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# Purple Potato Extract Promotes Intestinal Epithelial Differentiation and Barrier Function by Activating AMP-activated Protein Kinase

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## Abstract

**Scope—**Perturbation of gut epithelial barrier function induces inflammation and other health problems that originate from the gut. Purple potato contains a high content of beneficial polyphenolic compounds. The objective of this study was to evaluate the effect of purple potato extract (PPE) on intestinal differentiation and barrier function, and explore its underlying mechanism using Caco-2 cells and *ex vivo* cultured gut tissues.

**Methods and results—**PPE increased transepithelial electrical resistance and decreased FITC-dextran paracellular flux in Caco-2 cells, which were associated with strengthened intestinal epithelial differentiation in both Caco-2 cells and *ex vivo* guts. Furthermore, PPE treatment enhanced AMP-activated Protein Kinase (AMPK) activity, concomitant with the increased expression of CDX2, a key transcriptional factor regulating intestinal epithelial differentiation. Knocking out AMPK using CRISPR/Cas9 system abolished the positive effects of PPE on intestinal epithelial differentiation and barrier function, in junction with the reduced expression of CDX2.

**Conclusion—**PPE improved gut epithelial differentiation and barrier function via activating AMPK, indicating that PPE, as well as associated purple potato consumption, could be used as a supportive dietary therapeutic strategy for improving gut epithelial health.

### Keywords

AMPK; Barrier function; Epithelial differentiation; Gut; Purple potato extract

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Author contributions

X.S. and M.Z. designed the study and wrote the manuscript. X.S. performed the experiments. X.S., M.Z., and M.D. analyzed and interpreted the data. M.D. and D.N. revised the manuscript.

Competing interests

The authors declare no competing or financial interests.

# 1 Introduction

Intestinal epithelium (IE) is a single layer of cells along the mucosal surface that absorb nutrients and secrete fluid. IE also functions as a frontier barrier protecting the mucosal integrity, which physically inhibits the penetration of harmful substances from the external environment [1]. The abnormal epithelial barrier function, also called leaky gut, is associated with many diseases and complications [2], including inflammatory bowel disease (IBD) [3], metabolic syndrome [4] and autoimmune disorders [5]. Thus, a proper intestinal barrier is essential for health. The major determinant of epithelial permeability is the tight junctions between paracellular enterocytes [6]. Tight junctions are developed during intestinal stem cells differentiating into epithelial cells [7]. Nutraceutical factors or therapeutic strategies that enhance intestinal epithelial differentiation can improve gut epithelial barrier function and health [8].

Accumulating evidence supports the beneficial effects of dietary phenolics in preventing leaky gut [9, 10]. Dietary intake of phenolic-rich food, such as green tea, grapes, and berries, strengthens intestinal barrier integrity and the formation of tight junctions [11] in an energy-dependent manner [12]. Furthermore, polyphenol extracts are known to activate adenosine monophosphate-activated protein kinase (AMPK) [9], an energy sensor that regulates tight junctions and epithelial permeability [13].

Purple potato is a source of polyphenols [14]. Purple potato extract (PPE) has health beneficial effects, such as antioxidative [15], anti-tumor [16], and antimicrobial [17] activity. However, the effects of PPE on gut barrier function and intestinal epithelial differentiation remain untested, and the mediatory role of AMPK in the improvement of epithelial barrier function by polyphenolics has only been sparsely explored [9]. It is also undefined whether AMPK mediates the beneficial health effects of phenolic-rich PPE. The objective of this study was to evaluate whether PPE has a beneficial effect on intestinal barrier function, and to further unveil the role of AMPK in linking PPE to gut epithelial differentiation and barrier function.

# 2 Materials and Methods

## 2.1 Purple potato extract

The purple potato extract (PPE) was prepared as described in the Supporting Information. Concentrated PPE was stored at −20°C until further use.

## 2.2 Total phenolic compounds determination

Total phenolic content was tested using Folin-Ciocalteu assay in a 96-well plate as described previously [18]. The brief method was described in the Supporting Information.

## 2.3 Cell culture

Caco-2 cells were seeded at a density of $2\times10^5$ per well on 12-well plates, and treated with PPE at various concentrations, as described in the Supporting Information.

### 2.4 MTT assay

Ten thousands of Caco-2 cells were seeded in each well of 96-well plates. Treated with 0, 5, 10, 20 or 40 μg/ml PPE for 48 hours after seeding, cells were incubated with 5mg/ml 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma, St. Louis, MO) for 4h. Formazan was resuspended in 100 μl DMSO, and the absorbance at 540 nm was measured using Synergy H1 microplate reader (BioTek, Winooski, VT)

### 2.5 *In vitro* epithelial barrier function assessment

Transepithelial electrical resistance (TEER) assay and the permeability of 4 kDa FITC-dextran (FD4) across the Caco-2 cell monolayer was measured [19], as described in the Supporting Information.

### 2.6 Alkaline phosphatase assay

Alkaline phosphatase (AP) assay was performed as previously described [19], which is briefly described in the Supporting Information.

### 2.7 Immunoblotting analysis

Immunoblotting analysis was performed according to the procedures as previously described [20]. Membranes were visualized using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE). Band density was normalized to β-actin. Antibody information is described in the Supporting Information.

### 2.8 Calcium switch assay

The analysis was conducted with calcium-free DMEM [19], as described in the Supporting Information.

### 2.9 *ex vivo* gut culture

C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice studies were performed in an approved protocol by the Institutional Animal Care and Use Committee (IACUC) at Washington State University. *Ex vivo* guts were isolated and cultured as described in the Supporting Information. Immunofluorescent staining and RT-qPCR were conducted as described in the Supporting Information.

### 2.10 AMPK knockout in Caco-2 cells

The pX330 CRISPR/Cas9 plasmids with PRKAA1 sgRNA (AMPK KO) and corresponding scramble control (SC) sequences were designed and purchased from GeneCopoeia (Rockville, MD). Caco-2 cells were transfected with AMPK KO or SC plasmids using Lipofectamine 3000 (Life Technologies) per manufacturer's instructions as briefly described in the Supporting Information. Then the transfected cells were seeded onto 12-well plates at $2\times10^5$ cells per well, treated with 0 or 10 μg/ml PPE for 4 days for immunoblotting analysis, AP assay, and *in vitro* epithelial barrier function assessment.

### 2.11 Statistical analysis

Statistical analyses were conducted as previously described [21]. For cell studies, at least three independent experiments were carried out. For *ex vivo* studies, each cultured gut was considered as an experimental unit for immunofluorescent staining. For RT-qPCR assays, five cultured guts collected together were considered as an experimental unit. Data are presented as means ± standard error of the means (SEM). Comparisons of multiple means were analyzed using one-way ANOVA, followed by Duncan's multiple comparison test. Data related to epithelial barrier function assessment were analyzed using repeated measures two-way ANOVA with post hoc Tukey's test. Differing letters denote statistical differences in multiple comparison tests. All other data were analyzed using one-way ANOVA and two-tailed Student's t-test. $P <= 0.05$ was considered to be statistically significant.

## 3 Results

### 3.1 PPE enhances intestinal barrier function

TEER was monitored during 21 days of differentiation. TEER increased with culture time, and its value increased faster in the treatment with 10 and 20 μg/ml PPE (Fig. 1A). FITC flux was reduced in Caco-2 cells treated with 10 and 20 μg/ml PPE (Fig. 1B). The permeability did not change with 40 μg/ml PPE at 1h, but decreased at 4h (Fig. 1B). 5 μg/ml PPE treatment had no significant effects on both TEER and FITC flux (Fig. 1A–B). PPE at 20 and 40 μg/ml caused a reduction in cell viability (Fig. 1C) that might explain the no difference of barrier function observed between Caco-2 cells without PPE treatment and Caco-2 cells treated with 40 μg/ml PPE (Fig. 1A). Since 10 μg/ml PPE increased intestinal barrier function, this concentration was selected to use in subsequent experiments.

### 3.2 PPE strengthens intestinal differentiation and tight junction assembly

In response to PPE, the epithelial differentiation markers, alkaline phosphatase (AP) activity as well as the contents of villin and E-cadherin, were enhanced (Fig. 1D, 1F). Additionally, PPE enhanced the protein content of occludin, zonula occludens-1 (ZO-1), claudin-1 and claudin-2 (Fig. 1E). Consistently, PPE enhanced the TEER post-calcium switch (Fig. 1G); fragmented strands of ZO-1 staining at cell borders were formed much faster in response to PPE treatment (Fig. 1H).

We further examined intestinal epithelial differentiation by immunofluorescence staining of *ex vivo* cultured guts. The outer epithelial layer was covered completely with E-Cadherin and cytokeratin-8 in *ex vivo* cultured guts treated with PPE, while a small number of the epithelial cells were still concentrated in CON gut lumen (Fig. 2A). Furthermore, PPE upregulated the mRNA expression of brush border enzymes (*Anpep and SI*), epithelial polarity markers (*Cdh1* and *Villin*), intestinal differentiation transcription factors (*Elf3* and *Hes1*) and the epithelial differentiation regulator (*p21*) in *ex vivo* cultured guts (Fig. 2B).

PPE treatment increased the protein content of CDX2 (Fig. 3A), a key transcription factor governing epithelial cell differentiation [22], in Caco-2 cells associated with enhanced phosphorylation of AMPK and ACC (Fig. 3A). Consistently, mRNA level of *Cdx2* was increased in *ex vivo* gut with PPE treatment (Fig. 3B); the IF staining and distribution of

CDX2 (Fig. 3C) was in accordance with the differentiation markers, cytokeratin 8 and E-cadherin (Fig. 2A).

### 3.3 AMPK mediates PPE-induced intestinal differentiation

To investigate the regulatory role of AMPK in mediating PPE and its beneficial effects on gut health, we used the CRISPR/Cas9 system to knockout (KO) AMPK in Caco-2 cells, as indicated by the inactivation of p-AMPK (Fig. 4A). AMPK KO reduced CDX2 expression, which was unable to be elevated by PPE (Fig. 4B). Similarly, AMPK KO abolished the stimulatory effect of PPE on intestinal differentiation (Fig. 4C–D). AMPK KO had deleterious effects on TEER and FITC flux in Caco-2 cells; PPE treatment slightly improved barrier function in Caco-2 cells with AMPK KO (Fig. 4E–F).

## 4 Discussion

Caco-2 cells differentiate after confluence, resembling epithelial differentiation *in vivo*, which has been widely used for studying epithelial differentiation and barrier function [23]. 10 μg/ml PPE significantly strengthened barrier function, while 40 μg/ml PPE had no effects, which could be due to cell damage or apoptosis, as indicated by impaired cell viability. In support of our findings, 17 $\mu$M chicory phenol extracts upregulated TEER in Caco-2 cells, while 34 $\mu$M downregulated [24]. 1 mg/ml baobab fruit extract enhanced barrier function in Caco-2 cells, while 5 mg/ml baobab fruit extract induced cellular damage [25]. Our results are further supported by other studies on the beneficial roles of polyphenolic compounds, such as quercetin [26], kaempferol [27] and luteolin [28], in the barrier integrity of Caco-2 cells. Furthermore, anthocyanins from Meoru fruit extracts increased TEER in HCT-116 cells [29], and dietary chlorogenic acid decreased intestinal permeability in rat [30].

The integrity of the intestinal barrier is attributed to the expression and distribution pattern of junctional proteins during intestinal differentiation [31]. Retinoic acid enhances AP activity associated with upregulated mRNA of claudin-2 in Caco-2 cells [32]. The mRNA expression of claudin-2 and claudin-4, and TEER increased significantly in corneal epithelial RCE1(5T5) cells once differentiation initiated [33]. PPE stimulated intestinal differentiation, and enhanced tight junction assembly during intestinal development. Consistently, quercetin enhanced the distribution of claudin-1 and claudin-4 in Caco-2 cells [34]. Kaempferol treatment increased the intensity of claudin-3 and occludin at the intercellular junctions of Caco-2 cells [27]. Polyphenol-rich propolis extract increased the mRNA expression of ZO-1 and occludin, and improved tight junction structure in Caco-2 cells [9]. Claudin-1, occludin and ZO-1 are positively correlated to barrier function, while claudin-2 is inversely proportional to TEER [35]. Claudin-2 is the target of CDX2 transcription factor in Caco-2 cells [36] and HIEC cells [37]. Claudin-2 protein was enhanced by the expression of CDX2 facilitated by a retroviral vector in Colo 205 cells that do not express endogenous Cdx2 genes [38]. This indicates that PPE increased claudin-2 protein content possibly due to the activation of CDX2. However, the overall effect of PPE resulted in strengthened intestinal barrier function.

As a metabolic regulator, AMPK favors the maintenance and formation of epithelial barrier and differentiation [39]. AMPK promoted intestinal barrier function and differentiation through promoting the expression of CDX2, a critical transcription factor for intestinal differentiation [19]. Both AMPK activation and CDX2 expression were enhanced in response to PPE treatment. AMPK deletion notably abated the favorable function of PPE on epithelial differentiation and barrier function, confirming the regulatory roles of AMPK in PPE-induced gut differentiation. Consistently, a theaflavin derivative activated AMPK associated with augmented claudin-1 and ZO-1 and improved barrier in Caco-2 cells, while compound C, an AMPK inhibitor, treatment diminished this beneficial effects [40].

Purple-flesh potato contains high levels of polyphenolic compounds, chlorogenic acid and anthocyanins [41]. Oral administration of chlorogenic acid enhances tight junctions and intestinal barrier in rats [30]. Chlorogenic acid treatment phosphorylated AMPK in muscular cells associated with the stimulated glucose transportation, while an AMPK mutation abrogated the effects of chlorogenic acid [42]. Anthocyanins strengthen tight junctions and increase TEER in HCT-116 cells [29]; anthocyanins from meoru activate AMPK in HT-29 cells [43]. These data suggest that the beneficial effects of PPE on intestinal differentiation and barrier function might result from synergistic effects of anthocyanins and chlorogenic acid.

In summary, PPE strengthens intestinal differentiation and barrier function associated with the enhanced expression of CDX2 through a mechanism mediated by AMPK. Our data deepen the current understanding about the link between polyphenolic compounds and intestinal epithelial differentiation, and provide new insights into the molecular mechanisms underlying the beneficial role of polyphenolic compounds in intestinal barrier function. Our finding is important because intestinal barrier function is altered in a number of pathophysiological conditions such as obesity, diabetes, and inflammation, indicating that PPE, as well as associated purple potato consumption, could be used as a supportive dietary strategy for improving gut epithelial health.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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## Abbreviations

| | |
|---|---|
| **AMPK** | AMP-activated protein kinase |
| **AP** | alkaline phosphatase |
| **IBD** | inflammatory bowel disease |
| **IE** | intestinal epithelial |

| | |
|---|---|
| **PPE** | purple potato extract |
| **SC** | scramble control |
| **TEER** | transepithelial electrical resistance |
| **ZO-1** | zonula occludens-1 |

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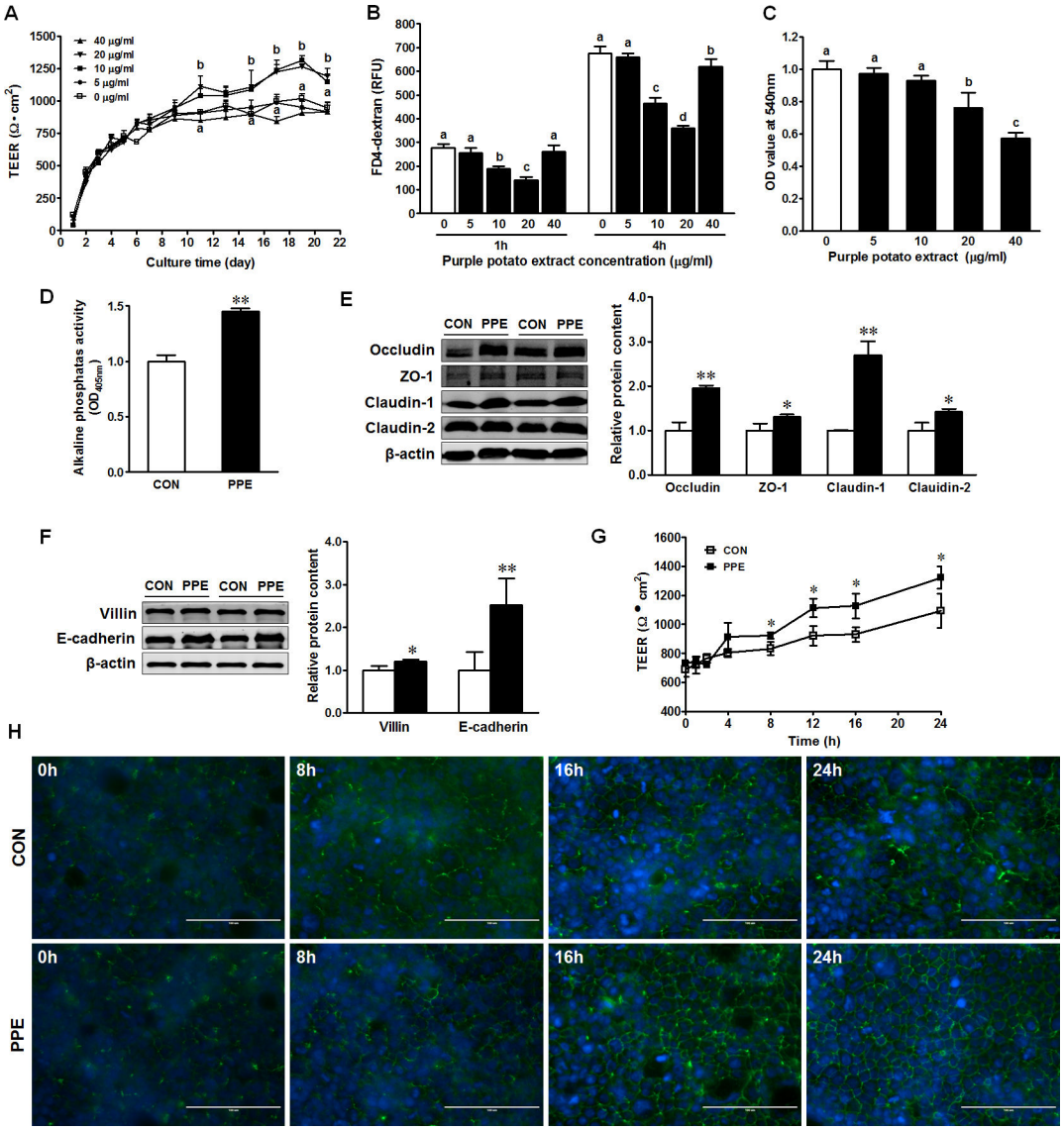


**Figure 1. PPE enhances intestinal epithelial differentiation, tight junction assembly and barrier function in Caco-2 cells.**

(A) Transepithelial electrical resistance (TEER) of Caco-2 cells treated with 0, 5, 10, 20 and 40 μg/ml PPE. (B) FD4-dextran paracellular intestinal epithelial permeability at 21 days post PPE treatment. (C) MTT analysis for cell survival in CON (□) and PPE (■) treated cells. (D) Activity of alkaline phosphatase. (E) Protein contents of occludin, ZO-1, claudin-1, and claudin-2. (F) Protein contents of E-cadherin and villin. (G) TEER of Caco-2 cells after calcium switch. (H) Immunofluorescent staining of tight junction protein ZO-1 pre- and post- calcium switch assay. Caco-2 cells were grown to confluence and subjected to a calcium switch assay. Cells were fixed at various time points (0, 8, 16 and 24 h) after the restoration of $Ca^{2+}$ containing medium. Scale bar is 100 μm. Mean ± SEM, n = 3, *: $P < 0.05$; **: $P < 0.01$. Differing letters denote statistical differences in multiple comparison tests.

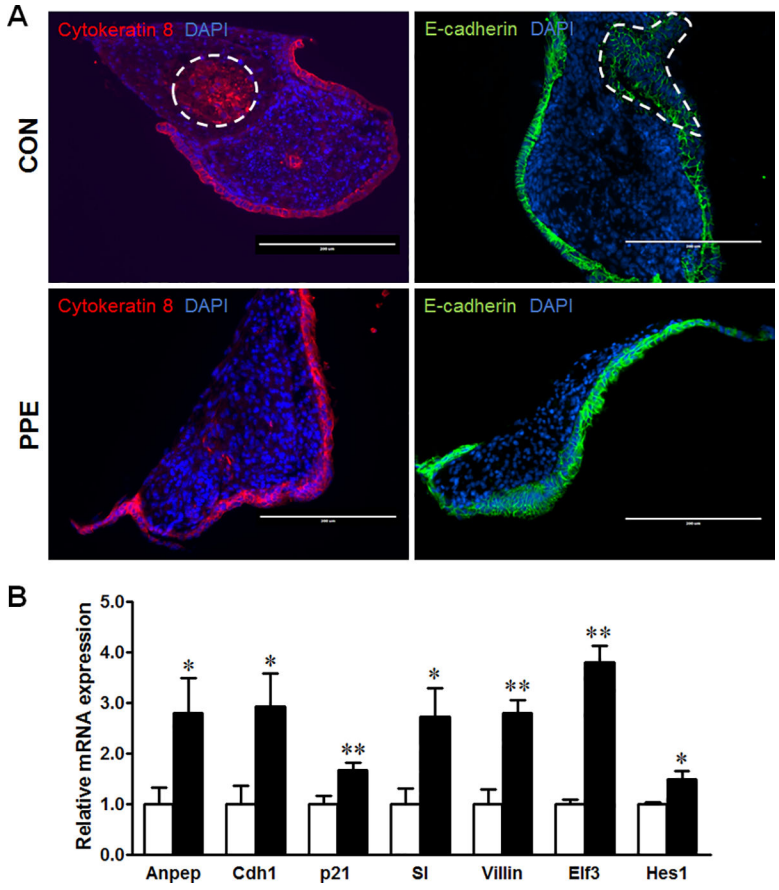


**Figure 2. PPE promotes intestinal differentiation in *ex vivo* guts.**

Intestine from E13.5 embryos was isolated, dissected, cultured to form *ex vivo* guts, and treated with 0 or 10 μg/ml PPE. (A) Immunofluorescent staining of cytokeratin 8 and E-cadherin. Scale bar is 200 μm. Dash lines indicate *ex vivo* gut lumen. (B) mRNA expression of *Anpep, Cdh1, p21, SI, Villin, Elf3* and *Hes1* in CON (□) and PPE (■) treated cells. Mean ± SEM, n = 4, *: $P < 0.05$; **: $P < 0.01$.

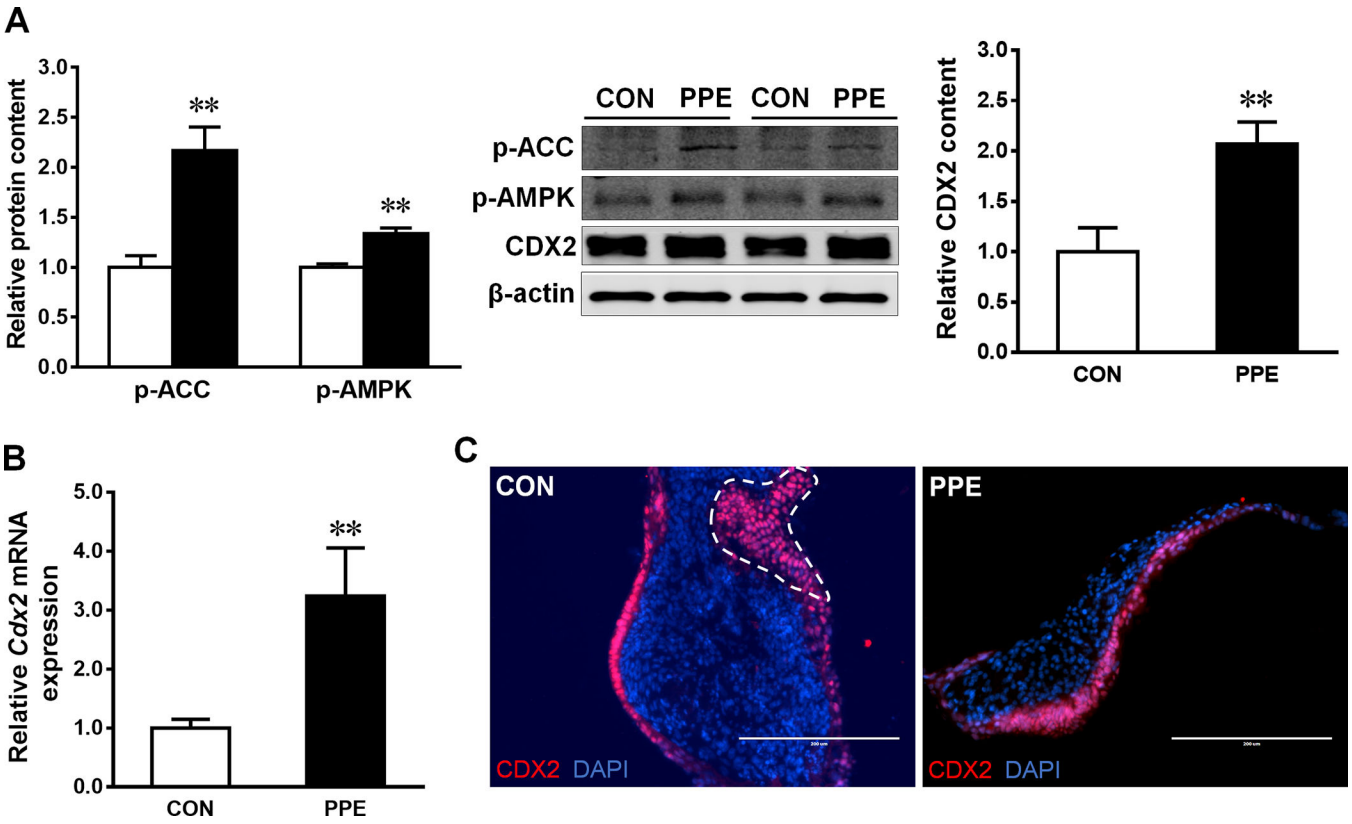


**Figure 3. PPE activates AMPK and CDX2 expression.**

(A) Protein contents of p-AMPK, p-ACC and CDX2 in CON (□) and PPE (■) treated Caco-2 cells. (B) mRNA expression of *Cdx2* in *ex vivo* guts. (C) Immunofluorescent staining of CDX2 in *ex vivo* guts. Scale bar is 200 μm. Dash line indicates *ex vivo* gut lumen. Mean ± SEM, n = 3, *: $P < 0.05$; **: $P < 0.01$.

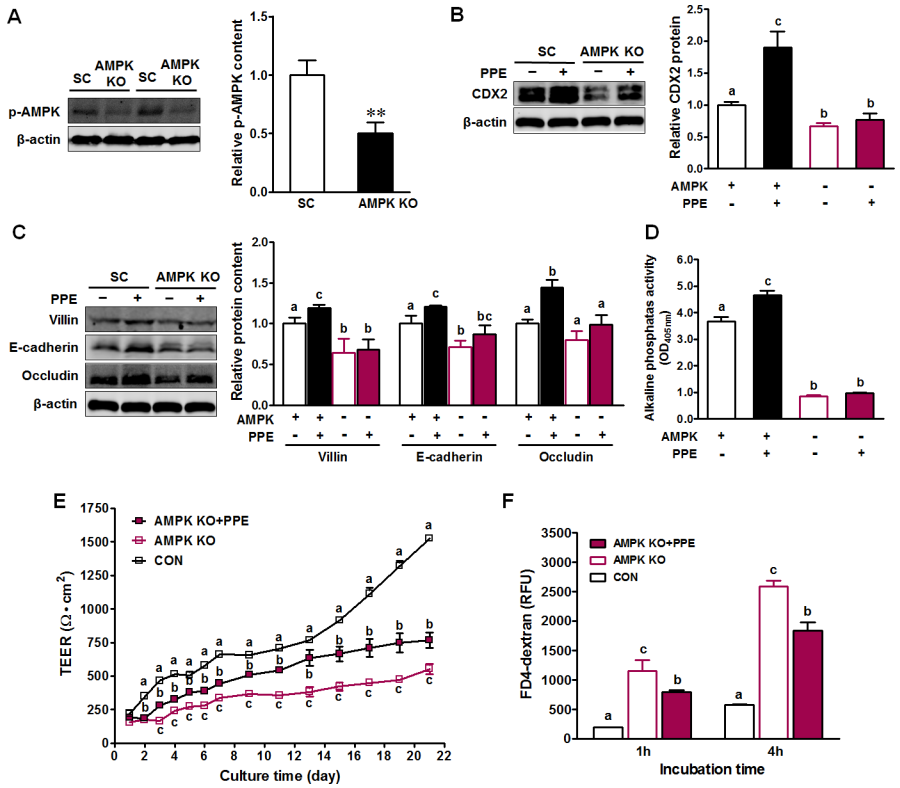


**Figure 4. AMPK mediates PPE-enhanced intestinal epithelial differentiation and barrier function.**

Caco-2 cells were transfected with CRISPR/Cas9 plasmids with PRKAA1 sgRNA (AMPK KO) or corresponding scramble control (SC) sequences to delete AMPK (−) or not (+), then treated with 0 (−) or 10 μg/ml PPE (+). (A) Protein content of p-AMPK. (B) Protein contents of CDX2. (C) Protein contents of villin, E-cadherin and occludin. (D) Activity of alkaline phosphatase. (E) Transepithelial electrical resistance of Caco-2 cells. (F) FD4-dextran intestinal epithelial permeability at 21-day post incubation. CON: Caco-2 cells without PPE treatment; AMPK KO: Caco-2 cells with AMPK KO; AMPK KO + PPE: Caco-2 cells with AMPK KO treated with 10 μg/ml PPE. Mean ± SEM, n = 3. Differing letters denote statistical differences in multiple comparison tests.