Original Article
Probiotic-derived p8 protein induce apoptosis via regulation of RNF152 in colorectal cancer cells

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Abstract: Worldwide, colorectal cancer (CRC) is one of the most common cancers and is a leading cause of cancer-related deaths. Accumulating evidence suggests that probiotics suppress the development of various cancers including CRC. Recently, we reported a *Lactobacillus rhamnosus* (LR)-derived 8 kDa protein (p8) that displayed anti-cancer properties in CRC cells. However, the precise anti-cancer mechanism of p8 and its target genes has not been fully examined. In the present study, we reveal that p8 leads to apoptotic cells and cleaved PARP1 expression in a mouse xenograft model of CRC. Additionally, we identified Ring finger protein 152 (RNF152) as a putative target of p8 using RNA-sequencing. Furthermore, the expression levels of RNF152 were increased following *in vivo* and *in vitro* treatment with p8. We also found that p8 leads to the accumulation of cleaved PARP1 in CRC cells. These results suggest that p8 induces apoptosis via regulation of RNF152, thus inhibiting the development of CRC.

Keywords: Probiotics, p8, RNF152, colorectal cancer, RNA sequencing

Introduction

Colorectal cancer (CRC), the most common cancers worldwide, is a leading cause of cancer-related death in men and women globally. There are more than 1 million new CRC cases diagnosed per year and 600,000 CRC-related deaths. Globally, the number of new CRC patients has slowly but steadily increased during the past several decades [1, 2]. Surgery with or without radiotherapy is typically performed to improve outcomes for patients with CRC. Nevertheless, many CRC patients develop metastases, which result in poor survival. Moreover, traditional chemotherapeutic CRC treatments cause severe side effects (e.g., reduction of white blood cells or platelets, hair loss, vomiting). Therefore, many investigators are currently investigating novel therapeutic strategies for CRC to increase treatment effectiveness and reduce side effects.

Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host [3]. Many studies have reported that probiotics provide beneficial effects to humans by modulating the immune system and gut barrier function and through anti-inflammatory pathways. Accumulating evidence from both human-cell line and mouse studies demonstrate that probiotics may also suppress the progression of various types of tumors (e.g., gastrointestinal, cervical, lung, breast, melanomas, hepatic) to varying degrees [4]. For example, *Lactobacillus* inhibits proliferation and induces apoptosis in CRC cells *in vivo* and *in vitro* [5, 6]. *Enterococcus* and *Lactococcus* show strong cytotoxicity effects and induce apoptosis in breast, lung, and cervical cancer cells [7, 8]. In addition, treatment with *Bifidobacterium* more effectively decreased tumor size in mice bearing melanoma compared with control treatment [9]. Taken together, these results suggest that probiotics can be selected for cancer prevention or/and treatment through their anti-cancer activities.

Recently, we isolated a *Lactobacillus rhamnosus* (LR)-derived 8 kDa protein (p8), which demonstrated anti-cancer properties in CRC cells. In addition, oral administration of *Pediococcus pentosaceus* strain SL4 (PP) harboring a p8 significantly inhibited tumor growth in a mouse xenograft model [10]. Moreover, side effects associated with this treatment were minimal, confirming that p8 had little toxicity. These

results show that p8 has the potential to be a new treatment for CRC. Nevertheless, the precise anti-cancer mechanism of p8 has not been identified. In this study, we observed that p8 induced apoptosis of CRC cells in a mouse xenograft model. Moreover, we analyzed gene expression using RNA-sequencing to identify targets of p8 and demonstrated that the expression of RNF152 was increased following treatment with p8 in CRC cells. These findings suggest that p8 induces apoptosis via regulation of RNF152, thus inhibiting CRC development.

Materials and methods

Bacterial strains and culture

Lactobacillus rhamnosus (LR, KCTC 12202BP) and *Pediococcus pentosaceus* strain (PP, KCTC 10297BP) were isolated from human feces obtained in Korea and Korean fermented vegetable product (kimchi), respectively. LR, PP, PP-p8, and PP-Ev were maintained in MRS broth (Difco, Detroit, MI, USA) at 37°C.

Animals

BALB/cAnN.Cg-Foxn1nu/CrlNarl mice (5 weeks) were bred with constant temperature (23°C), humidity (50%), and ventilation (10-12 times per hour). In accordance with the study schedule, mice were sacrificed with CO_2 overdose. The Institutional Animal Care and Use Committee at CellBiotech approved all animal experiments.

Plasmid Construction harboring DDS-p8 for use in the Pediococcus pentosaceus SL4 system

To generate PP-p8 for use in a mouse xenograft model, pPK-usp45-COSp8 (701 bp), pPK-palr-alr (1399 bp), and pPK-usp45-orip8 (701 bp) were sequentially cloned into pCBT24-2 (KCCM12182P) yielding pCBT24-2-pPK-usp45-COSp8-pPK-usp45-orip8-palr-alr. This plasmid was then transformed into PP cells.

Mouse xenograft model

DLD-1 colorectal cancer cells were used to generate a human-derived colon cancer xenograft model. Cells were inoculated subcutaneously in mice (2×10^6 cells in PBS) to initiate tumor development. After seven days, tumor volume

(mean: approximately 100-150 mm³) and weigh were measured. Treatments were initiated seven days post tumor inoculation with oral administration of 3.5×10^{11} CFU/Kg PP-Ev (empty vector) or PP-p8-W/L DDS (W: wet pellet, L: lyophilization) or 60 mg/Kg of 5-fluorouracil, respectively. Treatments were administered five times per week for six weeks.

Immunohistochemistry

Paraffin sections of human colon tissue (normal and tumor) were purchased from GeneTex. Each tissue section of human colon or mouse xenograft tissue was fixed. and performed antigen retrieval. After blocking and incubation with antibodies against P8 (1:100, Abfrontier, Korea), RNF152 (1:100, Santa Cruz), or Cleaved PARP1 (1:100, Abcam) overnight at 4°C, Signal detection was achieved using a Mouse and Rabbit Specific HRP/DAB (ABC) Detection IHC kit (Abcam). The signals were detected with a BX51 fluorescence microscope (Olympus), and the relative IHC intensity was determined using ImageJ software.

TUNEL assay

Each tissue section was immersed in xylene and ethanol for deparaffinization and rehydration. The TUNEL assay was then used to quantify the number of apoptotic cells using a TUNEL Assay Kit - HRP-DAB (Abcam, Cambridge, UK) following manufacturer's instructions. The Slides were observed with a BX51 fluorescence microscope (Olympus, Japan).

Cell culture

DLD-1 and HCT116 cells were obtained from the Korean Cell Line Bank. Both cells were maintained in RPMI-1640 medium (Invitrogen) containing 10% fetal bovine serum in a 37°C incubator. For the P8-target analysis, DLD-1 and HCT116 were plated at a density of 5×10^4 cells per well. After 24 h, either r-p8 protein (20 μM) or RNF152 siRNA (100 nm, Genepharma, China) was added to cells and harvested 48 h after treatment for extraction of total RNA or protein.

Purification of recombinant p8 protein from E. coli

Recombinant p8 (r-p8) protein was purified as described previously [10]. Briefly, full-length p8

was cloned into the pET-22b expression vector and transformed into *E. coli* strain C41(DE3) (Novagen, Madison, WI). Cells were cultured in M9 medium with 0.5 mM IPTG for 4 h and then purified by removing the 6 × His tag using TEV protease.

RNA sequencing

Total pooling of RNA that combined three individual samples was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA quality was determined using Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). After random fragmentation, cDNA was synthesized using random primers. PCR products were then ligated to Illumina sequencing adapters and selected to 200~400 bp. Sequencing was conducted by Macrogen (Korea).

RT-PCR and Real-time PCR

To prepare total RNA from CRC cells and mouse xenograft tissues, Trizol reagent (Invitrogen) was used. The PrimeScript 1st Strand cDNA Synthesis kit (Takara, Japan) was used for cDNA synthesis. RT-PCR and Real-time PCR were carried out using a MasterCycler X50a (Eppendorf, Germany) and a LightCycler480 (Roche, Basel, Switzerland), respectively. Expression levels were analyzed using the comparative ΔΔCt method [11]. Sequences of primers were as follows: RNF152 F, 5'-ACACC-TGCTGTTCAGTGTGC-3' and RNF152 R, 5'-GTGT-GTTCGGAAGTGTGTGG-3'; GAPDH F, 5'-ATGTT-CGTCATGGGTGTGAA-3' and GAPDH R, 5'-GTC-TTCTGGGTGGCAGTGAT-3'.

Western blot analysis

Total protein of CRC cells and mouse xenograft tissues were isolated using a RIPA buffer (Elpis-Biotech, Korea). Each protein was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Then it was incubated with a rabbit polyclonal RNF152 antibody (1:200, Santa Cruz, CA) or a mouse monoclonal β-Actin antibody (1:2500, Santa Cruz). The signal was detected using Pico EPD Western blot detection reagents (Elpis-Biotech).

Cell proliferation assay

To assess proliferation, DLD-1 and HCT116 cells were treated with r-p8 protein (20 µM) or RNF152 siRNA (100 nm). The relative viability of the cells was counted using a Cell Counting Kit-8 (Dogen, Korea). Absorbance was measured using a multifunctional microplate reader SpectraMax M5 (Molecular Devices, CA, USA).

Statistical analysis

Student's t-test was used to evaluation of Statistical significance. P<0.05 was regarded as statistically significant.

Results

Oral administration of PP-p8 induces CRC cell apoptosis in a mouse xenograft model

First, the anti-cancer effects of two forms of PP-p8 [wet pellet (PP-p8-W) and lyophilized (PP-p8-L)] were characterized in a mouse xenograft model. We observed a significant inhibition of tumor growth following administration of PP-p8 DDS compared with the control group (PP-Ev, Empty vector, **Figure 1A**). Mean tumor weights and volumes were significantly reduced following both wet pellet and lyophilized oral administration of PP-p8 compared with tumors in control mice (**Figure 1B**, **1C**).

To determine if the anti-cancer effect of PP-p8 was associated with uptake of p8 into CRC cells, we performed immunostaining using a p8-specific antibody. The P8 protein was detected in the cytosol of xenograft tumor tissue (**Figure 1D**).

Next, a TUNEL assay was performed to determine whether the anti-cancer activity of PP-p8 is mediated by apoptosis. As shown in **Figure 1E** and **1F**, the number of apoptotic cells was markedly increased in each group administered PP-p8. Taken together, these data indicate that uptake of p8 directly inhibits CRC cell growth by causing apoptosis.

*RNA sequencing reveals the gene expression profile of human CRC cells treated with r-p8*

To identify potential targets of p8 in CRC cells, genes that were differentially expressed in DLD-1 cells treated with r-p8 were characterized using RNA sequencing. Total of 30 differentially expressed genes were found in r-p8 treated DLD-1 cells (**Figure 2A**, **2B**). Briefly, 18 genes were increased and 12 genes were decreased in the r-p8 treated DLD-1 cells compared to control (**Tables 1** and **2**). In the current study, we focused on the regulation of RNF152 because i) its expression changed in r-p8 treat-
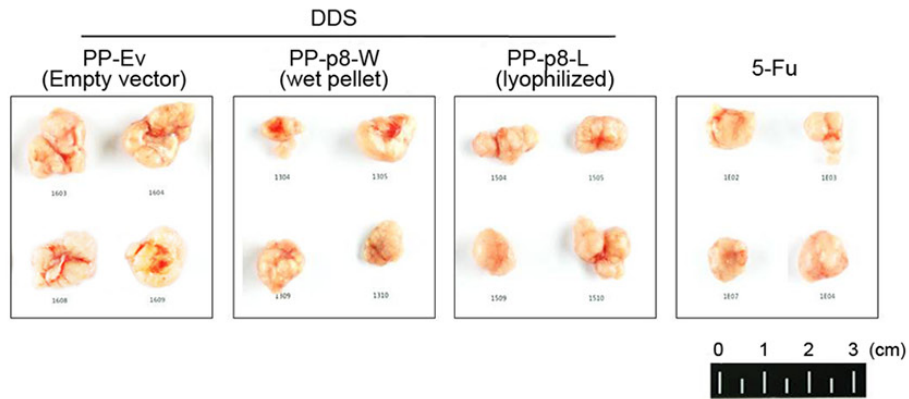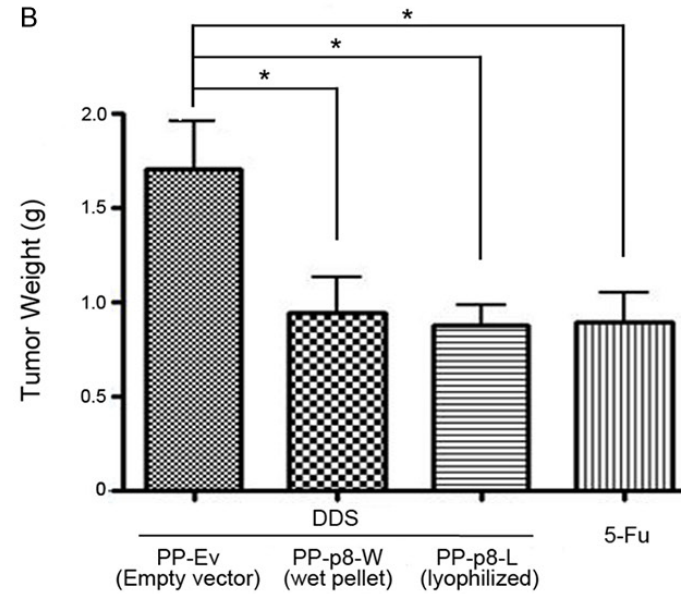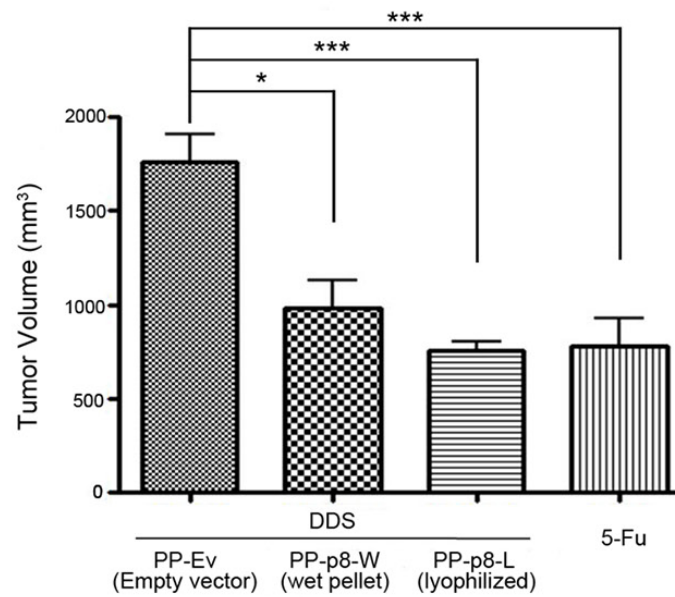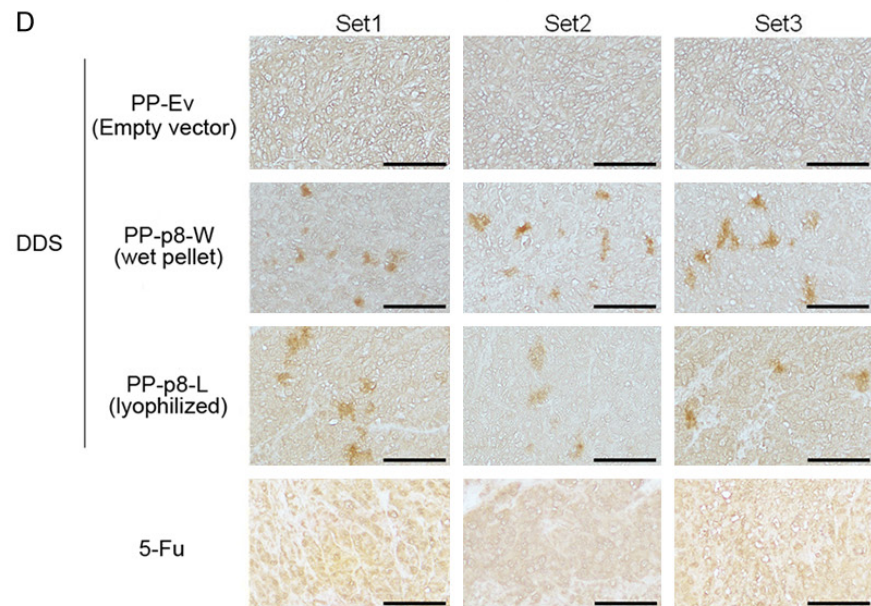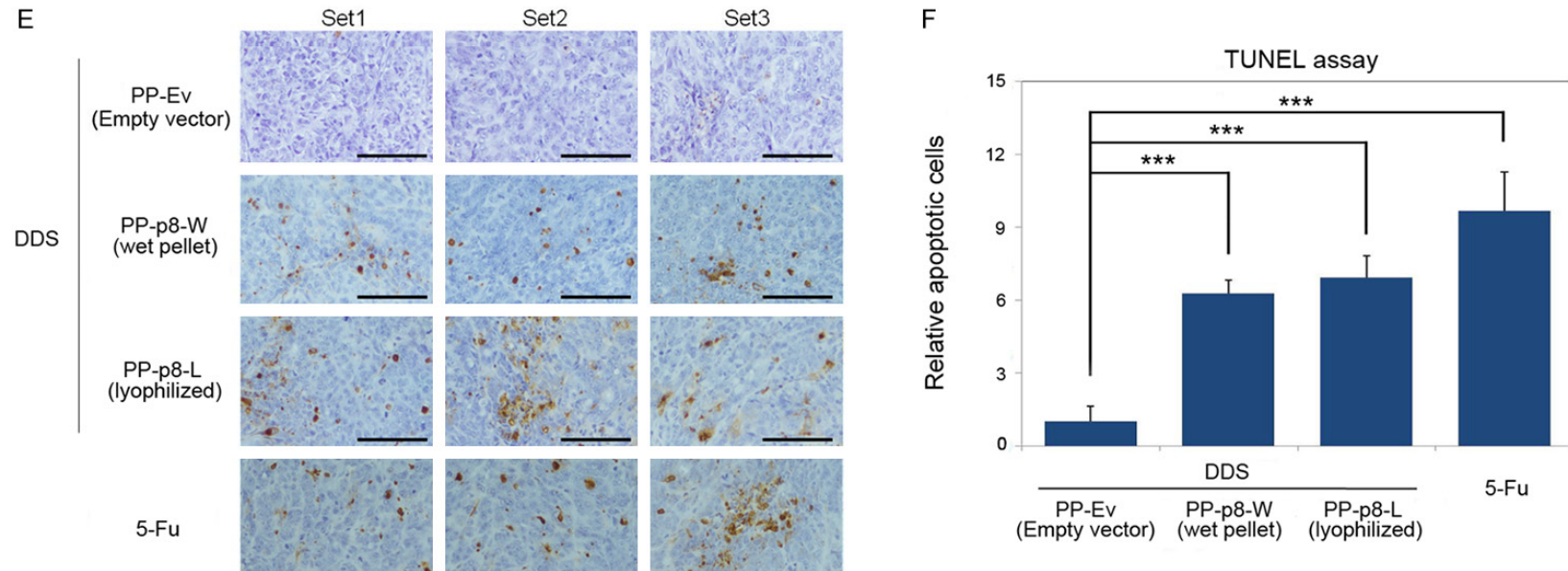
Figure 1. Oral administration of PP-p8 leads to apoptosis of CRC cells *in vivo*. A-C. Tumor growth was significantly inhibited following PP-p8 DDS administration compared to mice administered a control treatment. Ev: empty vector, W: original broth (cell pellet), L: lyophilization. D. P8 protein was detected by immunohistochemistry in the cytosol of xenograft tumor tissue cells. E, F. The TUNEL assay revealed that the numbers of apoptotic CRC cells in xenograft tumor tissues were increased following administration of PP-p8. Scale bar = 100 μm.
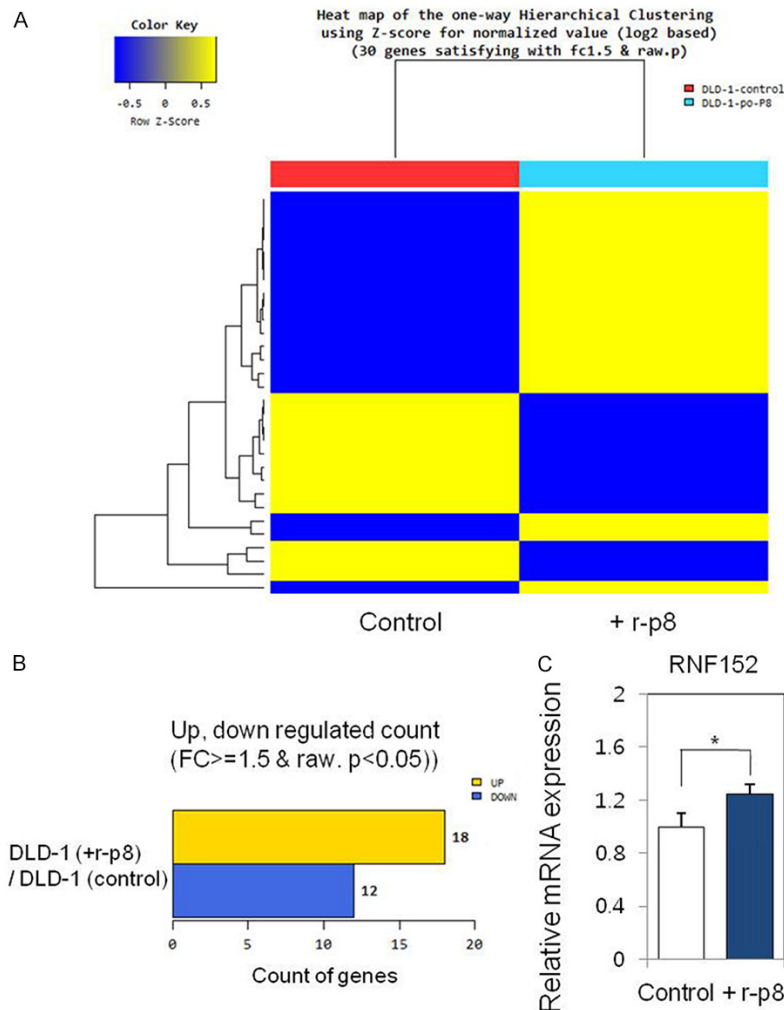
Figure 2. RNA sequencing to generate gene expression profiles in human CRC cells treated with recombinant p8 (r-p8). A. Cluster analysis of RNA-sequencing data revealed differential gene expression between DLD-1 cells treated with r-p8 (20 μM) and control. B. Summary of RNA-sequencing data analysis. The expression levels of 30 genes were altered following treatment with r-p8. C. Validation of RNF152 expression determined using RNA-sequencing by Real-time PCR. RNF152 mRNA was significantly increased following treatment with r-p8 (20 μM) in DLD-1 cells. Results are presented as the average of three independent experiments conducted in duplicate. *P<0.05.

ed DLD-1 cells and ii) it has been reported to have a role in the inhibition of CRC by inducing apoptosis [12]. The RNA sequencing results were confirmed by real-time PCR performed using the same RNA templates; the expression of RNF152 was significantly up-regulated in DLD-1 cells treated with r-p8 compared with control (**Figure 2C**).

RNF152 expression is up-regulated in human CRC

A recent report demonstrated that down-regulation of RNF152 is associated with poor prog-

nosis in patients with CRC [12]. To further confirm the expression of RNF152 in CRC patients, we analyzed data from four CRC cohorts using the Gene Expression Omnibus (GEO) database (GSE44076, GSE41657, GSE21510, and GSE32323). Consistent with previous reports, down-regulation of RNF152 expression was detected in all four CRC cohorts compared with normal tissue (**Figure 3A**). Next, we carried out immunohistochemistry to detect levels of RNF152 to further confirm the GEO data. As shown in **Figure 3B** and **3C**, the expression of RNF152 was decreased in CRC samples compared to non-tumor. These results further support the notion that decreased expression of RNF-152 is related to the development of CRC.

P8 regulates RNF152 expression in human CRC cell lines and a mouse xenograft model of CRC

To investigate whether r-p8 up-regulates RNF152 expression in CRC cells, we investigated the relative RNF152 mRNA expression in DLD-1 and HCT116 cells treated with r-p8 using real-time PCR. As shown in **Figure 4A**, treatment of r-p8 increased RNF152 expression in both DLD-1 and HCT116 cells compared with control cells. Similarly, western blot analysis demonstrated that RNF152 protein expression was up-regulated in both cell lines following treatment with r-p8 (**Figure 4B**, **4C**).

Previously, we reported that r-p8 entered CRC cells through endocytosis [10]. To further examine whether r-p8 up-regulates RNF152 expression through endocytosis, we investigated the expression of RNF152 at the mRNA and protein levels by administering r-p8 after inhibition of endocytosis using MitMAB. These experiments

Table 1. Up-regulated genes in the DLD-1 cells with r-p8 treatment compared with control (>1.5-fold, *P*<0.05)

Gene Symbol	Fold change	*p*-value
NUTM2A	77.673045	0.01615447
SNORA74D	77.673045	0.01615447
MXRA8	70.005740	0.02277515
SMIM17	62.338436	0.03276425
LOC101059915	62.338436	0.03276425
LOC101927854	62.338436	0.03276425
ENKUR	54.671131	0.04821909
TAS2R43	54.671131	0.04821909
SH2D4B	54.671131	0.04821909
MIR7113	54.671131	0.04821909
RNASEK-C17orf49	11.571471	0.02670602
FLJ38576	9.114757	0.04579963
CRYM-AS1	7.842345	0.02732206
MIR6087	6.288681	0.00779145
NPW	6.210115	0.03224147
C3	5.147757	0.04202812
RNF152	4.693715	0.01190955
CXCL1	4.473120	0.0310489

Table 2. Down-regulated genes in the DLD-1 cells with r-p8 treatment compared with control (>1.5-fold, *P*<0.05)

Gene Symbol	Fold change	*p*-value
LOC441178	-5.127781	0.04469739
NPIPB8	-8.065211	0.00420389
SNORD34	-11.693893	0.03478097
MIR4784	-13.623646	0.01622558
HBA2	-18.448027	0.0054041
HBB	-27.758883	2.6127E-05
RN7SL3	-40.640184	0.00014793
PIR-FIGF	-59.540142	0.04821909
LOC101927070	-59.540142	0.04821909
PIGZ	-67.903020	0.03276425
ZFP91-CNTF	-67.903020	0.03276425
ANKRD6	-84.628774	0.01615447

indicated that neither RNF152 mRNA nor protein was altered by r-p8 when endocytosis was suppressed (**Figure 4D-F**).

Next, we investigated whether treatment with p8 similarly leads to an up-regulation of RNF152 expression *in vivo*. The relative expression of RNF152 mRNA and protein were evaluated in a DLD-1 derived xenograft model of CRC following oral administration of PP-p8.

Consistent with *in vitro* data, administration of PP-p8 increased the expression of both RNF152 mRNA and protein (**Figure 5A**, **5B**). Interestingly, RNF152 up-regulation was also detected in mice treated with 5-Fluorouracil. Immunohistochemistry was carried out using an anti-RNF152 antibody to further characterize the up-regulation of RNF152 following administration of PP-p8 in xenograft tumor tissue. We observed that xenograft tissue of mice orally administered PP-p8 had increased levels of RNF152 compared with tumors in control mice (**Figure 5C**). These results suggest that treatment with p8 up-regulates the expression of RNF152 *in vivo* and *in vitro*.
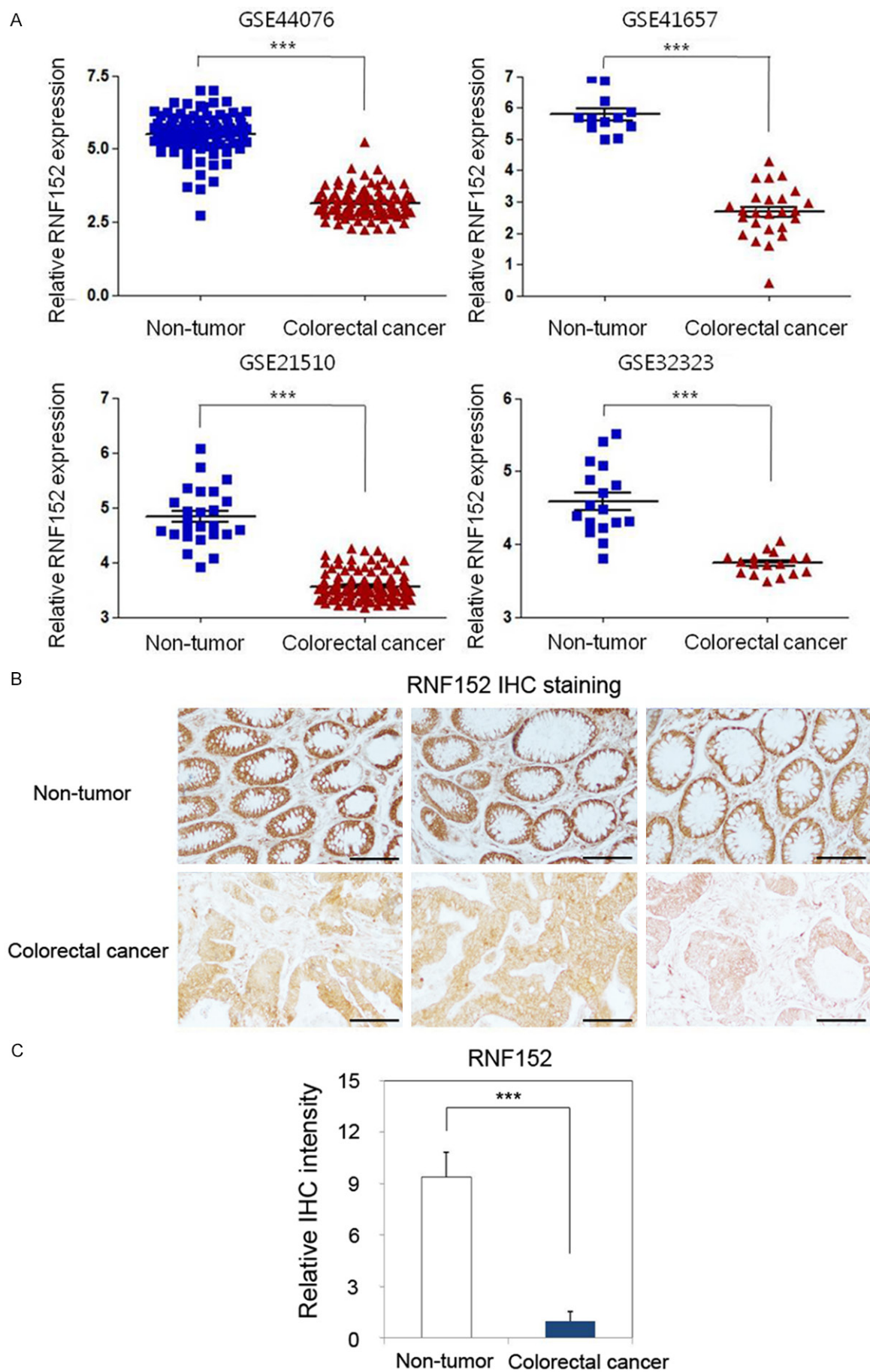
P8 leads to the accumulation of cleaved PARP1

Several recent studies have reported that RNF152 has pro-apoptotic activity mediated by inducing PARP1 degradation [12], therefore we quantified the levels of cleaved PARP1 in a DLD-1-derived xenograft model following oral administration of PP-p8. Immunohistochemistry revealed an increased abundance of cleaved PARP1 in xenograft tumor tissues compared with control tissues of mice treated with PP-p8 (**Figure 6A**). In addition, we also showed that cleaved PARP1 protein expression was up-regulated in both DLD-1 and HCT116 cells following treatment with r-p8 (**Figure 6B**, **6C**). Our data suggest that p8 induces the RNF152-cleaved PARP1 signal pathway, resulting in apoptosis of CRC cells *in vivo*.

Knockdown of RNF152 expression partially rescued by r-p8

Next, we tested whether knockdown of RNF152 could be rescued by treatment with r-p8. First, the expression of both RNF152 mRNA and protein was determined using RNF152 siRNA in DLD-1 and HCT116 cells. Knockdown of RNF152 expression was detected in CRC cells treated with RNF152 siRNA and compared with control cells (**Figure 7A-C**). The results revealed that treatment of r-p8 partially rescued the down-regulation of RNF152 and cleaved PARP1 by RNF152 knockdown (**Figure 7D-F**). Moreover, co-treatment with r-p8 and RNF152 siRNA showed inhibition of cell proliferation when compared to siRNA treatment alone (**Figure 7G**, **7H**). These data demonstrated that RNF152 mediates the inhibitory effect of r-p8.

A

GSE44076

GSE41657

GSE21510

GSE32323

B

RNF152 IHC staining

Non-tumor

Colorectal cancer

C

RNF152

Figure 3. Down-regulation of RNF152 in patients with CRC. A. Gene Expression Omnibus (GEO) database analysis (accession numbers: GSE44076, GSE41657, GSE21510, and GSE32323) revealed that RNF152 expression was significantly decreased in CRC tumors compared with non-tumor tissues. The *p*-value was calculated using the Mann-Whitney test. ***P<0.001. B, C. Further confirmation of lower expression of RNF152 in CRC tissue compared to matching non-tumor tissue using IHC staining. Scale bar = 40 µm. ***P<0.001.
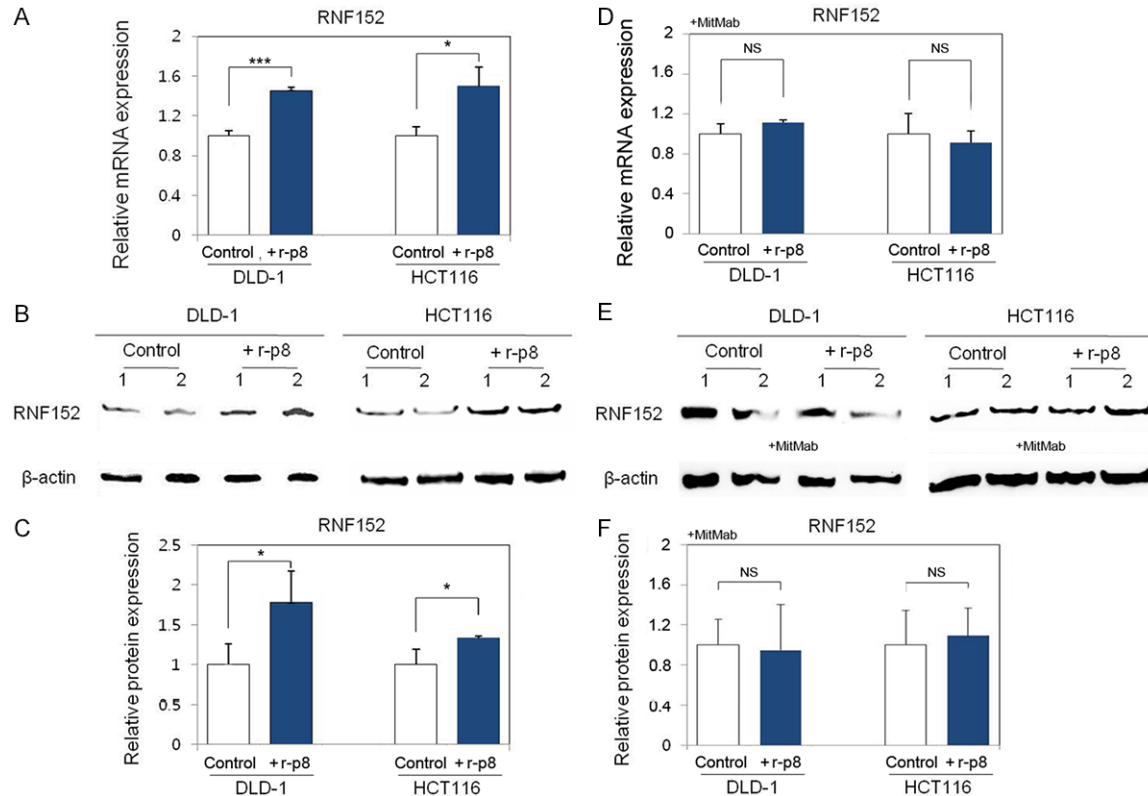

Figure 4. Treatment with r-p8 induces mRNA and protein expression of RNF152 in CRC cell lines. (A) Real-time PCR revealed that RNF152 mRNA expression was significantly increased following treatment with r-p8 (20 µM) in DLD1 and HCT116 cell lines. Results are reported as the average of three independent experiments. (B, C) Western blot analysis revealed that treatment with r-p8 (20 µM) significantly increased RNF152 protein expression in DLD1 and HCT116 cell lines. β-actin was used as a loading control. (D-F) The RNF152 expression of CRC cells that were treated with r-p8 (20 µM) with or without an endocytosis inhibitor (MiTMAB: 10 µM). Neither RNF152 mRNA (D) nor protein (E, F) was changed by r-p8 when endocytosis was inhibited. Quantitative analysis of the expression levels of RNF152 by IMAGE J software. *P<0.05; ***P<0.001.

Discussion

The development of probiotic-based treatments has been investigated for multiple types of cancer [4, 13]. In CRC, the mechanisms of probiotic-based anti-cancer activity are known to include: i) the induction of apoptotic responses, ii) protecting the intestinal mucosal barrier, iii) the inhibition of carcinogenic agents, and iv) altering the enzymatic activity of pathogenic bacteria [14, 15]. Many studies have shown that several strains of probiotics inhibit the proliferation of CRC cells and induce cell death *in vitro* and *in vivo* using these mechanisms [16].

For example, *Enterococcus faecium* RM11 and *Lactobacillus fermentum* RM28 suppress CRC cell proliferation [17, 18]. Similarly, *Lactobacillus delbrueckii* induces apoptosis of SW620 cells by increasing the expression of caspase-3 [19]. Interestingly, PP also conveys higher rates of anti-proliferation of CRC cells. Taken together, this suggests that several probiotic strains, including PP, have anti-CRC effects. Additionally, findings in the current study indicate that oral administration of PP-p8 induces apoptosis of CRC cells, thereby inhibiting tumor growth in mouse xenograft models. Considering the anti-tumor effect of PP-p8 compared to PP-Ev, it
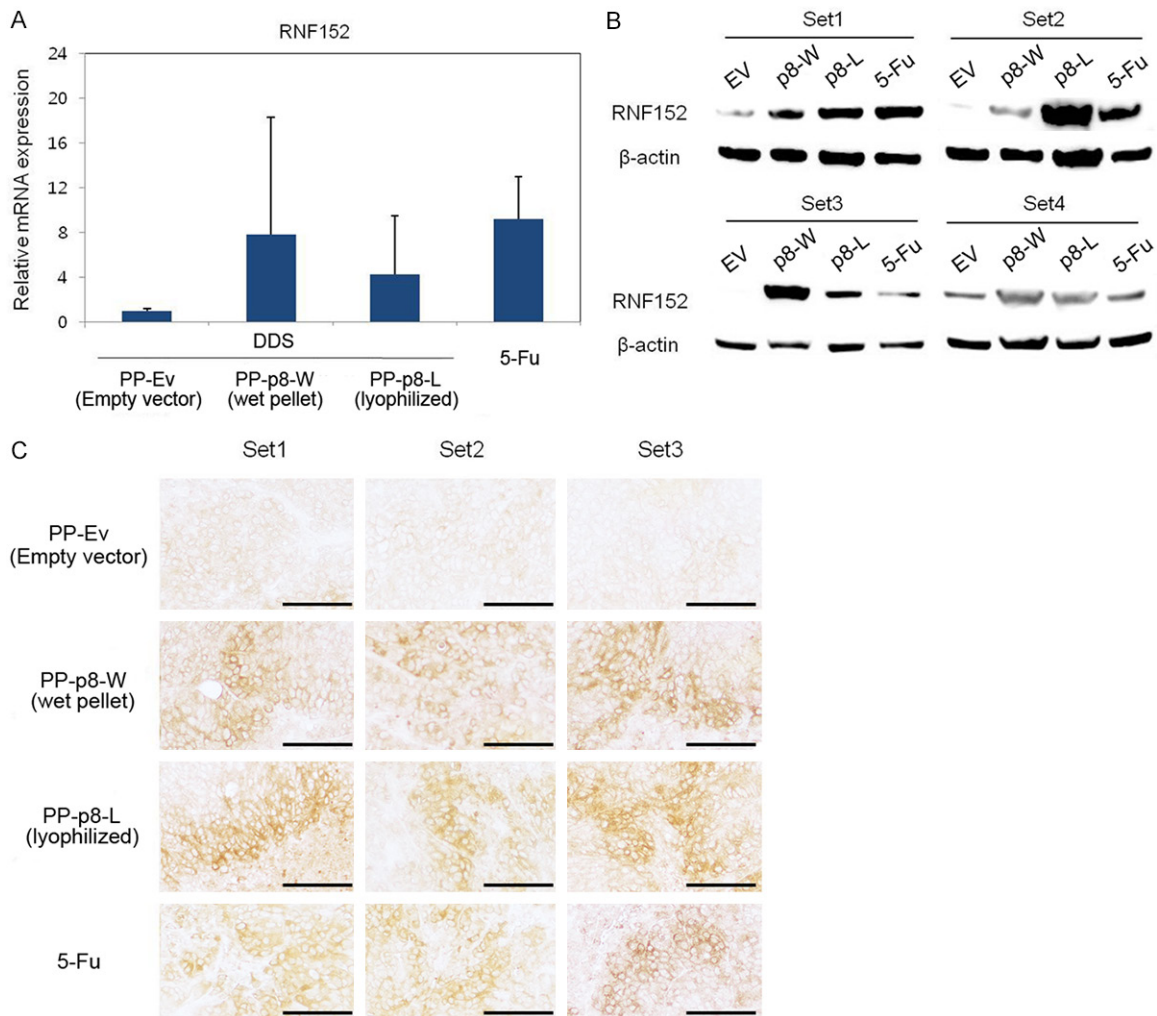
Figure 5. Up-regulation of RNF152 following oral administration of PP-p8 DDS *in vivo*. A. The mRNA expression of RNF152 was increased following oral administration of PP-p8 compared to control (PP-Ev-DDS). B. Consistent with mRNA expression, RNF152 protein was increased following oral administration of PP-p8 DDS *in vivo*. C. Immunohistochemistry revealed that RNF152 was expressed in mouse xenograft tissue following oral administration of PP-p8 DDS compared to tumors in mice in the control group. Scale bar = 100 μm.

seems likely that p8 is a critical factor that allows PP to have strong anti-cancer effects on CRC cells.

RNF152, a novel member of the RING finger protein family, is a lysosomal-localized E3 ligase regulating lysosome-related apoptosis by degradation and translocation of substrate proteins [20, 21]. Recent studies have shown that over-expression of RNF152 promotes apoptosis of Hela cells, inhibits CRC cell proliferation, and induces apoptosis via regulating mechanistic target of rapamycin complex 1 (mTORC1) in an E3 ligase activity-dependent manner [12, 20]. In line with previous data, we observed that RNF152 expression was decreased in

human patients with CRC. Moreover, results revealed increases in the expression of RNF152 and cleaved-PARP1 in a xenograft model of CRC following oral administration of PP-p8. Similar to our DDS model, RNF152 overexpression similarly caused inhibition of tumor growth with strong expression of cleaved-PARP1 in xenograft mice [12]. PARP, one of the targets of apoptosis, is cleaved when the apoptotic signal pathway is activated. In addition, an increase in the number of apoptotic cells was detected *in vivo* using the TUNEL assay following oral administration of PP-p8 as shown in **Figure 1D**. Both previous and current data demonstrate that r-p8 entered CRC cells through endocytosis and, like RNF152, was mainly localized to
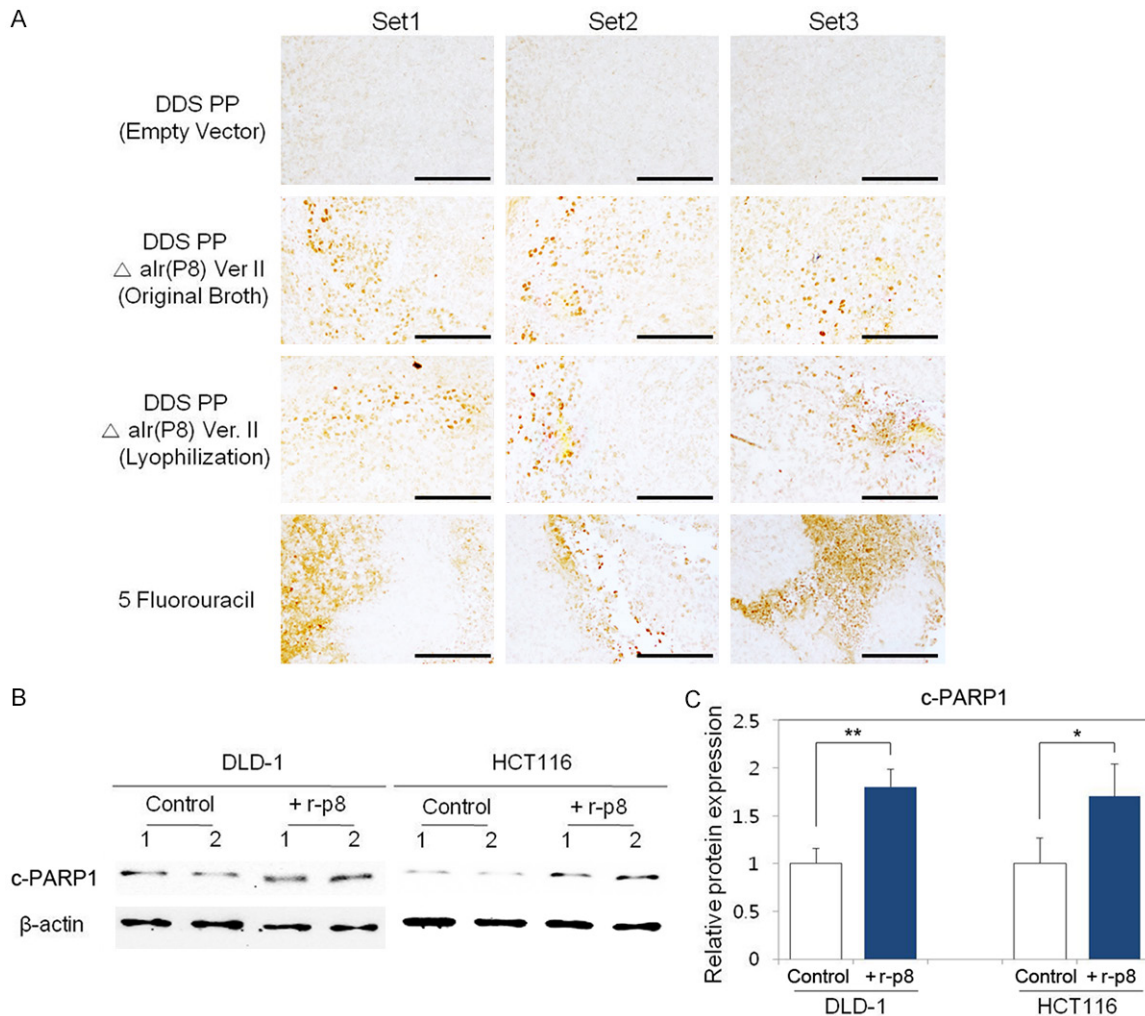
Figure 6. Increased expression of cleaved PARP1 by p8 *in vivo* and *in vitro*. A. High levels of cleaved PARP1(c-PARP1) were observed in the xenograft tumor tissue of mice treated with PP-p8 DDS. Scale bar = 100 μm. B, C. Treatment with r-p8 (20 μM) significantly increased cleaved PARP1 expression in DLD1 and HCT116 cell lines. β-actin was used as a loading control. *P<0.05; **P<0.01.

the cytoplasm [10]. Collectively, these results suggest that a primary mechanism of r-p8 in CRC cells is to induce apoptosis by increasing the abundance of RNF152 in the cytoplasm, subsequently increasing PARP degradation in the nucleus. Furthermore, we detected RNF152 up-regulation in mice treated with 5-Fluorouracil as shown in **Figure 5**. This result supports the possibility that the increased expression of RNF152 by PP-p8 is indirectly regulated by a specific intermediate factor. Therefore, further investigations will be needed to clarify the regulation of RNF152 by p8 in the suppression of CRC progression.

Interestingly, the anti-cancer property of p8 was detected in low amounts *in vitro* compared with *in vivo* [10]. Previous reports have shown that knockdown of RNF152 in CRC cells does not result in a significant increase in apoptosis due to the low levels of endogenous RNF152 in CRC cells [12]. In the current study, we found that treatment of CRC cells with exogenous r-p8 increased RNF152 expression by less than 2-fold. Moreover, we observed that p53 expression was highly increased by PP-p8 in a mouse xenograft model, an effect which was not observed *in vitro*, confirming that r-p8 does induce apoptosis slightly *in vitro*. Therefore, we speculate that the low therapeutic potential of r-p8 *in vitro* is caused by the low penetrative efficacy of r-p8 which is incapable of sufficiently affecting the expression of RNF152 to induce apoptosis.
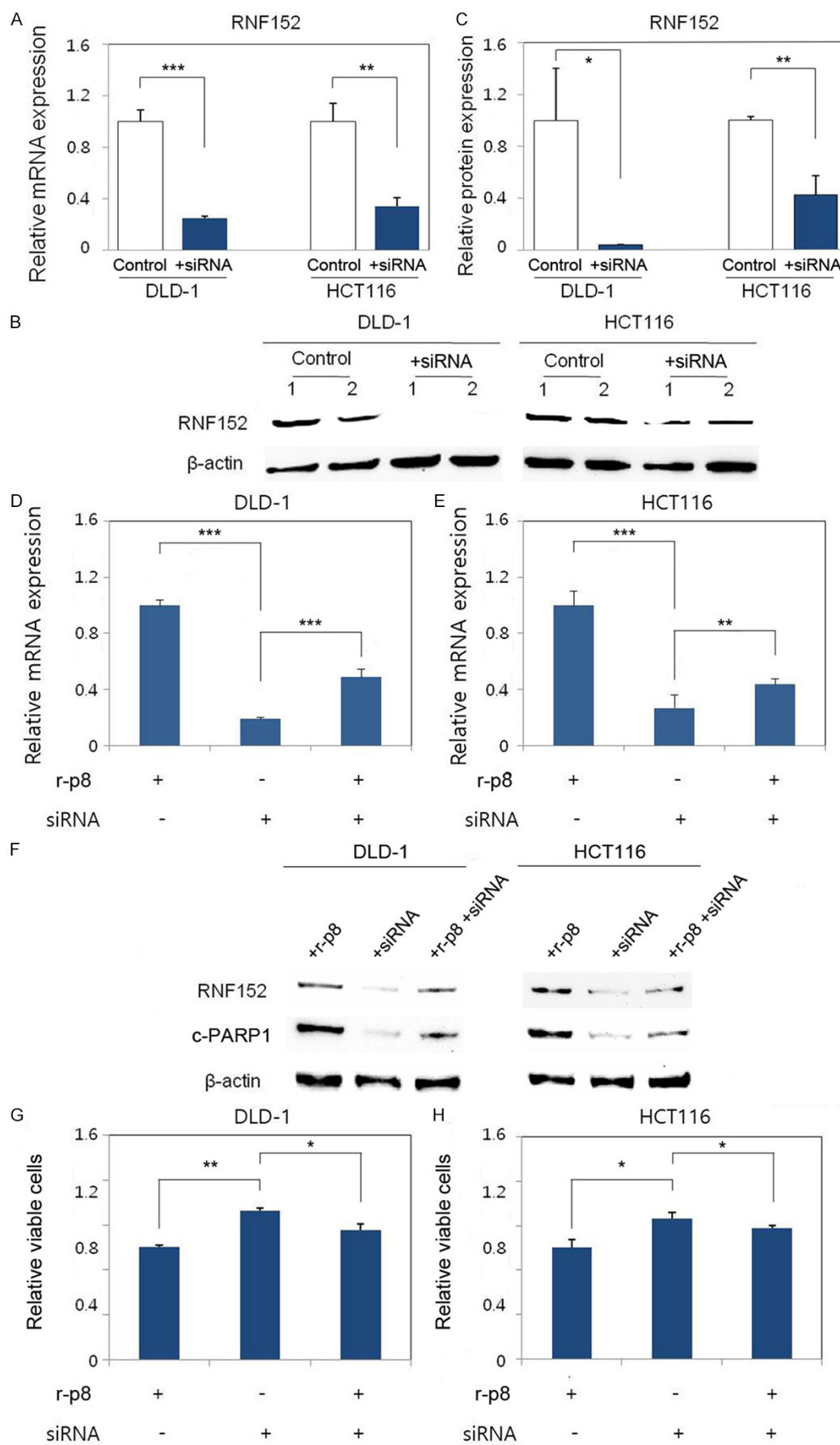
Figure 7. Knockdown of RNF152 expression partially rescued by r-p8. (A-C) Real-time PCR (A) and western blot (B, C) analysis indicated that RNF152 expression was significantly inhibited in RNF152 siRNA (100 nM) treated DLD1 and HCT116 cell lines. (D-F) Treatment of r-p8 partially rescued the down-regulation of RNF152 and cleaved PARP1 by RNF152 knockdown. (G, H) CCK assay revealed that co-treatment with r-p8 and RNF152 siRNA inhibited CRC cell proliferation compared to siRNA treatment alone. Results are the average of three independent experiments. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Additionally, using data from the Cancer Genome Atlas (TCGA) database (http://fire-browse.org), we determined that the expression of RNF152 was reduced in various cancers (e.g., bladder, colon, kidney, liver, lung, prostate, rectum, stomach) (Supplementary Figure 1). These data raise the possibility that down-regulation of RNF152 is a one of critical factor contributing to the development of cancers of the gastrointestinal tract and other cancers. Further investigations will be needed to understand the role of RNF152 in the inhibition of carcinoma progression.

Lyophilization is widely used for the long-term preservation of substances used for a variety of applications, including those related to medicine [22]. Moreover, lyophilization is expected to: i) be suitable for easy handling of small proteins and living cells and ii) allow for storage of these samples at room temperature [23, 24]. In this study, we demonstrated that lyophilization of PP-p8 (PP-p8-L), like the living wet cell pellet (PP-p8-W), has similar anti-cancer effects *in vivo*. Therefore, lyophilization of PP-p8 is a potentially effective option for treating CRC patients, an approach that would simplify the storage and transport of any future treatment.

Collectively, the current study suggests that the probiotic-derived p8 protein induces apoptosis of CRC cells via up-regulation of RNF152. Although further researches will be needed to examine the actual relationship between p8 and RNF152 in patients with CRC, these finding provide a better understanding of the p8 protein and be helpful in developing new treatment of CRC.

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### Disclosure of conflict of interest

None.

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Up-regulation of RNF152 by p8 induce CRC apoptosis
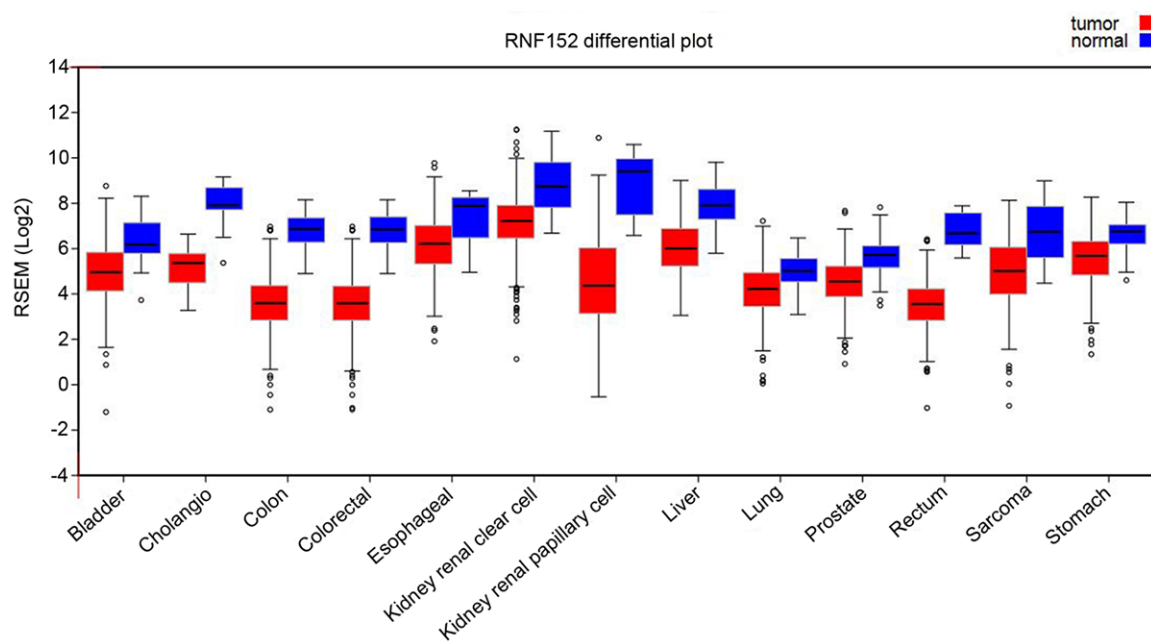
Supplementary Figure 1. RNF152 expression in various human cancers. RNF152 expression pattern was analyzed in various tumor tissues using the TCGA database (http://firebrowse.org). RNF152 expression was decreased in various cancers, including bladder, colon, esophageal, kidney, liver, lung, prostate, rectum, and stomach carcinoma. RSEM, RNA-Seq by Expectation-Maximization. Dots represent outliers. Boxes represent the first quartile, median, and third quartile.