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AMP-activated protein kinase is a positive regulator of poly(ADP-ribose) polymerase

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Abstract

AMPK acts as a cellular fuel gauge and responds to decreased cellular energy status by inhibiting ATP-consuming pathways and increasing ATP-synthesis. The aim of this study was to examine the role of AMPK in modulating poly(ADP-ribose) polymerase (PARP), a nuclear enzyme involved in maintaining chromatin structure and DNA repair. HT-29 cells infected with constitutively active AMPK demonstrated increased PARP automodification and an increase in bioNAD incorporation. AMPK and PARP co-immunoprecipitated under basal conditions and in response to H_2O_2, suggesting a physical interaction under both resting and stress-induced conditions. Incubation of PARP with purified AMPK resulted in the phosphorylation of PARP; and the inclusion of AMP as an AMPK activator potentiated PARP phosphorylation. Using immobilized PARP, the incorporation of bioNAD by PARP was dramatically increased following the addition of AMPK. These data suggest a novel role for AMPK in regulating PARP activity through a direct interaction involving phosphorylation.

Keywords: PARP; AMPK; Intestine; DNA; HT-29

Maintaining sufficient stores of ATP is a fundamental requirement for cell survival. AMP-activated protein kinase (AMPK) appears to act as a master switch able to maintain energy balance within cells [1]. Present in all eukaryotic cells, AMPK is a serine/threonine kinase that exists as a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits [2]. Cellular reductions in ATP and alterations in cellular AMP:ATP ratio activate AMPK, which acts to correct the energy imbalance by inhibiting ATP-consuming pathways and increasing ATP-synthesis [1]. In various pathophysiological conditions, reactive oxidants cause DNA strand breakage and subsequent activation of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [3]. PARP is a nuclear protein involved in DNA repair; an 18-membered-superfamily of PARP enzymes synthesizes poly(ADP-ribose) (PAR), a reversible post-translational protein modification implicated in the regulation of a number of biological functions [4]. Poly(ADP-ribosyl)ation of PARP-target proteins serves as an important signal to additional proteins involved in DNA repair; in essence, it is the recruitment signal that collates and activates the DNA repair machinery. PARP activation is a crucial component to the maintenance of genomic integrity; however, chronic activation of PARP can lead to a depletion of cellular NAD, and a subsequent decline in cellular ATP levels [3]. Here, we describe that a physical interaction occurs between PARP and AMPK within intestinal epithelial cells, and that phosphorylation of PARP by AMPK increases PARP activity.

Materials and methods

Cell culture and treatments. HT-29 cells were obtained from American Type Culture Collection (Rockwell, MA) and cultured in RPMI 1640 (Gibco, Burlington, ON) supplemented with 10% heat-inactivated fetal calf serum (Cansera, Rexdale, ON), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 2% sodium bicarbonate, 1× penicillin–streptomycin (Gibco, Burlington, ON), and 10 mmol/L Hepes. Confluent monolayers

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(passage 30–45) were used in all experiments. Where indicated, cells were infected for 18 hR with adenoviral vectors at a multiplicity of infection (MOI) of 50, or as otherwise indicated in figure legends. The Ad5GFP vector encoding for green fluorescent protein was used as a viral negative control. The adenoviruses were washed off and cells returned to growth media for subsequent experimentation.

Western blotting. For Western blot analysis, HT-29 cells were lysed in Mono Q buffer (50 mM β-glycerophosphate, 1 mM EGTA, 0.5% Triton X-100, 2 mM $MgCl_2$) and cleared by centrifugation, and 50 μg of total cellular lysate protein was subjected to electrophoresis on 10% SDS–polyacrylamide gels. Anti-PADPR (abcam, Cambridge, MA), anti-AMPK (Cell Signaling, Beverly, MA), and two anti-PARP (Cell Signaling, Beverly, MA and Upstate Biotechnology, Charlottesville, VA) antibodies were used to detect immunoreactive poly(ADP-ribose), total and adenoviral AMPK, and full-length and cleaved PARP, respectively, using an enhanced chemiluminescence light-detecting kit (Amersham, Arlington Heights, IL). In order to confirm equal loading of protein, all Western blots were stained with Ponceau S.

Immunoprecipitation. For co-immunoprecipitation analysis HT-29 cells were lysed in RIPA buffer (50 mM Tris–HCl, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, Na_3VO_4, 1 mM NaF, 0.25% Na-deoxycholate, 1% NP-40, and 1 μg/mL aprotinin, leupeptin, and pepstatin) and cleared by centrifugation. The supernatant fraction was precleared with protein-G agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h. The lysate was incubated with anti-PARP antibody (Upstate Biotechnology, Charlottesville, VA, 10 μg/mg protein) overnight at 4 °C followed by immunoprecipitation with 50 μL protein-G agarose beads (50:50 slurry). The beads were washed and bound proteins eluted by boiling of samples (5 m) in 50 μL protein sample buffer (2% β-mercaptoethanol, 10% glycerol, 4% SDS, 100 mM Tris–HCl, and trace bromophenol blue, pH 6.8). Immunoprecipitated proteins were resolved by SDS–PAGE and transferred to PVDF. Blots were probed using the immunoprecipitating antibody as well as others as indicated.

Cell-free AMPK assay. The cell-free AMPK assay was performed using purified AMP-activated protein kinase (Upstate Biotechnology) and either SAMS peptide (HMRSAMSGLHLVKRR, Alberta Peptide Institute, Edmonton, AB) or purified, recombinant PARP (Trevigen) as substrate. The kinase reaction was performed according to (Upstate Biotechnology) the manufacturer's directions. Briefly, purified enzyme (at concentrations as indicated in Fig. 3, where 1 U = 1 nmol phosphate incorporated into 200 μM SAMS peptide/min) was diluted in reaction buffer (20 mM Hepes–NaOH, pH 7.0, 0.4 mM DTT, 0.01% Brij-35, and ±300 μM AMP) containing 1 μCi/μL [γ-^{32}P]ATP (complexed with 5 mM $MgCl_2$ and 200 μM unlabelled ATP). SAMS peptide was used at a concentration of 100 μM, while PARP was substituted as substrate at a concentration equivalent to 2 U (where 1 U is defined as the amount of enzyme required to incorporate 100 pmol of poly(ADP) from NAD into acid-insoluble form in 1 min). Once prepared, samples were incubated for 15 min at 30 °C. Where SAMS peptide was used as kinase, substrate samples were spotted on P81 filter papers and washed 3× in 0.75% phosphoric acid, 1× with acetone and allowed to dry prior to immersion in scintillation cocktail. Where PARP was used as substrate, sample proteins were resolved by SDS–PAGE. Incorporation of radio-label into SAMS peptide was determined using a Beckman-coulter scintillation counter, and where PARP was substituted as substrate, a typhoon 8600 phosphorimager (Amersham Biosciences, Piscataway, NJ).

PARP cELISA. The detection of poly(ADP-ribose) polymerase activation by cell ELISA (cELISA) is described in detail by Bakondi et al. [5]. Briefly, HT-29 cells were seeded in a 96-well tissue culture plate, and 18 h prior to beginning the cELISA infected with appropriate adenoviral vectors (see Cell culture and treatments). Cells were incubated for 1 h (37 °C) in PARP reaction buffer [56 mM Hepes, 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, and 10 μM biotinylated NAD^+ (Trevigen)]. Following incubation, monolayers were washed 1× with PBS and fixed with ice-cold 95% EtOH for 30 min (−20 °C). Monolayers were washed 1× with PBS and blocked for 1 h (37 °C) with 1% BSA. Block was aspirated and replaced with streptavidin–HRP (1:20,000) and incubated for 30 min (37 °C). Monolayers were washed 3× with PBS and TMBS added. After 15 min incubation (RT), the reaction was stopped with the addition of 1 M H_2SO_4, and absorbance determined at 450 nm.

bioNAD assay. The biotinylated NAD assay for the detection of PARP auto-poly(ADP-ribosyl)ation is described in detail by Putt and Hergenrother [6]. Briefly, PARP-1 enzyme (Trevigen, Gaithersburg, MD) is immobilized in a 96-well EIA/RIA plate (Costar, Corning, NY) in a dilution buffer (50 mM Tris–HCl, pH 8.0, 20 μM $ZnCl_2$, and 4 mM $MgCl_2$) and incubated overnight (4 °C). Following incubation, wells are washed 3× with PBS and incubated for 2 h (4 °C) in reaction buffer [dilution buffer supplemented with 1 mM DTT, 100 μM NAD, and 25 μM biotinylated NAD (Trevigen) ±12.5 μg/mL sheared DNA, ± treatments], as indicated. Following this incubation, wells are washed 3× with PBS and incubated for 1 h (37 °C) with streptavidin–HRP (1:20,000). Following this incubation, wells are washed 3× and TMBS added. After 15 min incubation (RT), the reaction was stopped with the addition of 1 M H_2SO_4, and absorbance determined at 450 nm.

Statistical analysis. Data are expressed as means ± SE of ⩾ triplicate samples. Statistical significance was assessed using ANOVA, and where appropriate, post hoc analysis (LSD), (Sigmastat, Systat Software, Inc., Point Richmond, CA).

Results

Overexpression of AMPK increases PARP activity

The catalytic action of PARP involves the poly-ADP ribosylation of various nuclear proteins, including PARP itself, a process termed automodification [7]. Constitutively active AMPK (Ad.CA-AMPK) was overexpressed in HT-29 cells, using an adenoviral gene delivery approach in order to examine if PARP is regulated by AMPK. Cells infected with Ad.CA-AMPK demonstrated an MOI-dependent increase in PARP automodification as compared with control cells expressing green fluorescent protein (Ad.GFP, Fig. 1A). PARP activity in these cells was assessed using a cell-based ELISA, or cELISA [5]. The results from this experiment confirmed the Western blot findings, with Ad.CA-AMPK overexpression resulting in a significant (≈2.8-fold) increase in bioNAD incorporation versus the uninfected epithelial cells (Fig. 1B). Of note, infection with the Ad.GFP also increased bioNAD incorporation, presumably through indirect means, and likely as an artifact of viral infection.

PARP and AMPK physically interact within epithelial cells

To evaluate whether the regulatory relationship between AMPK and PARP was being mediated through direct interaction, HT-29 cells were treated with an increasing concentration of H_2O_2 and lysates were prepared for immunoprecipitation. A treatment with H_2O_2 was included to determine whether the regulation of PARP by AMPK was genotoxin-dependent (H_2O_2 is an activator of both PARP and AMPK), and the inclusion of this parameter to the experiment had the added effect of ensuring that PARP was in fact the immunoprecipitate in our pull-down, as we were also able to immunoblot against automodified PARP, or PAR (Fig. 2). The results from this experiment revealed two important pieces of data, the first being the recognition of AMPK and PARP as co-immunoprecipitates,

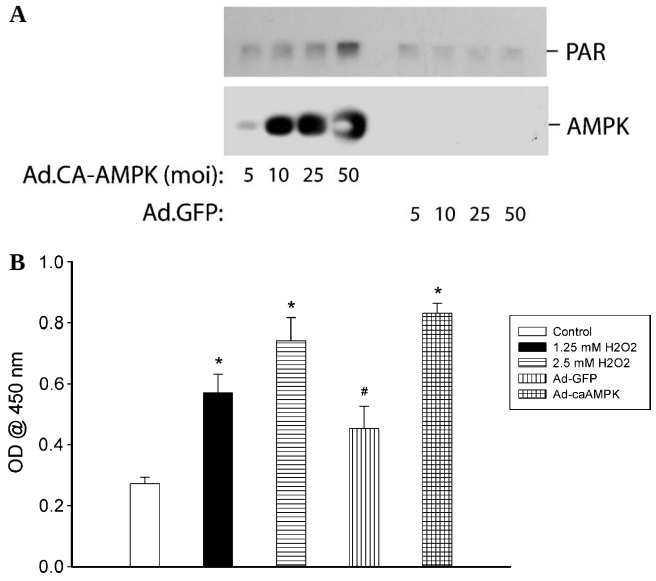

Fig. 1. AMPK is a positive regulator of PARP activity in intestinal epithelial cells. (A) HT-29 intestinal epithelial cells were transduced with an increasing MOI of adenoviruses encoding for a constitutively active AMPK variant (Ad.CA-AMPK) or, as an infection control, GFP (Ad.GFP). Epithelial monolayers were incubated with the indicated viral titres for 18 h, washed twice with PBS, and incubated for an additional 6 h in complete media (RPMI 1640 w/10% FBS). Following this incubation, cells were collected in lysis buffer and prepared for SDS–PAGE and subsequent Western blot analysis. The PVDF membrane was probed first for poly(ADP-ribose) (PAR) as a measure of PARP activity and second for AMPK, to visualize the protein product of adenoviral-mediated gene transfer. (B) HT-29 cells were transduced as described at a MOI of 50. Following transduction, monolayers were incubated with a reaction buffer containing biotinylated NAD (bioNAD) as substrate for ADP-ribosylation reactions. Subsequent to this incubation, monolayers were washed with PBS and fixed in 95% ethanol prior to incubation with streptavidin–HRP. Incorporation of bioNAD was quantified between groups by measuring optical density subsequent to incubation with an HRP substrate. $^{*}p < 0.01$, $^{\#}p < 0.05$ vs untreated cells.

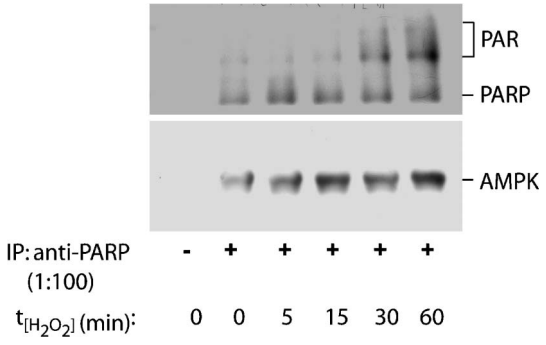

Fig. 2. AMPK and PARP co-immunoprecipitate from HT-29 intestinal epithelial cell lysate, independent of exogenous activation. HT-29 intestinal epithelial cell monolayers were treated with a fixed concentration of H_2O_2 (5 mM) for 0–60 min and subsequently harvested in a lysis buffer amenable to immunoprecipitation. Protein concentrations between groups were standardized to 2 mg/mL total protein concentration and PARP was immunoprecipitated from the lysate. Samples were subjected to SDS–PAGE and Western blot analysis. The PVDF membrane was probed first for PARP and subsequently for PAR and AMPK.

a strong indication that these proteins interact physically within the cell, and the second being that the interaction between PARP and AMPK likely occurs in the resting cell, and not solely as a stress response to exogenous stimuli, as co-immunoprecipitation of the proteins was evident irrespective of treatment with H_2O_2.

AMPK regulation of PARP involves kinase activity

With good evidence that the regulation of PARP by AMPK involved their physical interaction, we next focused our investigation towards the nature of that interaction. To determine if regulation of PARP activity by AMPK involved kinase activity, an in vitro kinase assay employing purified, recombinant PARP and AMPK was developed. Fig. 3A demonstrates the specificity of the assay using an engineered AMPK substrate, SAMS peptide. As is routinely reported, the inclusion of the AMPK activator AMP (300 μM) increased kinase activity. When purified, recombinant PARP was substituted for SAMS peptide as substrate, increasing phosphorylation of PARP was evident through an increasing range of concentrations of AMPK (Fig. 3B). Furthermore, phosphorylation of PARP was increased when the assay conditions were modified to include the canonical AMPK activator AMP (300 μM, Fig. 3B).

PARP is a substrate for AMPK phosphorylation

To determine the functional consequences of PARP regulation by AMPK, an enzymatic assay for PARP [6] was utilized [6]. PARP was immobilized and incubated with and without AMPK, and in the presence and absence of ATP (125 μM). As with previous findings, our results clearly indicate that the regulation of PARP by AMPK is of a positive nature, as the incorporation of bioNAD by PARP was dramatically increased with the inclusion of AMPK (Fig. 3C). Using sheared salmon-sperm DNA as a positive control, we were able to demonstrate a further 2-fold increase in activity over control values, but the increase in activity was only observable upon the inclusion of ATP in the reaction buffer. We take this finding to be further indication that PARP is a substrate for AMPK phosphorylation, and that this phosphorylation dramatically increases PARP activity.

Prolonged AMPK activity induces apoptosis

The finding that AMPK positively regulated PARP activity was paradoxical, given that a recognized endpoint to PARP activation is the depletion of cellular ATP [3]. However, while the chief mandate for AMPK is the preservation and/or restoration of cellular ATP levels, to accomplish this task at the expense of maintaining genomic integrity would seem counterintuitive. To investigate the effects of chronic activation of AMPK, we employed the pharmacological activator of AMPK

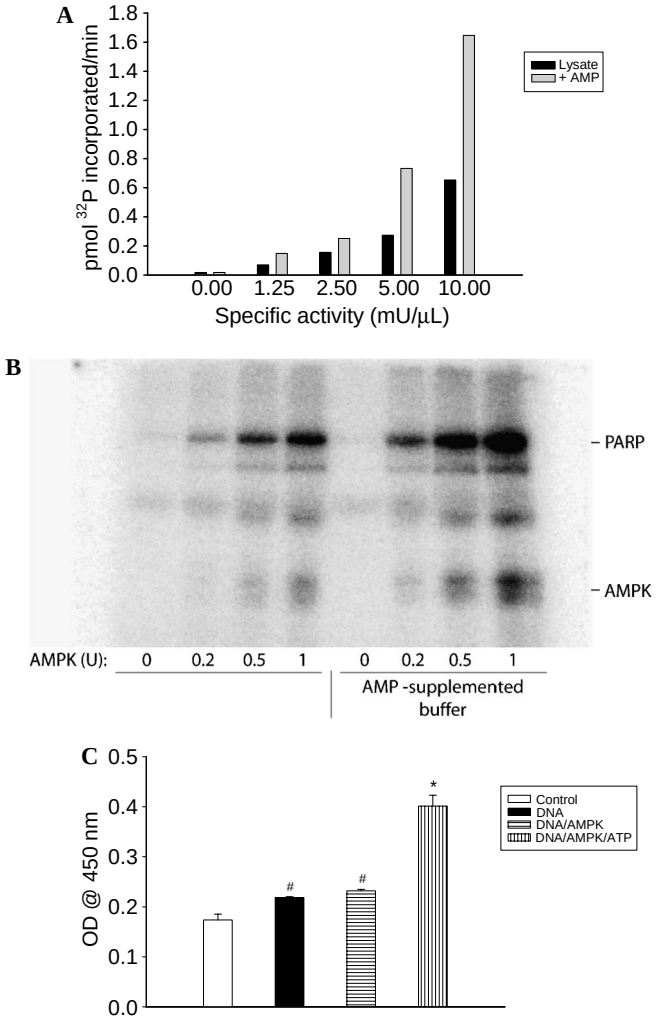

Fig. 3. PARP is a substrate for AMPK-dependent phosphorylation, and activation of AMPK potentiates this reaction. (A) As a positive control for the in vitro kinase assay (B), purified AMPK in increasing concentrations (0–10 mU/μL) was incubated with the synthetic AMPK substrate SAMS peptide in the presence or absence of 300 μM AMP as activator. Incorporation of [γ-^{32}P]ATP was assessed by spotting the substrate peptide-containing reaction mixture onto filter papers and measuring radioactive decay by liquid scintillation counting. (B) The experiment described in (A) was repeated with purified, recombinant PARP substituted as substrate. The unlabelled band at the vertical midpoint of the gel is BSA, included in the reaction mixture as a negative control. Following incubation, the protein mixture was separated by SDS–PAGE and incorporation of [γ-^{32}P]ATP was visualized using a typhoon phosphoimager. The incorporation of [γ-^{32}P]ATP was increased with the addition of 300 μM AMP. Autophosphorylation of AMPK is also observed under the conditions of the assay. (C) A cell-free enzymatic assay for PARP activity by the chemical quantification of NAD was modified to include purified AMPK. Purified, recombinant PARP was immobilized and combined with sheared DNA as an activator in the presence and absence of 10 mU AMPK. Incorporation of bioNAD by PARP was quantified between groups by measuring optical density subsequent to incubation with an HRP substrate. $^{*}p < 0.01$, $^{\#}p < 0.05$ vs untreated cells.

5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, 1 mM) to assess the impact of sustained AMPK activation in HT-29 epithelial cells. By Western blot analysis we were able to demonstrate the cleavage of PARP in these

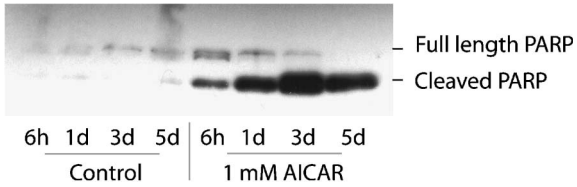

Fig. 4. Sustained treatment with AICAR induces apoptosis in HT-29 epithelial cells. HT-29 intestinal epithelial cells were treated with AICAR (1 mM) for the time periods indicated (0–5 d) and subsequently harvested in a lysis buffer. Total lysate proteins were separated by SDS–PAGE and analysed by Western blot. PVDF membranes were probed using an antibody recognizing the full-length PARP protein, as well as the caspase cleavage product.

cells in a manner that increased with the duration of AICAR treatment (Fig. 4). PARP cleavage is a classic marker of caspase-dependent apoptosis, thus these findings would suggest that chronic activation of AMPK leads to increased apoptosis.

Discussion

In the present study, we have demonstrated that AMPK and PARP share a direct, functional association in intestinal epithelial cells, and further, AMPK regulates PARP activity through a phosphorylation reaction. The paradoxical finding that AMPK positively regulates PARP activity suggests that AMPK may act as a molecular switch, capable of modulating the cellular response between survival and cell death. Jones et al. [8] have very recently reported findings that would indicate this to be the case. In their study, AMPK induced a p53-dependent metabolic checkpoint, with persistent activation of AMPK leading to accelerated p53-dependent cellular senescence. Within this context, AMPK can be viewed as a regulator of cell division that coordinates cellular proliferation with energy availability. The involvement of AMPK in the induction of apoptosis has been reported in other cell types, including hepatocytes [9] and pancreatic β cells [10]. Interestingly, and consistent with our observation in intestinal epithelial cells, both of these reports specify a requirement for sustained or prolonged AMPK activation in the induction of regulated cell death. The notion that AMPK acts acutely to ''rescue'' the cell from ATP-depletion and subsequent cell death is in keeping with its role as described extensively in the literature. However, sustained activation of the kinase, presumably accompanied by the resultant long-term inhibition of transcription and protein synthesis, would undoubtedly be deleterious to the cell, and at that point apoptosis over necrosis would be the preferred outcome.

An alternative explanation for the activation of PARP activity by AMPK lies in a possible role for ADP-ribosylation in providing a pool of ATP. In that the putative role for AMPK is in the restoration of cellular ATP levels, it is possible that activation of PARP contributes to this restoration. Oei and Zeigler [11] have reported provocative

findings that would suggest that ADP-ribosylation might in fact create a protected, nuclear pool of ATP through the coordinated action of poly(ADP-ribose) glycohydrolase (PARG). There is a dramatic decrease in cellular ATP concentration following exposure to genotoxic agents [12] and a role of poly(ADP-ribosyl)ation may be to facilitate the extraction of energy from NAD for its immediate use for DNA repair. It is intriguing to speculate that AMPK may play a role in this process; by engaging PARP, AMPK would in effect create a "pool" of ATP, exclusively reserved for the privileged process of DNA repair.

AMPK is regulated upstream by LKB1 which is a serine/threonine kinase involved in numerous and disparate cellular processes, including maintaining epithelial cellular polarity [13]. Germline mutations of the LKB1 gene, located on chromosome 19p13.3, have been identified in the majority of Peutz–Jeghers syndrome (PJS) patients [14]. These patients are at great risk of developing malignant tumours, predominantly in the intestine but also in other tissues including breast, lung, uterus, and ovary. Estimates of the risk to PJS patients of developing some form of cancer range as high as 93% [15]. Impairment in the polarisation of the intestinal epithelium, such as is seen with the loss of functional LKB1 [16], could contribute to the transformed phenotype of the intestinal epithelium seen in PJS. An alternative and perhaps complementary mechanism for the pathogenesis of PJS may lie with a dysregulation of AMPK, and subsequently a lack of stimulation of PARP activity. PARP activity is linked with DNA repair, chromatin structure, and the distribution of genetic material to daughter cells [17]. It has been shown that PARP binds single- and double-strand DNA breaks and is involved in the recruitment of DNA repair elements functioning in base excision repair [18,19]. Thus, in the physiological setting of the intestine, where dietary, bacterial, and immune cell-derived genotoxic stressors are abundant, one could surmise that a loss of function of LKB1 might be translated into a deficiency in DNA repair through an inhibited AMPK signal to PARP. Interestingly, while rates of spontaneous mutant frequencies in the livers of $PARP^{-/-}$ and $PARP^{+/+}$ mice are not different, treatment with an alkylating agent increases the frequency of mutations in $PARP^{-/-}$ animals [20]. Furthermore, elevated susceptibility to colon carcinogenesis induced by an alkylating agent in $PARP^{-/-}$ mice has been established [21], and these tumours involved an alteration of Wnt-β-catenin signalling [21], consistent with previously published results linking LKB1 with this critical oncogenic pathway [22,23]. These findings are intriguing when considered in light of the pathogenesis of PJS, where a germ-line mutation is first and primarily manifested in the gastrointestinal tract. The intestine is in constant exposure to a variety of carcinogens that would necessitate efficient DNA repair machinery. Intriguingly, an absence of stimulation of PARP activity in patients predisposed to colon cancer has previously been demonstrated [24].

In conclusion, our results suggest a new and important role for AMPK in regulating PARP activity and maintaining genomic integrity, and further, that impairment of AMPK–PARP interactions might contribute to the pathogenesis of Peutz–Jeghers syndrome.

Acknowledgments

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