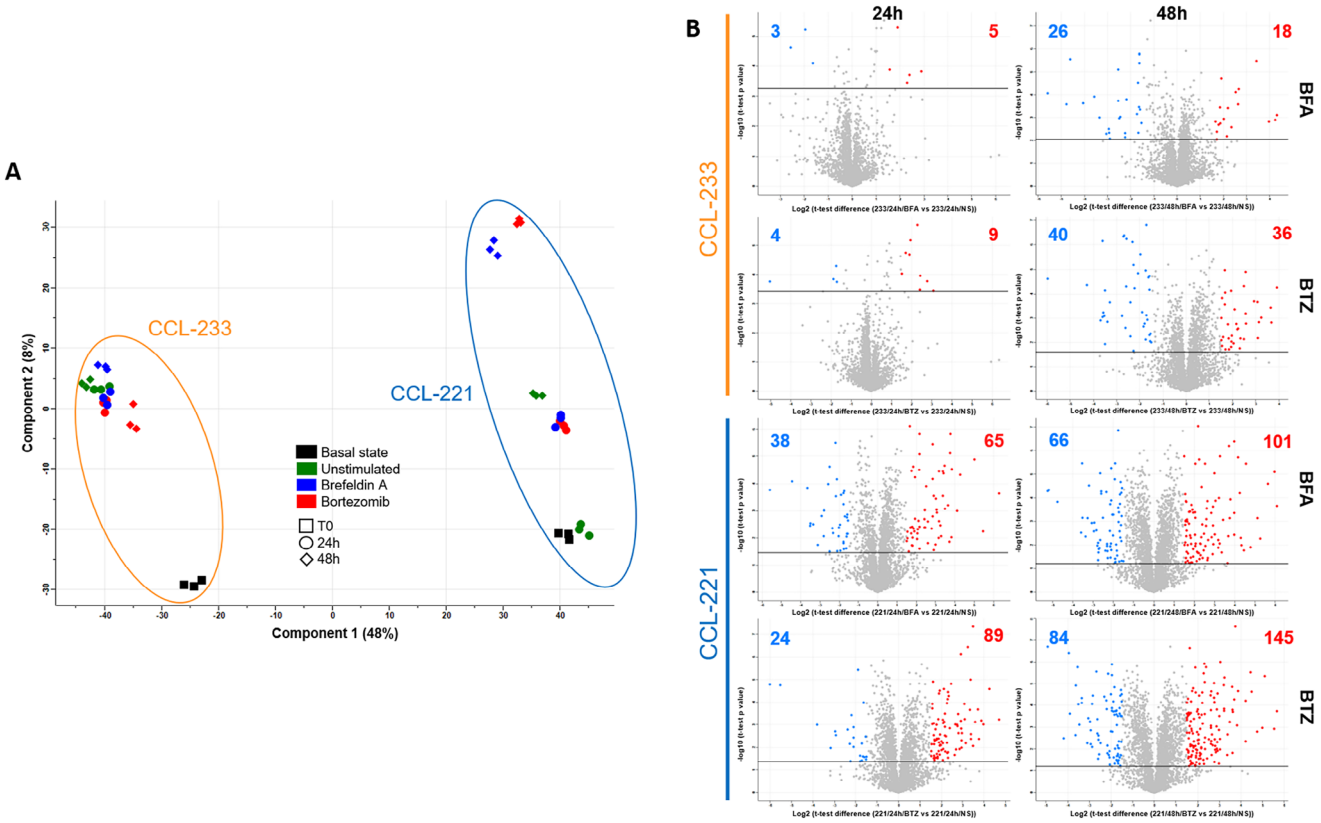

FIGURE 3 | Perseus analysis of proteins identified in CCL-233 and CCL-221 cells after 24 or 48 h of stimulation with brefeldin A (BFA) or bortezomib (BTZ). (A) Principal component analysis of the CCL-233 and CCL-221 cells at basal state (black), unstimulated (green), with BFA (blue) or BTZ (red). (B) Volcano plots representing proteins differentially expressed in CCL-233 cells (up panel) and CCL-221 cells (down panel) after induction of the UPR compared to the baseline condition (Student's *t*-test, permutated based-FDR adjusted $p < 0.05$). Only significant proteins with $|\log_2(\text{Fold Change})| \geq 1.5$ are coloured. Proteins overexpressed in BFA or BTZ condition are represented in red and proteins underexpressed are represented in blue.

the UPR, suggesting a pathway to restore homeostasis in the ER by decreasing protein translation, promoting mRNA degradation, increasing the synthesis of chaperone proteins to promote protein folding, and degrading unfolded or excess proteins [32]. Yet, the induction of cellular stress on CCL-233 cells seemed to have little impact on cell biology, even on UPR, autophagy and apoptosis pathways.

To support these previous observations with GO terms enrichment, a focus on the UPR, the autophagy and the apoptosis was realised in the basal state with the same methodology. For CCL-233 cells, only two proteins involved in the UPR and apoptosis were identified in the condition with BTZ after 48 h of stimulation. Ubiquilin-1 (UBQLN1), involved in many mechanisms and pathways leading to protein degradation, was overexpressed and cathepsin C (CTSC) implicated in the proteolysis was underexpressed. In these cells with our experimental conditions, the analysis did not reveal a clear induction of UPR pathways, nor autophagy and apoptosis, contrary to what was observed for CCL-221 cells. All UPR, autophagy and apoptosis proteins identified in CCL-221 cells are summarised in Table 2. Most of the proteins overexpressed after UPR induction with BFA were proteins involved in the UPR pathway, like chaperones BiP (HSPA5) and DnaJ homolog subfamily C member 5 (DNAJC5), transport proteins from the ER to the Golgi apparatus as protein transport protein SEC24D (SEC24D) and protein SEC 13 homolog (SEC13), and proteins of the endoplasmic reticulum-associated degradation (ERAD) pathway as endoplasmic reticulum lectin 1 (ERLEC1), synoviolin 1 (SYVN-1), homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 protein (HERPUD1) and protein sel-1 homolog 1 (SEL1L). For autophagy, endophilin-B1 (SH3GLB1) and Ras-related protein R-Ras2 (RRAS2) proteins were overexpressed and involved in the regulation of the autophagy pathway. For the apoptosis pathway, cathepsin L (CTSL) was overexpressed and implicated in antigen processing, and caspase 3 (CASP3) was underexpressed and involved in the activation of the apoptosis. For BTZ, most of the proteins were also involved in the UPR pathway like BiP, co-chaperones as heat shock 70 kDa protein 1B (HSPA1B), heat shock 70 kDa protein 6 (HSPA6) and DnaJ homolog subfamily A member 1 (DNAJA1), proteins of the ERAD pathway as HERPUD1 and SEL1L, and transport protein from the ER to the Golgi apparatus (SEC13). For the autophagy pathway, the same two proteins overexpressed with BFA, SH3GLB1 and RRAS2, were present in addition to sequestosome-1 (SQSTM1) protein which acts as a bridge between polyubiquitinated proteins and autophagosomes, and protein kinase C delta type (PRKCD) and 5′-AMP-activated protein kinase catalytic subunit alpha-1 (PRKAA1) which are involved respectively in the promotion of the apoptosis and the transcription. Finally, proteins of the apoptosis were also modulated. The proteins overexpressed were CTSL, dual specificity mitogen-activated protein kinase kinase

TABLE 2 | Proteins of the unfolded protein response (UPR), the autophagy and the apoptosis identified in CCL-221 cells after 24 and 48 h of stimulation with brefeldin A (BFA) or bortezomib (BTZ). Proteins overexpressed in BFA and BTZ conditions are identified in red and proteins underexpressed are identified in green with a cut-off at $|\log_2(\text{Fold Change})| \geq 1.5$ (adjusted $p < 0.05$).

	CCL-221			
	BFA		BTZ	
	24 h	48 h	24 h	48 h
UPR	ERLEC1; SYVN1; SEC24D; HERPUD1; HSPA5 (BiP)	SEL1L; ERLEC1; HERPUD1; HSPA5 (BiP); DNAJC5; SEC13; HEBP1	HSPA1B; HERPUD1; HSPA6; DNAJB1	HERPUD1; HSPA1B; SEL1L; HSPA6; DNAJB1; HSPA5 (BiP); SEC13; DNAJA1
Autophagy	SH3GLB1	RRAS2	SH3GLB1; SQSTM1	SQSTM1; PRKCD; RRAS2; PRKAA1
Apoptosis	CTSL	CASP3	CTSL	MAP2K1; PARP4; CASP3; CTSH; CTSB

1 (MAP2K1) protein implicated in the ERK pathway and poly (ADP-ribose) polymerase 1 (PARP1) protein involved in ribosylation. CASP3 and two cathepsins (CTSB and CTSH) involved in the degradation of proteins were underexpressed (Table 2).

4 | Discussion

CRC is the result of the accumulation of multiple and diverse genetic mutations. Depending on their molecular state (mutations, metabolism, stress …), the cancer cells expressed different tumour profiles that can define the stages of carcinogenesis [33, 34]. The global proteome expressed by the studied cancer cell lines was different. CCL-233 cells from tumour grades I–II revealed a proteome profile more oriented toward metabolic profile than CCL-221 cells from tumour grade III which had a profile more dedicated to transcription. These results are in line with the study of Frejno et al. who defined different molecular signatures and subtypes of CRC from the integration of multi-omics data. They showed that CCL-233 cells had 'a full proteome subtype (FPS) A' with a 'high metabolism, low cell cycle, microsatellite stable' signature while CCL-221 had a 'FPS C' with a 'high immune response, low metabolism, microsatellite instable' signature [15]. The differences in molecular profiles, described in the literature and observed in our study, illustrate the cellular processes dynamic in cancer cells during tumorigenesis. Cancer cells undergo changes to promote proliferation and survival due to a complete overhaul in their metabolism arising from transcriptional reprogramming [35]. The cellular changes included an increase in the capacity of the cells to synthesise new molecules and control gene expression to promote the tumour growth [5]. The identification of molecular patterns in cancer cells is a promising way to determine resistance to cancer treatment and develop precision oncology [36, 37]. Recent studies showed that characterization for molecular subtypes, mainly by genomic and proteomic, could help to model response to drugs and so on predict resistance to treatment and identify new therapeutic targets. Indeed, high-throughput techniques like LC-MS/MS, DNA microarray or RNA sequencing have identified in CRC patient tumours new potential protein biomarkers for the diagnosis and predicted the effect of a large panel of drug treatments [14, 38].

Numerous studies have shown that cancer cells are submitted to extrinsic and intrinsic stresses leading to ER stress [7]. Despite the overexpression of apoptosis proteins, CCL-233 cells could be cultivated in vitro which means that they were de facto resistant to apoptosis like numerous cancer cells by the activation of anti-apoptotic signals under stress conditions [39]. Interestingly, some proteins differentially expressed in CCL-221 cells were proteins involved in the UPR pathway compared to CCL-233 cells. These observations suggested that the CCL-221 cells might suffer from an ER stress and have an active UPR at basal state due to an accumulation of proteins not folded or misfolded.

The activation of the UPR showed differences at proteomic level between the two cell lines and allowed us to identify proteins involved in key cellular processes involved in cancer. UPR, autophagy and apoptosis-associated protein expression is well identified by LC-MS/MS. CCL-233 cells seemed to respond less and later to the UPR induction than CCL-221 cells. For CCL-233 cells, only two proteins were identified after 48 h of induction of the UPR only with BTZ which suggested that cells did not react to the UPR induction or they were able to resist the induction of ER stress in our experimental conditions. For CCL-221 cells, the greater response to UPR induction might be related to the presence of active UPR activity at the basal state. Both cell lines expressed protein profiles in favour of cell survival, commonly found in cancer resistance to apoptosis [40]. In addition, the absence of an overexpression of pro-apoptotic markers might suggest that the times of stimulation and/or the doses of BFA and BTZ used were maybe not sufficient to induce the terminal UPR or cells were in resolving their cellular stress. Another hypothesis to explain the lack of autophagy and apoptosis proteins identified could be related to the limitation of the LC-MS/MS technique used. Shotgun proteomic allows us to quickly identify proteins from complex mixtures like cell lysate but small and low abundant proteins were not detected. Thus, to complete our approach, it would be interesting to extend the time of stimulation and increase doses of UPR inducers, but also to add to our protocol a fractionation step of whole cell extracts before LC-MS/MS to identify the greater amount of proteins, especially those of UPR, autophagy and apoptosis. The addition of a normal colon epithelial cell line would strengthen our experimental design to compare the activation state of the UPR

between tumoural and normal cellular backgrounds. Regarding enrichment analysis, we did not use the quantified proteins as a background for correction. The reference gene list from *Homo sapiens* rather than the global list of identified proteins may lead to an overestimation of some GO terms and PC. However, we selected only significant proteins with a $|\log_2(FC)| \geq 1.5$ for enrichments and kept GO terms and PC with a p value < 0.05 and a q-value < 0.05 to mitigate this risk.

Our study also identified numerous chaperones, enzymes (cathepsins, caspases) and proteins involved in signalling pathways activated and altered in an inflammatory context (NFκB/JNK pathway, JAK/STAT pathway). Recent reviews highlighted the influence of the ER stress expressed in cancer cells on the tumour microenvironment (TME), inflammation and its potential transmission to other cells like immune cells. Tumour cells expressing a UPR activation could lead to the secretion of inflammatory regulators as cytokines or proteolytic enzymes as cathepsins released in the TME and interact with immune cells [41, 42]. Caspases were reported in cancer to be dysregulated and could lead to inflammation and tissue damage. Some of them are pro-apoptotic or anti-apoptotic and the dysregulation of their expression could determine the fate of cancer cells. Therefore, in cancer, caspases could be responsible for apoptosis and lead to the exposition and the release of intracellular content that may trigger specific immune system responses [43]. Cathepsins are involved in normal protein degradation and inflammatory processes. The dysregulation of these enzymes could lead to disturbance in these mechanisms and lead to cellular stress and tumour promotion [44]. ER stress could be also transmitted to non-tumour cells or immune cells through soluble factors such as tumour-derived extracellular vesicles and proteins [8, 45].

Using a simple in vitro model based on the comparison between two CRC cell lines, we demonstrated that the activation state of the UPR was different in baseline and after stimulation with UPR inducers. These data could explain the failure of some therapeutic strategies based on the up or down-regulation of key effectors in UPR pathways in these cancers. Moreover, these cells could be used to explore the role of cellular stress in the TME, the impact of UPR activation on immune cells, the mechanisms involved and find potential therapeutic targets to prevent tumour promotion, for example, by setting up a co-culture with immune cells.

5 | Conclusion

This study based on quantitative proteomics showed that CRC epithelial cell lines classified as early or late tumour grades had differences in their proteome illustrated by different metabolic profiles including the UPR activation state under or not an induced cellular stress. According to many studies, cancer cells are more or less already stressed which can condition the efficiency of treatments based on ER stress induction and lead to therapeutic failures. However, the crosstalk between UPR, autophagy and apoptosis pathways are not completely understood yet, especially in cancer field and need still investigations to develop more precise and efficient treatments.

Author Contributions

S.V., F.B., F.R., C.R., D.L., S.D., and V.S. conceptualised the project. S.V., F.B., S.F., and L.G. performed experiments. S.V. analysed the data and performed statistics. C.R., D.L., S.D., and V.S. supervised the project. S.V., S.D., and V.S. wrote the original draft. All authors reviewed and edited the manuscript.

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Conflicts of Interest

David Launay reports consulting fees from Takeda, CSL Behring, Biocryst and CSL Behring; outside the submitted work. Vincent Sobanski reports consulting and speaking fees from Boehringer Ingelheim, Grifols and Ultragenyx; and research support from CSL Behring, Grifols; outside the submitted work. The other authors declare no conflicts of interest.

Data Availability Statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD030505. Original data from GO terms analysis are available from the corresponding author.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.