PRIMA-1met Induces Autophagy in Colorectal Cancer Cells With Different p53 Status

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Primary research

Keywords: colorectal cancer (CRC), p53, PRIMA-1met, autophagy, oncotherapy

Posted Date: September 16th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-73569/v1

Abstract

Background: PRIMA-1^{met} (APR246), a methylated form of PRIMA-1 (p53-reactivation and induction of massive apoptosis-1, APR-017), targets mutant p53 for restoring its wild-type structure and function. We previously demonstrated that PRIMA-1^{met} was efficient in suppressing the growth of colorectal cancer (CRC) cells in a p53-independent manner, and distinctly induced apoptosis mediated by up-regulation of Noxa in p53-mutant cell lines. Here we aimed to the effect of PRIMA-1^{met} on autophagy in different CRC cell lines, to further investigate mechanisms underlying the inhibitory effect in cells with different p53 status.

Methods: 3 CRC cell lines with wild-type p53, 5 lines with mutant p53 and 1 line without p53 were obtained for this study. Using western blotting, acridine orange staining, and transmission electron microscopy detection, we assessed autophagy flux in different cells treated with PRIMA-1^{met}, and detected expression of mTOR/AMPK-ULK1-Vps34 autophagic signaling cascade. We also evaluated cell proliferation of cells with PRIMA-1^{met} treatment by cell counting Kit-8 proliferation assay, compared to combination of PRIAM-1^{met} and 3-Methyladenine. Furthermore, we knocked down Noxa gene by siRNA in different CRC cells, to assess LC3 conversion after administration of PRIMA-1^{met}. Values were expressed as mean + standard error of the mean. Comparison between groups of data was made using one-way analysis of variance.

Results: In this study, we showed that PRIMA-1^{met} induced autophagy in CRC cells independent on p53 status. PRIMA-1^{met} not only promoted autophagic vesicles (AVs) formation and AV-lysosome fusion, but also increased lysosomal degradation. Mechanistically, activation of mTOR/AMPK-ULK1-Vps34 autophagic signaling cascade was important for PRIMA-1^{met}-induced autophagy. Furthermore, autophagy played a crucial role in the inhibitory effect of PRIMA-1^{met} only in cells harboring wild-type p53, which was closely related to the increased Noxa.

Conclusions: Our results indicated that PRIMA-1^{met} induced autophagy in CRC cells regardless of p53 status via activating mTOR/AMPK-ULK1-Vps34 signaling cascade. However, induced autophagy was relevant to the cytotoxicity of PRIMA-1^{met} in cells carrying wild-type p53, along with up-regulation of Noxa. Implying that, PRIMA-1^{met}-based therapy could be an effective strategy for CRC.

Trail registration: Not applicable.

Introduction

Colorectal cancer (CRC, cancer of colon and rectum) is a worldwide health problem that is the second and the third most common cancer in women and men (www.wcrf.org), respectively. There were over 1.8 million new CRC cases and 0.88 million deaths in 2018[1]. In recent years, the incidence of CRC, especially rectal cancer, showed an increasing trend in youngers [2]. Early diagnosis and radical surgery

effectively improve the 5-year survival rate, however, there is still a lack of efficacious measures for advanced patients of stage III and stage IV.

Activation of oncogenes and inactivation/defect of tumor suppressor genes (TSGs) are crucial for the tumorigenesis of CRC. p53, a transcriptional factor, regulates processes of cell cycle arrest, apoptosis, DNA repair, and tumor angiogenesis via mediating numerous targeted genes. Importantly, p53 mutation occurring in about 40–50% CRC patients [3], is associated with resistance to current regimens and poor prognosis. PRIMA-1 (p53-reactivation and induction of massive apoptosis-1, APR-017), a low molecule compound ($C_9H_5NO_3$), was discovered to restore the sequence-specific DNA binding domain of mutant p53 by forming adducts with thiols for recovering the wild-type structure and function to induce cell apoptosis, thus selectively killing cancer cells with mutant p53 [4, 5]. PRIMA-1^{met} (APR246) as a methylated analog of PRIMA-1, is more effective on p53-mutant cells, and further certified to inhibit the growth of cells without mutant p53 [5], [6–9]. In our previous study, we have demonstrated that PRIMA-1^{met} suppressed CRC cell proliferation, migration, invasion, and colony formation independently on p53 status. Nevertheless, PRIMA-1^{met} induced robust apoptosis in cells carrying mutant p53 via up-regulation of proapoptotic Noxa [10]. However, mechanisms underlying the cytotoxicity of PRIMA-1^{met} in different CRC cell lines is still not entirely unknown.

Autophagy is a “self-eating” response to intracellular and environmental stimuli, such as starvation, nutrient deprivation, energy exhaustion, through regulatory factors that consist of the products of autophagy-related genes (ATGs) occurring in both normal and cancer cells. Once receiving nutrient or energy signals, regulators of autophagy induce the process, involving the formation of double-membraned autophagosome, fusion with lysosomes with cargoes, degradation, and recycling. By the process, intracellular proteins and organelles are able to be degraded in lysosomes and released into the cytoplasm for biosynthesis and metabolism recycling[11]. Autophagy originally acted as a tumor suppressive process based on the deletion of autophagy gene Beclin-1 (ATG6) in 40–75% human breast, ovarian and prostate cancers [12, 13]. Recent investigation has indicated that autophagy exerted an inhibiting influence on tumor initiation and progression via suppressing chronic inflammation, DNA damage, and genomic instability of tumor environment [14]. Thus, some pivotal points of autophagic pathways, such as mTOR (mammalian target of rapamycin), AMPK (Adenosine monophosphate-activated protein kinase), Akt (protein kinase B), PI3K (phosphoinositide 3-kinases) class III, Beclin-1, p53, etc, become promising targets for anti-cancer studies.

In this study, we shed new light on the effect of PRIMA-1^{met} on autophagy in different CRC cell lines, to further investigate mechanisms underlying the inhibitory effect in cells with different types of p53. Our results showed that PRIMA-1^{met} induced autophagy in different CRC cells irrespectively of p53 status via activating mTOR/AMPK-ULK1-Vps34 signaling cascade. Furthermore, up-regulated autophagy played a crucial role in the suppressed effect of PRIMA-1^{met} on cells carrying wild-type p53, which was related to up-regulation of Noxa. These findings further indicated deeper mechanisms of PRIMA-1^{met} for CRC.

Materials And Methods

Cell lines and drugs

CRC cell lines used in this study with wild-type or mutant p53 were obtained from Dr. Zhang Ting (Cancer Institute, Affiliated Hospital of Jiangnan University, China). The details were as follows: LOVO (wild-type p53), RKO (wild-type p53), HCT116 (wild-type p53), DLD-1 (mutant p53-S241F), SW480 (mutant p53-R237H), SW620 (mutant p53-R237H), HCT15 (mutant p53-P153A), and CaCO2 (mutant p53-E204X). HCT116 null (p53 -/-) cells were provided from Dr. Jimmy Chao (Bioprocessing Technology Institute, A*Star, Singapore). Cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium, Hyclone, Logan, USA), except SW620 in RPMI-1640 Medium (Hyclone, Logan, USA), with 10% FBS (fetal bovine serum, Gibco life technology, New York, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) in a humidified incubator with 5% CO_2 at 37°C. PRIMA-1^{met} (APR-246, Santa Cruz Biotechnology, CA) dissolved in DMSO (Dimethyl sulfoxide) at the concentration of 50 mM and 3-Methyladenine (3-MA, Absin Bioscience Inc, Shanghai, China) dissolved in PBS (phosphate buffer saline) as 67.04 mM were both stored at -20°C. Working solutions were diluted to appropriate concentrations with culture medium. Same amount of DMSO as control reagent was added in experiments.

Western blotting analysis

Different CRC cells were seeded at the density of 2×10^6 cells per 100 mm dish overnight, and treated with DMSO, different doses of PRIMA-1^{met} or 3-MA (800 μM). After incubated for 24 hours, cells were harvested, lysed and analyzed by western blotting. Primary antibodies used in this study were against β-Actin (AC026, ABclonal Technology, Wuhan, China), LC3 (NB100-2220, Novus Biologicals, US), Noxa (OP180, EMD Millipore, Gibbstown, NJ), p62 (#8025), ULK1 (#8054), phospho-ULK1 (Ser757) (#14202), mTOR (#2983), phospho-mTOR (Ser2448) (#5536), AMPK (#2532), phospho-AMPK (Thr172) (#2535) and phospho-PI3K Class III (Ser249) (#13857) (Cell Signaling Technology, Danvers, MA) diluted in blocking solution at the ratio of 1:1000. Second antibodies were goat-anti-rabbit IgG HRP (AS014), and goat-anti-mouse IgG HRP (AS003, ABclonal Technology, Wuhan, China) diluted in blocking solutions at the ratio of 1:5000. Expression level of proteins was estimated with gray value calculated using ImageJ version 1.8.0 (National Institutes of Health, USA) and presented in percentage normalized to β-Actin.

Acridine orange (AO) staining

A panel of CRC cells with different p53 status (HCT116^{wt}, RKO, CaCO2, SW480 and HCT116^{ko}) were seeded in 24-well plates containing cell slides at the density of 6×10^4 cells with 1 ml medium per well overnight, and treated with DMSO or 30 μM PRIMA-1^{met} for 24 hours. AO (Absin Bioscience Inc, Shanghai, China) solution was added to each well at a concentration of 1 μg/ml, followed by incubation at 37°C for 15 minutes. Rinsed with PBS twice, slides were taken out and inverted onto carry sheet glass. The slides of cells were observed and taken pictures under confocal microscopy with the filter excited at 488 nm and blocked at 515 nm (Hitachi, Japan).

Transmission electron microscopy (TEM) detection

RKO and HCT15 cells were seeded in 100 mm dishes at the density of 2×10^6 cells per dish overnight, and then treated with DMSO, 30 μM PRIMA-1^{met} or 60 μM PRIMA-1^{met}. After 24 hours, cell pellets were harvested, and fixed with 4% glutaraldehyde solution at 4°C for 4 hours, then followed by 2-hour fixation with 1% osmic acid at 20°C. Specimens were dehydrated by 50–100% ethanol and 100% acetone, and embedded with Epon 812. Cut into 60–80 nm, slides were stained with both 2% uranyl acetate and lead citrate to observe AVs with double membrane and autolysosomes under a TEM (Hitachi, Japan).

Cell counting Kit-8 (CCK-8) proliferation assay

After 24 hours of seeding in a 96-well plate at the density of 7000 cells/well with 100 μl medium, cells were treated with DMSO, PRIMA-1^{met} (45 μM for $HCT116^{wt}$, 50 μM for RKO, 55 μM for DLD-1 and HCT15), 3-MA (800 μM) or combination, and incubated for 48 hours. Cell Counting Kit-8 (CCK-8) proliferation assay (Dojindo, Shanghai, China) was used for measuring the cell proliferation by quantitating WST-8 formazan dye which was proportional to living cells according to previous description[15]. The reading value of DMSO control sample was set as 100%, as well as values of other samples were calculated for relative proliferation to DMSO control. Each cell line was obtained three independent experiments.

siRNA transfection

DLD-1 and $HCT116^{wt}$ cells were seeded in 6-well plates at 6×10^5 cells with 2 ml medium per well and cultured for 24 hours. Mixture containing 110 pmol of Noxa siRNA (sc-37305, Santa Cruz Biotechnology) or control siRNA (sc-36869, Santa Cruz Biotechnology), 200 μl jetPRIME® buffer (Polyplus-Transfection, Illkirch, France), and 4 μl jetPRIME® siRNA transfection reagent (Polyplus-Transfection, Illkirch, France) was vortexed 10 seconds, spun down and incubated for 10 minutes at room temperature. Then the mixture was added to each well. After 24 hours, cells were harvested for further experiments.

Statistical analysis

The statistical analysis was performed by SPSS version 19.0. Values were presented as mean + standard error of the mean (SEM). Comparisons between groups of data were made by one-way analysis of variance (ANOVA) and succedent Tukey's test. $p < 0.05$ was considered statistically significant.

Results

PRIAM-1^{met} induced autophagy flux in different CRC cells

In order to investigate the effects of PRIMA-1^{met} on autophagy flux, different CRC cells were exposed to PRIMA-1^{met} for further detection by western blotting. Following 24-hour treatment, increases of LC3-II expression were found in 3 lines carrying wild-type p53, 4 out the 5 p53-mutant lines (exclusive of DLD-1), and 1 p53-deleted cell line ($p < 0.01$, Fig. 1A, 2A, and 3A). Conversely, reduction of p62 expression was observed in $HCT116^{ko}$, RKO, HCT15, DLD-1, SW480, and SW620 cell line ($p < 0.01$, Fig. 1B, 2B, and 3B).

These results suggested that PRIMA-1^{met} promoted the dissociation of plasma LC3 (LC3-I) to membranous LC3 (LC3-II) for AV-membrane extension as well as degradation of cargoes (p62) in autolysosome. To further determine whether PRIMA-1^{met} induced autophagy flux in CRC cells, acridine orange (AO) staining was performed on HCT116^{wt}, RKO, SW480, CaCO2 and HCT116^{ko} cells with 24-hour treatment, showing that treated cells revealed stronger red fluorescence than DMSO controls under the confocal microscope (Fig. 1C, 2C, and 3C). However, AO had the affinity for acidic vesicular organelles (AVOs) including not only autolysosome but also lysosome. For deeply distinguishing, autolysosomes were observed in PRIMA-1^{met}-treated RKO, HCT15 cells under a transmission electron microscope (TEM). As shown in Fig. 4, autolysosomes/autophagosomes were increased significantly after PRIMA-1^{met} administration in both two cell lines compared with DMSO control, and even more prominently at high concentration ($p < 0.01$). Taken together, PRIMA-1^{met} promoted the whole process of AVs formation, cargoes removal, and degradation in autophagy flux that was irrespective of p53 status.

PRIMA-1^{met} regulated mTOR/AMPK-ULK1-Vps34 autophagic signaling cascade in different CRC cells

We next detected several molecules of autophagic signaling cascades using western blotting to dissect distinct mechanisms for the effect of PRIMA-1^{met} on autophagy in different CRC cells. Decrease in phospho-mTOR (a nutrient sensor) expression was found only in HCT116^{wt} and RKO cells with wild-type p53 after PRIMA-1^{met} treatment. On the contrary, increase in phospho-AMPK (an energy sensor) was observed in HCT116^{wt}, RKO, and HCT15 cells ($p < 0.01$, Fig. 5A). Furthermore, PRIMA-1^{met} administration up-regulated expression of phospho-ULK1 (Unc-51-Like Kinase 1) in RKO, DLD-1, and HCT15 cell lines, as well as PI3K Class III (the mammalian homolog of Vps34) in three lines of RKO, HCT116^{wt}, and HCT15 ($p < 0.01$, Fig. 5B). These findings indicated that PRIMA-1^{met} mediated mTOR/AMPK-ULK1-Vps34 signaling cascade for autophagy in CRC cells independent on p53 status.

PI3K inhibitor suppressed inhibitory effect of PRIMA-1^{met} in CRC cells with wild-type p53

To determine if the effects of PRIMA-1^{met} on autophagy made an impact on growth inhibition in CRC cell lines, PI3K inhibitor 3-methyladenine (3-MA) was used for further detection. CRC cells of four lines, including HCT116^{wt}, RKO, DLD-1, and HCT15, were exposed to PRIMA-1^{met}, 3-MA either alone or in combination for 48 hours, following by Cell Counting Kit-8 (CCK-8) proliferation assay. As expected, cell proliferation decreased in all cell lines with PRIMA-1^{met} alone ($p < 0.01$, Fig. 6A) while 3-MA exposure resulted in a rare suppression ($p > 0.05$, Fig. 6A). However, in the presence of combination, inhibition of cell growth was obviously weakened compared to PRIMA-1^{met} single in both HCT116^{wt} and RKO lines expressing wild-type p53 ($p < 0.01$, Fig. 6A). In contrast, there were no significant differences in cell proliferation between PRIMA-1^{met} and combination regimens in DLD-1 and HCT15 cell lines carrying

mutant p53 ($p > 0.05$, Fig. 6A). Decreased expression of LC3-II was confirmed by western blotting after 3-MA treatment in both HCT116^{wt} and RKO cells (Fig. 6B).

PRIMA-1^{met}-induced LC3 conversion in CRC cells with wild-type p53 was positively correlated to Noxa

We previously reported that up-regulation of Noxa, a pro-apoptotic molecule, was crucial for PRIMA-1^{met} to induce robust apoptosis in CRC cell lines with mutant p53 [10]. To investigate the role of Noxa in PRIMA-1^{met}- induced autophagy, we knocked down Noxa gene by siRNA in DLD-1 and HCT116^{wt} cell lines, and confirmed decreased expression by western blotting (Fig. 7). Notably, LC3-II expression was reduced in HCT116^{wt} cells after knocking down Noxa ($p < 0.01$), whereas knocked-down Noxa in p53-mutant DLD-1 cell line failed to produce significant impacts on LC3 conversion ($p > 0.05$) (Fig. 7).

Discussion

Autophagy proceeds through several phases, including initiation (prepare membrane to form AVs), membrane curative, LC3 family conjugation cascade, cargo loading, AV maturation, AV-lysosome fusion, lysosomal degradation and recycling[16]. This dynamic process is also called autophagy flux. In this study, several methods, such as western blotting, AO staining, and TEM observation, provided sufficient evidence for PRIMA-1^{met}-induced autophagy in CRC cell lines with different p53 status. PRIMA-1^{met} treatment not only up-regulated LC3 conversion and resulted in the increase of AVs, but also promoted degradation of cargoes combining with receptor p62. This effect was regardless of p53 status, but relative to different cell types. Furthermore, our results suggested a molecule mechanism underlying PRIMA-1^{met}-induced autophagy in CRC cells via mediating mTOR/AMPK-ULK1-Vps34 signaling cascade. ULK1, the mammalian homolog of ATG1, plays a convergent role in multiple signals that regulate autophagy, receiving nutrient and energy signals from the two upstream factors of AMPK and mTOR. AMPK activated under low energy condition with an elevated AMP/ATP ratio phosphorylates ULK1 [17]. On the other hand, mTOR as an autophagy inhibitor reversely regulates the phosphorylation of ULK1 by nutrient stress and even disrupts the interaction of ULK1, and AMPK [18]. Once activated, ULK1 complex that consisting of ULK1, ATG13, FIP200, and ATG101 promotes the initiation of AV formation [19], then further leads to activation of Vps34 complex, which including Vps34 (PI3K Class III), Beclin-1, Vps15, ATG14, for AV membrane curvature [20]. In this study, we found PRIMA-1^{met} up-regulated phosphorylation of AMPK, ULK1, and PI3K Class III simultaneously in CRC cells carrying both wild-type p53 and mutant p53. However, decrease in the phosphorylation of mTOR was only observed in cells with wild-type p53. It was worth nothing that PRIMA-1^{met} increased the phosphorylation of PI3K Class III but without ULK1 in the the HCT116^{wt}cell line, supposed due to activation by AMPK directly. These findings provided a pronounced link between PRIMA-1^{met} and autophagy in different CRC cell lines mediated by mTOR/AMPK-ULK1-Vps34 signaling cascade. 3-MA, targeting at Vps34 and PI3Kγ for inhibiting PI3K Class I permanently as well as PI3K Class III temporally, is a useful reagent to block the formation of the

autophagosome. Combined with 3-MA in PRIMA-1^{met} treatment, we suppressed inducible autophagy and further increased cell proliferation effectively in CRC cells expressing wild-type p53 compared to PRIMA-1^{met} alone. This result supported our hypothesis that the mechanism underlying the cytotoxicity of PRIMA-1^{met} in cells with wild-type p53 was relative to the induction of autophagy. Previous studies demonstrated the crosstalk between autophagy and apoptosis was required for regulating cell growth and survival especially the Bcl-2 family [21, 22]. Our study showed that reduced expression of Noxa by siRNA suppressed LC3 conversion in the HCT116^{wt} cell line, but not in the DLD-1 cell line. In other words, up-regulation of Noxa was supposed to influence cell autophagy after PRIMA-1^{met} treatment in CRC cells carrying wild-type p53. These findings confirmed that different mechanisms were involved in the inhibitory effect of PRIMA-1^{met} in CRC cells with different types of p53. PRIMA-1^{met} mainly promoted apoptosis in cells harboring mutant p53, whereas PRIMA-1^{met} induced autophagy in cells carrying wild-type p53 or null p53. Inducible autophagy including autophagic proteins was supposed to participate in apoptosis regulation through the complex network of molecular interactions between them in p53-mutant cells after PRIMA-1^{met} treatment.

In conclusion, our study elucidated that PRIMA-1^{met} induced autophagy in CRC cells via activating mTOR/AMPK-ULK1-Vps34 signaling cascade in a p53-independent manner (Fig. 8). Nevertheless, induced autophagy was closely related to the cytotoxicity of PRIMA-1^{met} in cells carrying wild-type p53, along with up-regulation of Noxa. According to the complex interaction between autophagy and apoptosis, a deeper insight into the correlation between them induced by PRIMA-1^{met} especially in p53-mutant cells is an attractive avenue for further investigation. Our new findings further elaborate a deeper understanding of mechanisms for the anti-tumor activity of PRIMA-1^{met} in CRC, that will support to PRIMA-1^{met}-based therapy as a strategy for improving advanced CRC patients' outcome.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets during and/or analysed during the current study available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by grants from National Natural Science Foundation of China (NSFC, 81603128) *, Gusu Health Layer Training Program for Young Top Talent (GSWS2019059), and Science and Technology Project of Suzhou (SYSD2018136).

Authors' contributions

Li XL, Zhou J, and Chen ZR were responsible for the experimental design; Li XL and Zhou J wrote the manuscript; Chen ZR revised the manuscript; Li XL, Xia CJ and Lu ZK performed experiments. All authors read and approved the final manuscript.

Acknowledgements

We gratefully acknowledge to Dr Jun-dong Zhou (The Affiliated Suzhou Hospital of Nanjing Medical University, China) and Dr Fang Lin (Soochow University, China) for their grateful help to this study.

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Figures

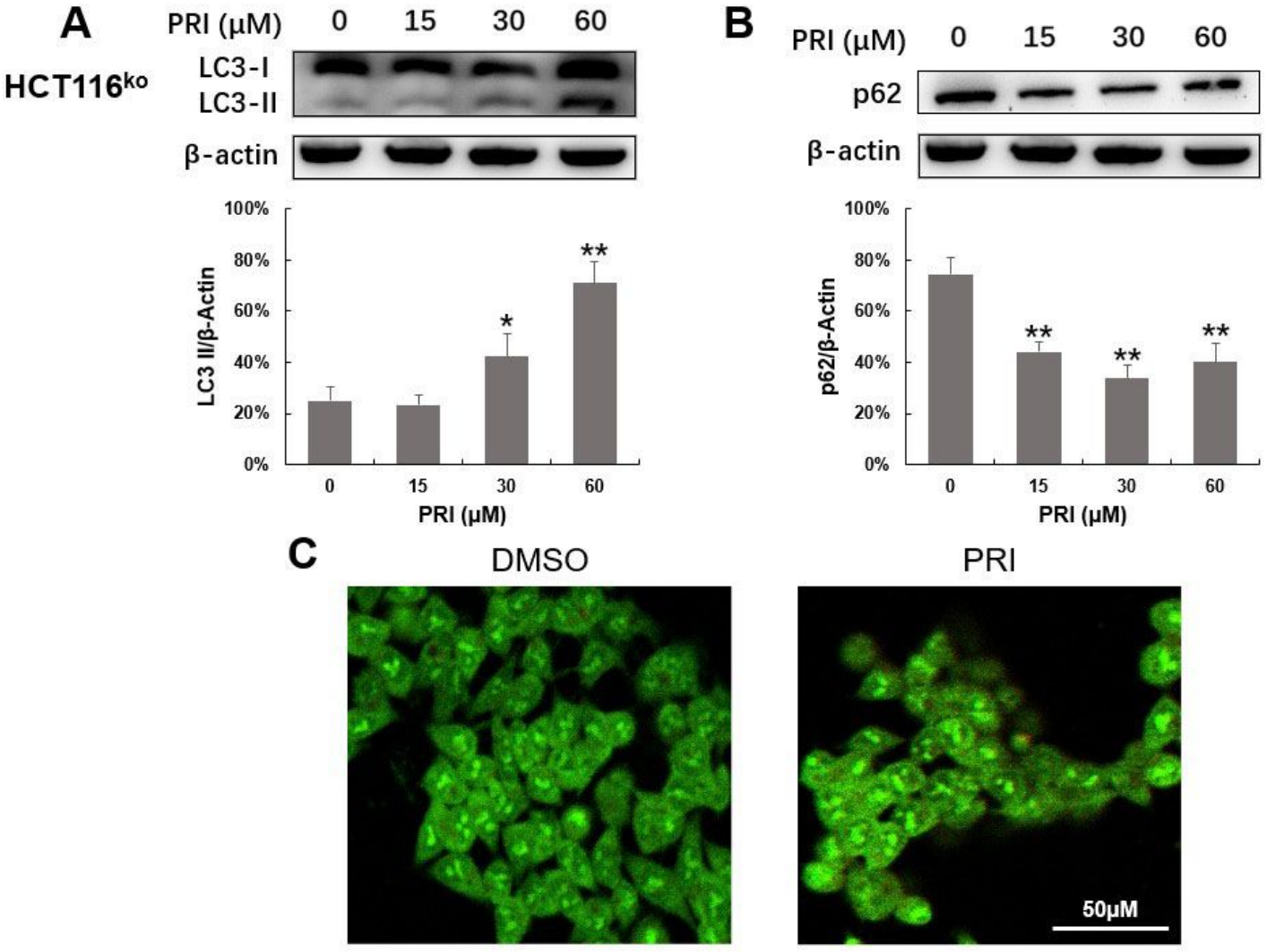

Figure 1

PRIMA-1met promoted autophagy flux in HCT116ko cell line without p53. A. HCT116ko cells were treated with PRIMA-1met (PRI) at concentrations of 0, 15, 30 and 60 μM for 24 hours. Western blotting showed LC3-I and LC3-II bands of these four cell lysates. The histogram of gray value was calculated using ImageJ version 1.8.0 from three independent experiments. The amount of LC3-II was normalized with respective β-Actin. Error bars showed standard error of mean (SEM). B. and expression level of p62. C. HCT116ko cells were staining with acridine orange (AO) after 24-hour treatment of DMSO or 30μM PRIMA-1met (PRI). The cytoplasm and nucleus revealed green fluorescence, as well as acidic vesicular organelles (AVOs) showed bright red. More red fluorescence was observed in cells with PRIMA-1met administration compared to DMSO control. *p<0.05, **p<0.01. (p>0.05, not significant)

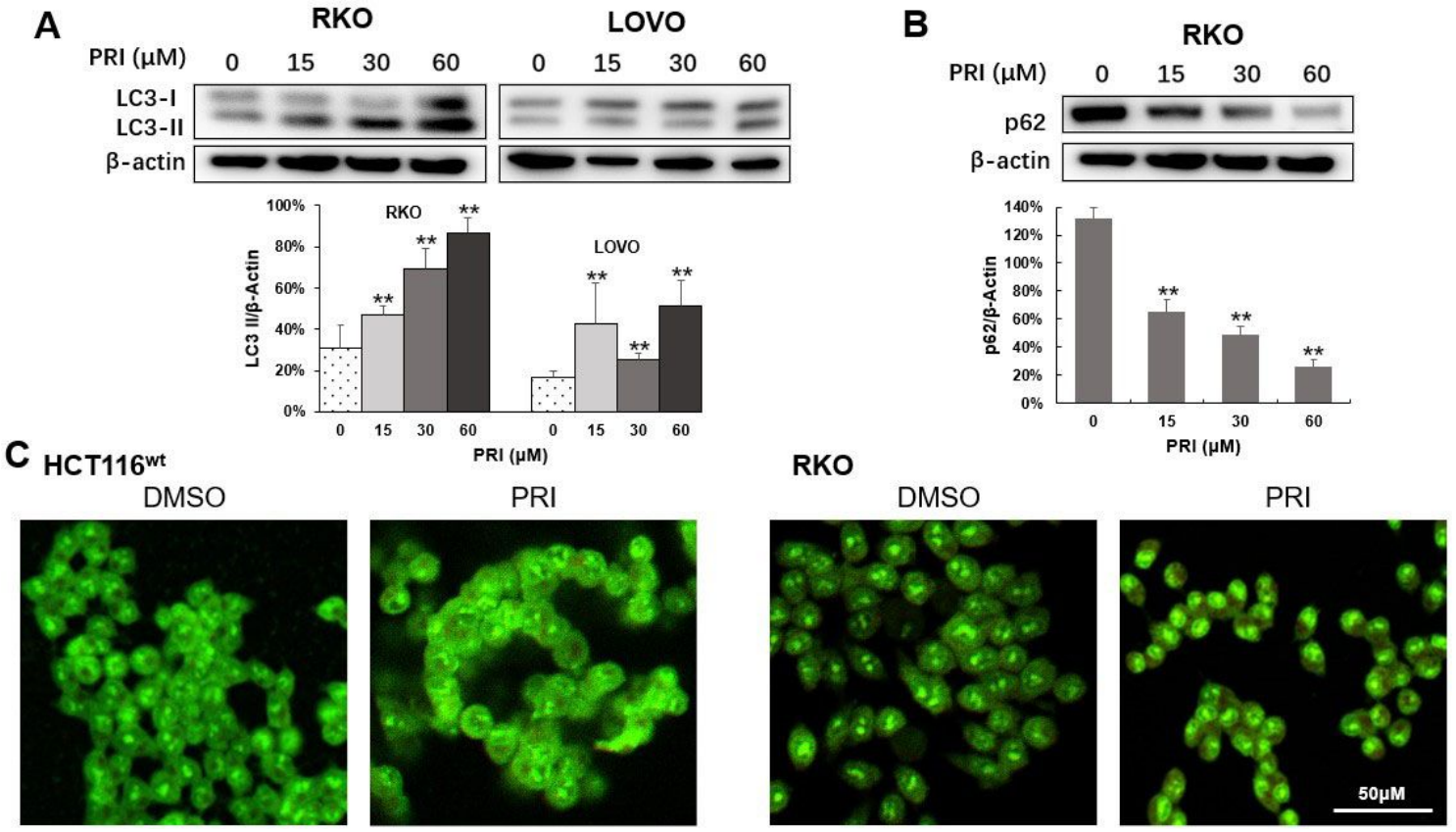

Figure 2

PRIMA-1met promoted autophagy flux in CRC cell line with wild-type p53. A. LC3-I and LC3-II bands of RKO and LOVO cell lysates with 24-hour treatment of PRIMA-1met (PRI) were showed by western blotting. The histogram of LC3-II amount was calculated after three independent experiments, and error bars showed SEM. B. Expression level of p62 was decreased in HCT116wt cells along with progressive increases in the concentrations of PRIMA-1met (PRI). N=3, error bars represented SEM. C. Both HCT116wt and RKO cells with treatment of 30μM PRIMA-1met revealed more red fluorescence from AVOs by AO staining. **p<0.01. (p>0.05, not significant)

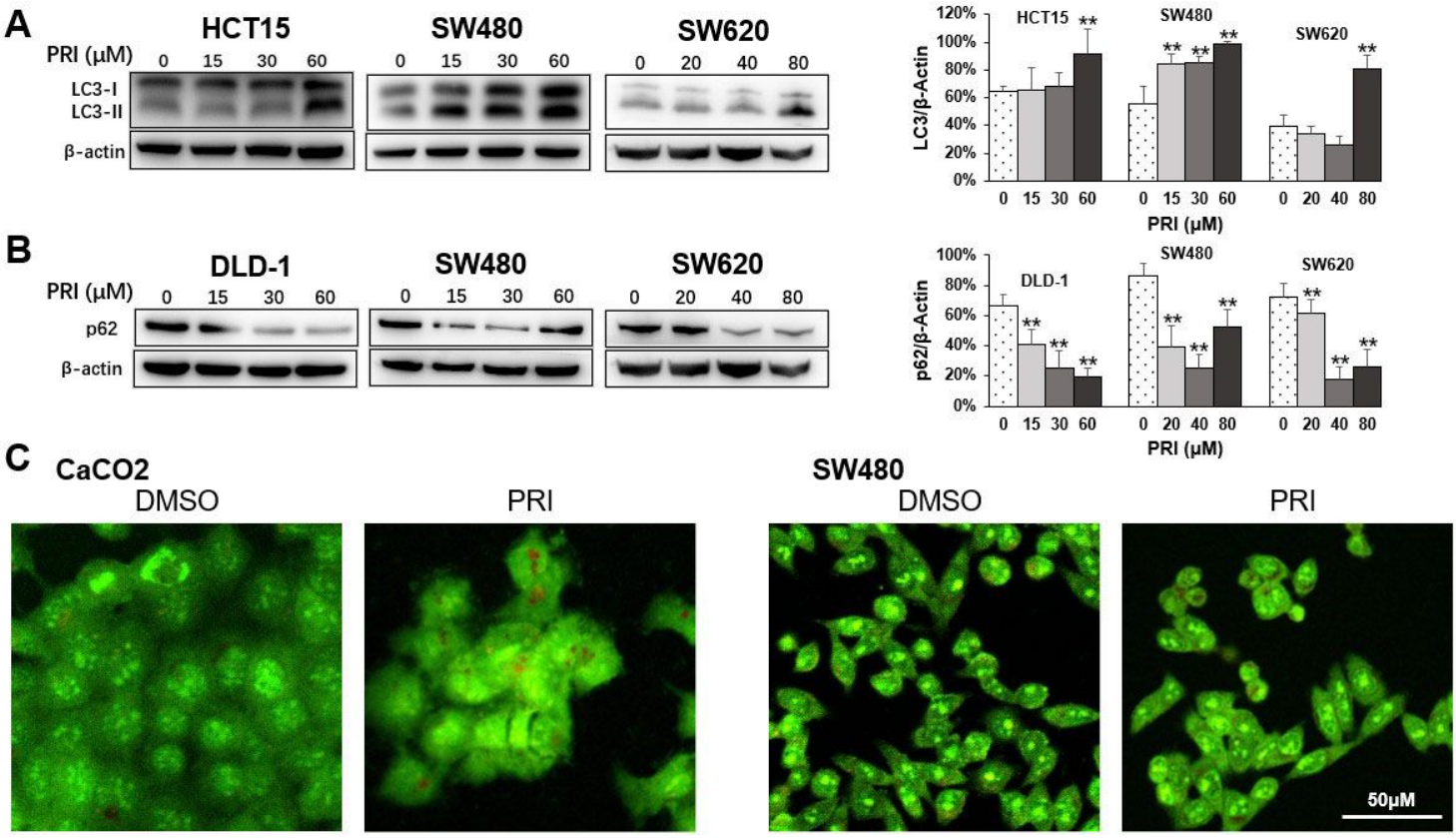

Figure 3

PRIMA-1met promoted autophagy flux in CRC cell line with mutant p53. A. The western blotting showed LC3-I and LC3-II bands of HCT15, SW480 and SW620 cell lysates with different concentrations of PRIMA-1met (PRI) for 24 hours. The histogram of gray value represented the amount of LC3-II normalized with respective β-Actin. N=3, error bars showed SEM. B. Expression level of p62 was decreased in DLD-1, SW480, and SW620 cell with 24-hour treatment of PRIMA-1met (PRI). N=3, error bars indicated SEM. C. More red fluorescence of AVOs was observed in both CaCO2 and SW480 cells treated with 30μM PRIMA-1met (PRI) by AO staining. **p<0.01. (p>0.05, not significant)

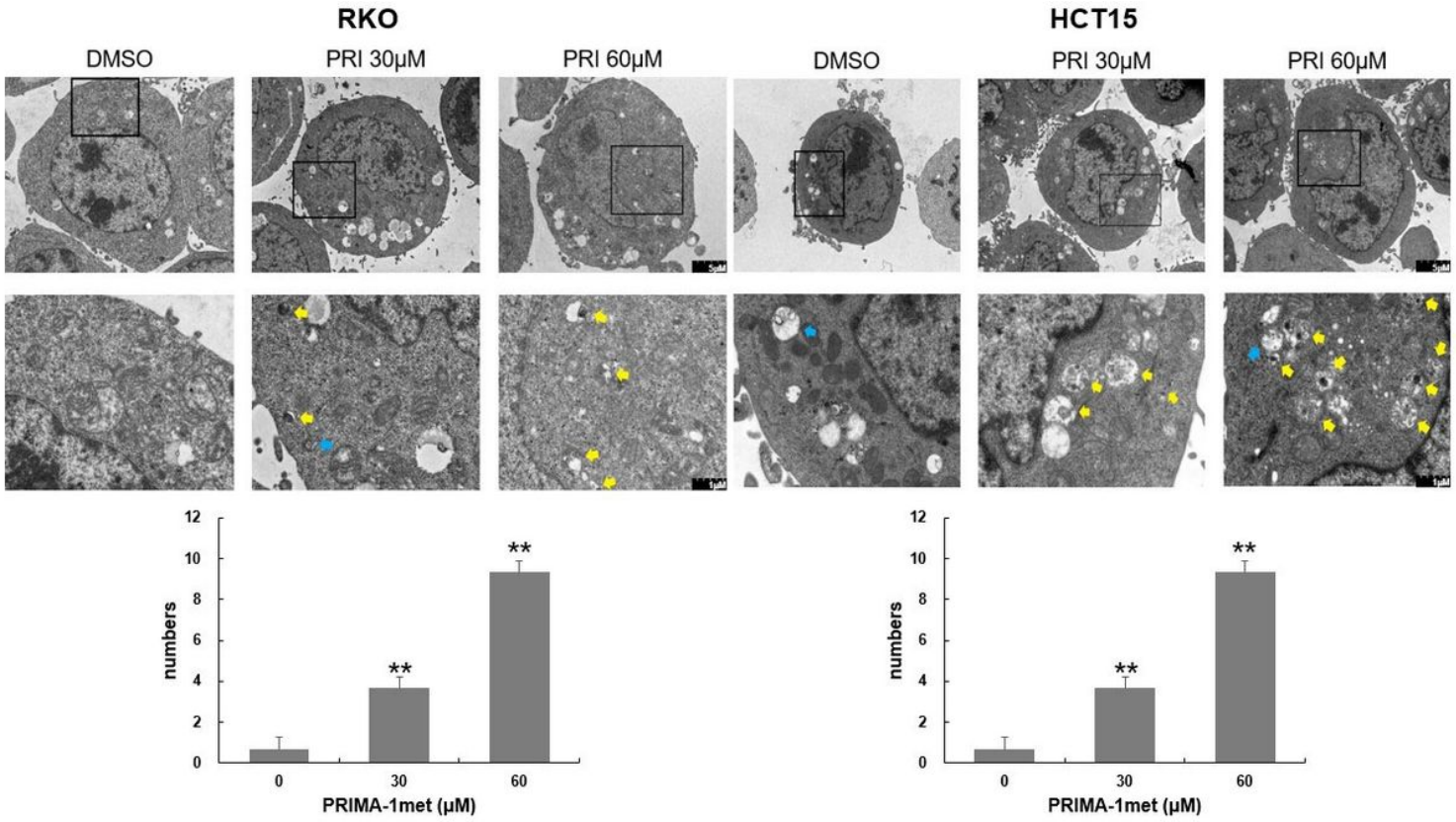

Figure 4

Autophagosomes and autolysosomes were detected by TEM after treatment of DMSO, 30μM PRIMA-1met (PRI) or 60μM PRIMA-1met (PRI) for 24 hours (blue arrow: autophagosome, yellow arrow: autolysosome). Increases of autophagosome and autolysosome were observed in both RKO and HCT15 cell lines with PRIMA-1met administration. The histogram of their numbers was obtained from three different cells in each treated sample, and error bars represented SEM. **p < 0.01. (p>0.05, not significant)

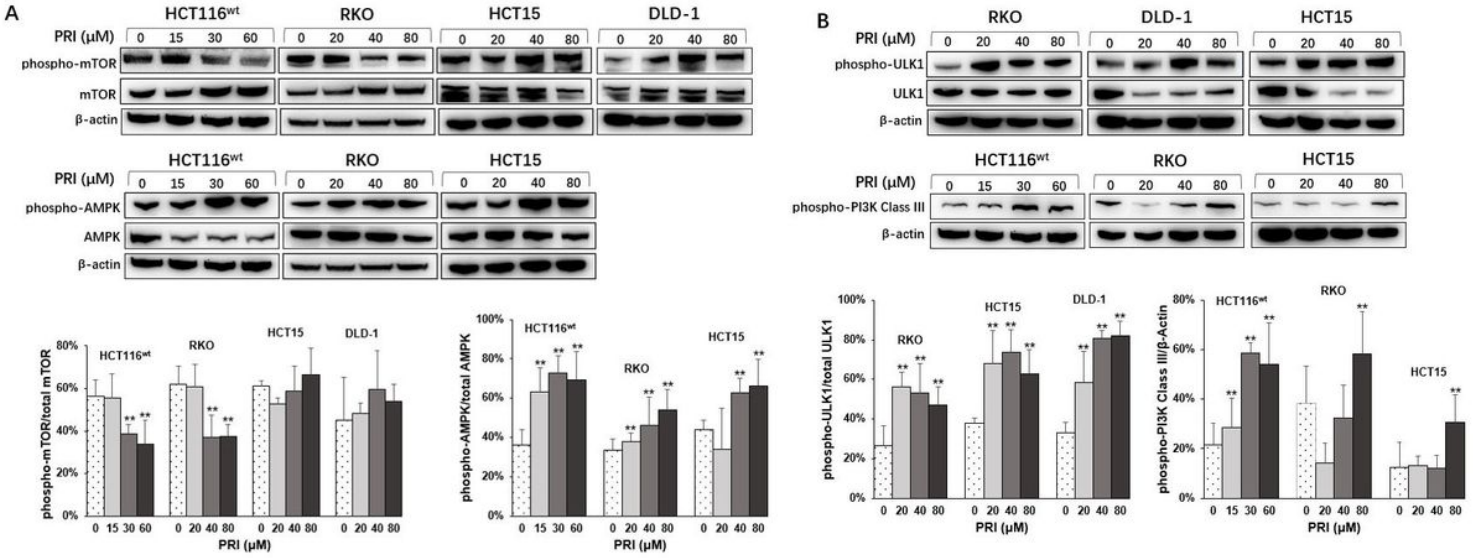

Figure 5

Autophagic proteins bands of CRC cell lysates with different concentrations of PRIMA-1met (PRI) for 24 hours were showed by western blotting. A. Expression level of phospho-mTOR was decreased as well as increases in mTOR in HCT116wt and RKO cell lines carrying wild-type p53. However, there were no significant differences in p53-mutant cell lines, HCT15 and DLD-1. The histogram represented the value of phospho-mTOR normalized with total mTOR (phospho-mTOR and mTOR). PRIMA-1met treatment up-regulated expression of phospho-AMPK, along with reduction of AMPK in HCT116wt, RKO and HCT15 cell lines. The histogram represented the value of phospho-AMPK normalized with total AMPK (phospho-AMPK and AMPK). Each experiment repeated three times, and error bars showed SEM. B. Expression of phospho-ULK1 was increased in RKO, DLD-1 and HCT15 cell lines after PRIMA-1met treatment, as well as reduction of ULK1 expression. The histogram represented the value of phospho-ULK1 normalized with total ULK1 (phospho-ULK1 and ULK1). Expression level of phospho-PI3K Class III was up-regulated by PRIMA-1met in HCT116wt, RKO and HCT15 cell lines. The amount of phospho-PI3K Class III was normalized with respective β-Actin in the histogram. N=3, error bars indicated SEM. **p<0.01. (p>0.05, not significant)

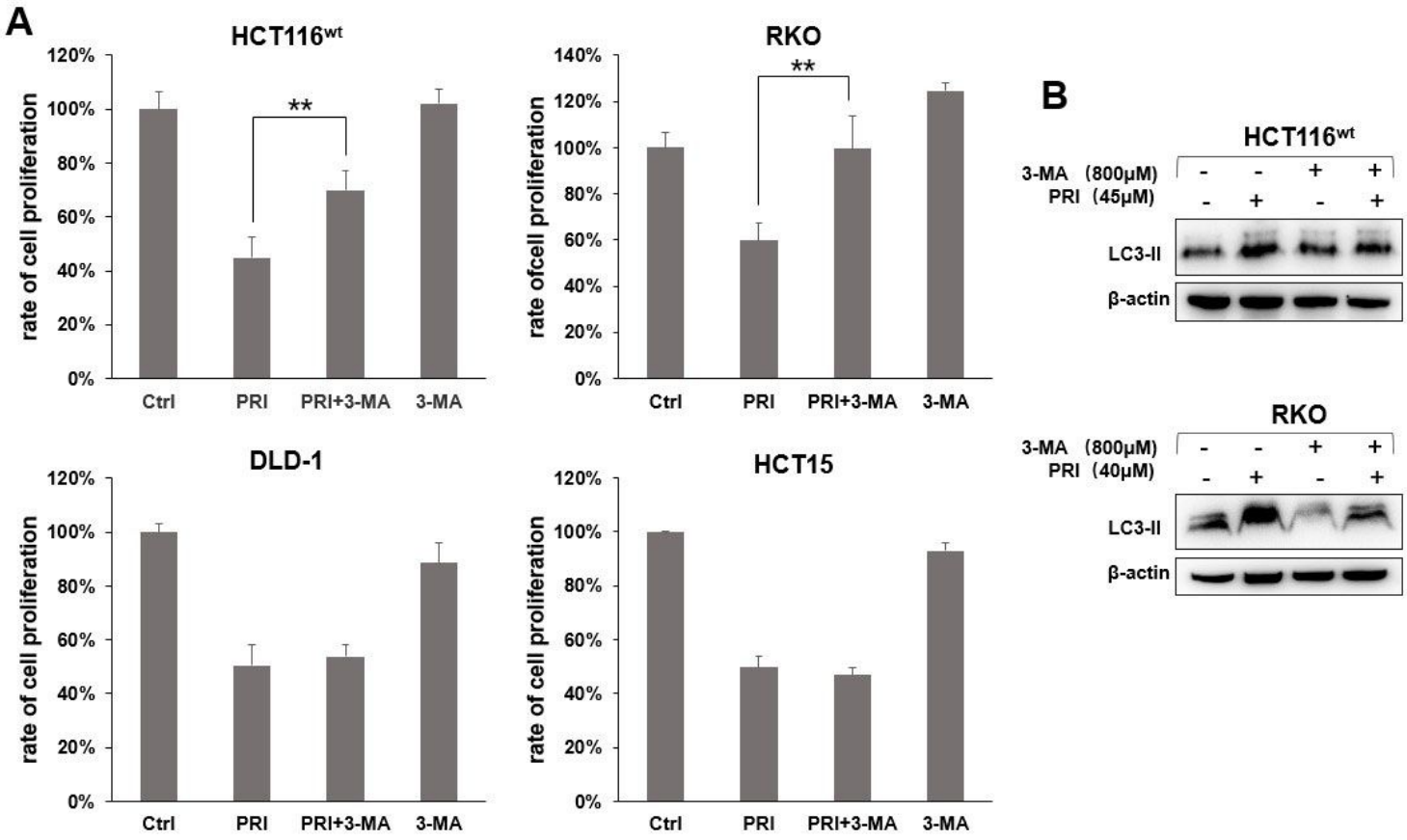

Figure 6

HCT116wt, RKO, DLD-1 and HCT15 cells were respectively treated with DMSO, PRIMA-1met (PRI) (45μM for HCT116wt, 40μM for RKO, 55μM for DLD-1 and HCT15), 3-MA (800μM) or two-drug combination. A. After 48 hours, CCK-8 assay was performed to estimate the cell proliferation of different cell lines with different treatments. The combined treatment showed a weaker suppression compared with PRIMA-1met alone in both HCT116wt and RKO lines. Each experiment was repeated for triplication, and error bars

represented SEM. **p<0.01. (p>0.05, not significant) B. LC3-II bands of HCT116wt and RKO cell lysates with different treatments were showed by western blotting, that confirming the decreased expression in HCT116wt and RKO cells after co-treatment compared to PRIMA-1met alone.

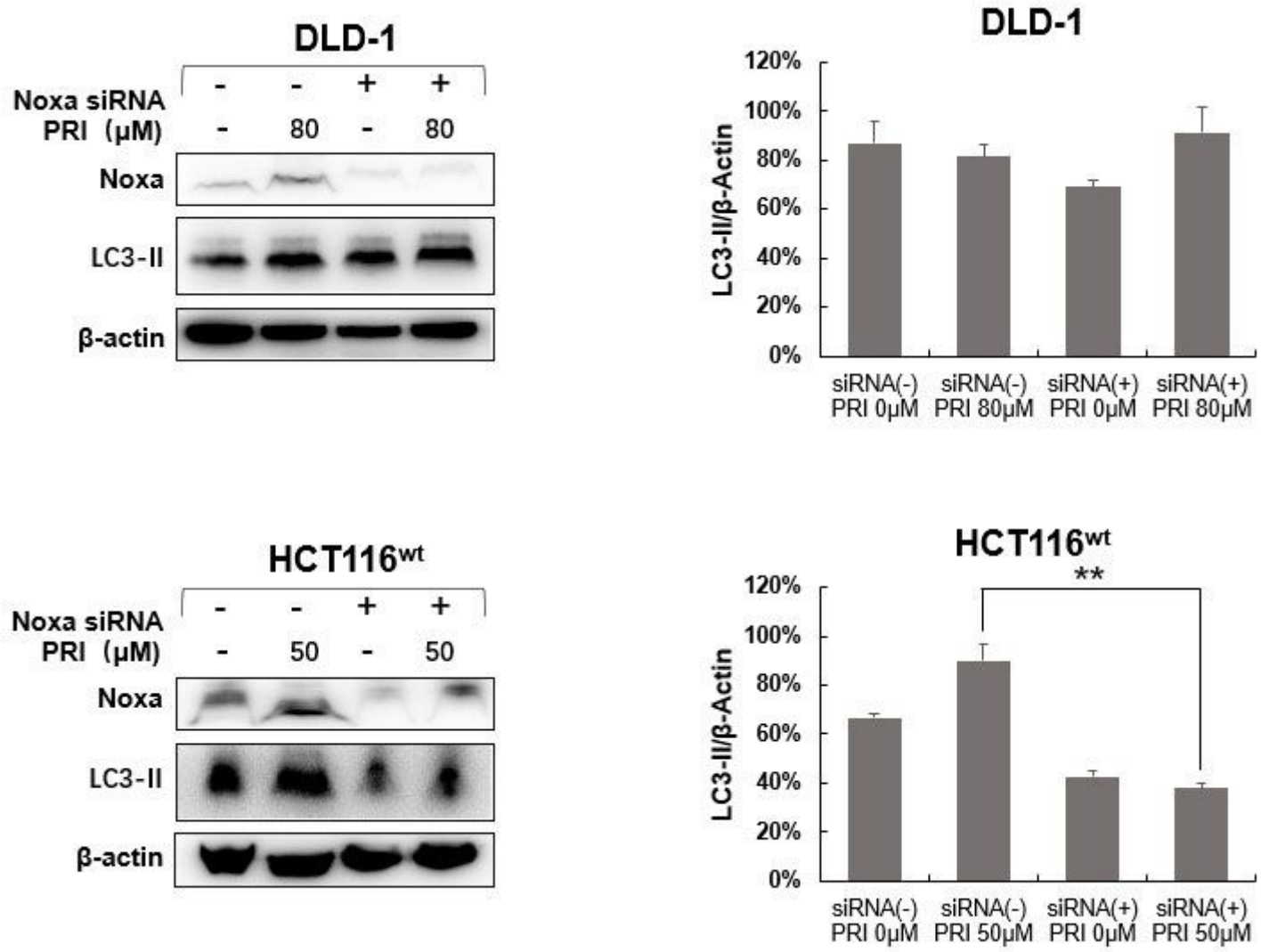

Figure 7

DLD-1 and HCT116wt cells were treated with or without PRIMA-1met (PRI) after transfection with control siRNA or Noxa siRNA. Western blotting showed Noxa bands to confirm the reduced protein level by Noxa-siRNA treatment in these 2 cell lines. Expression of LC3-II was suppressed in HCT116wt cell line with transfection of Noxa siRNA, but not in DLD-1 cell line. The amount of LC3-II was showed in the histogram normalized with β-Actin. N=3, error bars represented SEM. **p<0.01. (p>0.05, not significant)

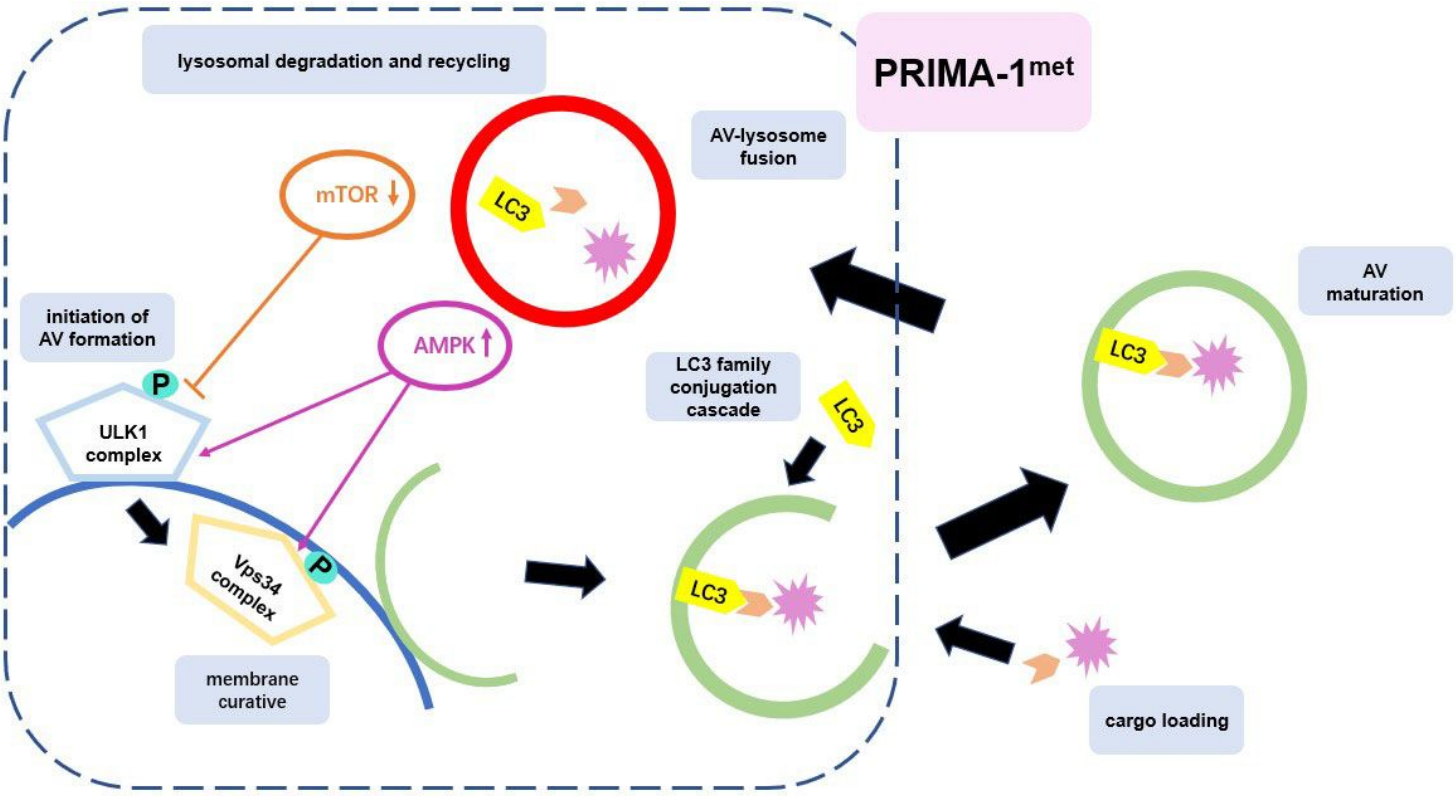

Figure 8

Autophagy pathway consists of sequential steps as reported before[16]. In our study, PRIMA-1met not only evidently induced autophagic vesicles (AVs) formation and AV-lysosome fusion, but also increased lysosomal degradation in CRC cell lines with different p53 status. PRIMA-1met up-regulated activation of AMPK in cells expressing wild-type p53 or mutant p53, whereas negatively regulated mTOR in cells with wild-type p53, and then further to increase activation of ULK1 and its downstream PI3K Class III (Vps34) regardless of p53 status.