



Research article

The involvement of Parkin-dependent mitophagy in the anti-cancer activity of Ginsenoside



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ABSTRACT

Colon cancer, the third most frequent occurred cancer, has high mortality and extremely poor prognosis. Ginsenoside, the active components of traditional Chinese herbal medicine Panax ginseng, exerts anti-tumor effect in various cancers, including colon cancer. However, the detailed molecular mechanism of Ginsenoside in the tumor suppression have not been fully elucidated. Here, we chose the representative ginsenoside Rg3 and reported for the first time that Rg3 induces mitophagy in human colon cancer cells, which is responsible for its anticancer effect. Rg3 treatment leads to mitochondria damage and the formation of mitophagosome; when autophagy is inhibited, the clearance of damaged mitochondria can be reversed. Next, our results showed that Rg3 treatment activates the PINK1-Parkin signaling pathway and recruits Parkin and ubiquitin proteins to mitochondria to induce mitophagy. GO analysis of Parkin targets showed that Parkin interacts with a large number of mitochondrial proteins and regulates the molecular function of mitochondria. The cellular energy metabolism enzyme GAPDH is validated as a novel substrate of Parkin, which is ubiquitinated by Parkin. Moreover, GAPDH participates in the Rg3-induced mitophagy and regulates the translocation of Parkin to mitochondria. Functionally, Rg3 exerts the inhibitory effect through regulating the nonglycolytic activity of GAPDH, which could be associated with the cellular oxidative stress. Thus, our results revealed GAPDH ubiquitination by Parkin as a crucial mechanism for mitophagy induction that contributes to the tumor-suppressive function of ginsenoside, which could be a novel treatment strategy for colon cancer.

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Abbreviations: BNIP3, BCL2 interacting protein 3; COX IV, cytochrome c oxidase IV; GSH, glutathione; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP60, heat shock protein 60 kDa; LC3, microtubule-associated proteins 1A/1B light chain 3; MFN2, mitofusion 2; PINK1, PTEN induced putative kinase 1; PARK2, Parkin; PHGDH, phosphoglycerate dehydrogenase; SQSTM1, sequestosome 1; TOMM20, translocase of outer mitochondrial membrane 20; TIM23, translocase of the inner membrane 23; VDACC1, voltage-dependent anion-selective channel protein 1.

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1. Introduction

Cancer is a life-threatening disease and has high mortality rate and less effective treatments [1]. At present, the clinical treatment of cancer generally involves several conventional therapies [2], including surgical resection, radiotherapy, chemotherapy, targeted therapy, immunotherapy etc. Although the integration of various therapies improve cancer patients' surviving time to some extent, the final effect is still not satisfactory. Nowadays, increasing evidence [3] indicate that natural products play an important role in the prevention and treatment of cancer. Natural products have advantages of efficacy, safety and economic impact and thus their unique therapeutic role has been highlighted. Due to chemical

diversity, natural products have been the major source for anti-cancer discoveries [4,5], including plant-derived active principles, some semi-synthetic and synthetic analogs etc.

Ginseng, the root of *Panax ginseng* Meyer, has been widely used in China for thousands years as a natural tonic. It is generally believed that the active compounds in *Panax ginseng* are ginsenosides and notoginsenosides [6,7], a diverse group of steroidal saponins. Several ginsenosides have been reported to possess various pharmacological and physiological activities [6], including anti-proliferative and anti-angiogenic activities in cancer. Among them, Rg3 is widely studied and applied to treat cancer as a natural product. The mechanisms responsible for the antitumor effect of Rg3 appear to be varied, including apoptosis and many signaling pathways [8], such as hypoxia-inducible factor 1 α (HIF-1 α), NF- κ B (nuclear factor kappa-B), Wnt/ β -catenin and AMPK (adenosine 5'-monophosphate-activated protein kinase) etc. In addition, Rg3 also serves as an adjuvant in conventional cancer therapies through improving the efficacy and abolishing adverse effects [9,10]. Due to the diversity of cancer models, the molecular modes of actions are various and still remain largely undefined.

Mitophagy belongs to selective autophagy and is an important mechanism for cellular mitochondrial quality control. In response to mitochondrial damage, the cleavage of PINK1 (PTEN induced putative kinase 1, one serine/threonine kinase) is attenuated and PINK1 becomes more stable and accumulates on the outer membrane of mitochondria to phosphorylate the E3 ubiquitin ligase Parkin [11]. After phosphorylation, Parkin is activated and recruited from cytosol to damaged mitochondria to initiate mitophagy [12]. Parkin is a tumor suppressor and its mutation occurs frequently in cancer [13]. In addition, other mitophagy receptors and adaptors such as BNIP3 (BCL2 interacting protein 3), BNIP3L/NIX, and p62/SQSTM1 are also impaired in cancer [14]. Dysregulated mitophagy results in the defect of mitochondria clearance, which plays an important role in carcinogenesis and tumor progression [15]. Thus, targeting mitophagy may be a novel strategy for cancer therapy. Several Ginsenosides have been reported to modulate cellular mitophagy level in various cells [16,17], including Rh2, Rd, Rg1, Rb1 etc. Mechanistically, PINK1 signaling is revealed to involve in this process. Through regulating mitophagy, these agents exert their anti-oxidative and anti-inflammatory activities in fatty liver disease, nutritional-stress induced injury, colitis etc. For example, Rh2 is able to mitigate oxidative stress and attenuate aging-related disease [18], including cancer, but mitophagy inhibition impairs the inhibitory effect of Rh2 on cancer cell growth. Thus, mitophagy may be novel mechanism of tumor suppression of ginsenosides and it seems necessary to investigate it.

In this study, we aimed to investigate the anticancer activity of ginsenoside and its potential molecular mechanism in colon cancer cells. We reported for the first time that the representative ginsenoside Rg3 inhibits colon cancer cell proliferation *in vitro* and this inhibitory effect is associated with mitophagy induction. The molecular mechanism of Rg3-caused mitophagy is revealed to the activation of PINK1-Parkin signaling pathway and Parkin-mediated GAPDH ubiquitination. GAPDH further regulates cell death through exerting its nonglycolytic activity. These findings shed light on a novel understanding that Parkin-dependent mitophagy is probably a novel mechanism for the tumor-suppression function of ginsenoside.

2. Results

2.1. Ginsenoside Rg3 induces mitophagy in human colon cancer cells

In various cancers, ginsenosides have been reported [19,20] to decrease the mitochondrial membrane potential, promote ROS

(reactive oxygen species) generation and trigger a series of apoptotic events. This implies that the damaged mitochondria could be eliminated through mitophagy. Here, we chose the representative ginsenoside Rg3 and examined whether Rg3 affects the mitophagy level in human colon cancer cells. As shown in Fig. 1A, in the TEM observation, the untreated HCT116 cells showed the integrity of cell membrane and healthy mitochondria, which was filled with well-developed cristae and cell nuclei. But under Rg3 treatment, severely swollen and disrupted mitochondria was observed in the autophagosome or autolysosome (Fig. 1A), demonstrating that Rg3 markedly damages mitochondria and initiates mitophagy in cells. In addition, we prepared the mitochondrial fractions of Rg3-treated cells and found that Rg3 treatment increased the levels of LC3-II (autophagosome marker) and SQSTM1/P62 (a selective autophagy adaptor) in mitochondria (Fig. 1B). In the meanwhile, higher ubiquitination level of mitochondrial proteins was also observed under Rg3 treatment. To further confirm the occurrence of mitophagy, we transfected HCT116 cells with a pH-sensitive fluorescent plasmid mito-Keima, which is often used to determine mitochondrial movement from the cytosol to the lysosome [21]. As shown in Fig. 1C, red spots appeared in the cytoplasm under Rg3 treatment, indicating that mitochondria tends to form autolysosomes.

Pharmacologic or genetic inhibition of autophagy was also performed to further examine the mitophagy level change. As shown in Fig. 1D, western blotting analysis showed that Rg3 treatment decreased the level of mitochondrial proteins, including VDAC1 (voltage-dependent anion-selective channel protein 1) and MFN2 (mitofusion 2). But in the presence of autophagy inhibitor CQ (chloroquine), the reduction of mitochondrial proteins was attenuated, indicating the impairment of Rg3-induced mitophagy. ULK1 (unc-51 like autophagy activating kinase 1) is critical for the induction of autophagy and is upregulated and translocates to fragmented mitochondria and induces mitophagy in response to hypoxia or mitochondrial uncouplers [22]. Here, we knocked down *ULK1* in HCT116 cells and observed that Rg3 treatment failed to diminish the level of mitochondrial proteins, including TIM23 (translocase of the inner membrane 23) and MFN2. Moreover, *ULK1* knockdown attenuated the growth-inhibitory effect of Rg3 on colon cancer cells, suggesting the tumor-suppressive role of mitophagy.

The involvement of the PINK1-Parkin signaling pathway in Rg3-induced mitophagy

The PINK1-Parkin pathway plays an important role in regulating mitophagy [23,24]. PINK1 phosphorylates and recruits Parkin to the damaged mitochondria surface, where Parkin ubiquitinates numerous mitochondrial proteins and initiates mitophagy [23]. Parkin, an E3 ubiquitin ligase, serves as tumor suppression and its inactivating somatic mutations have been reported in human malignancies [25,26]. Thus, mitophagy in general, could be a tumor suppression mechanism. Here, we examined the expression level of Parkin in a variety of human colon cancer cell lines and found that it was indeed downregulated compared with normal colon cells (Fig. 2A). GEPIA (gene expression profiling interactive analysis) also showed that the mRNA level of Parkin was lower in colon tumor tissues than that in nontumor tissues (Fig. 2B).

To elucidate the molecular mechanism of Rg3-induced mitophagy, we examined the effect of Rg3 on PINK1-Parkin signaling pathway and found that Rg3 treatment increased the expression levels of PINK1 and Parkin with time and dose (Fig. 2C and D). As a result, the enhancement of PINK1 stability resulted in the recruitment of Parkin to damaged mitochondria. Indeed, mitochondrial fractions of Rg3-treated cells also showed more accumulation of PINK1 and Parkin proteins (Fig. 2E), leading to the ubiquitination of mitochondrial proteins. In addition, we

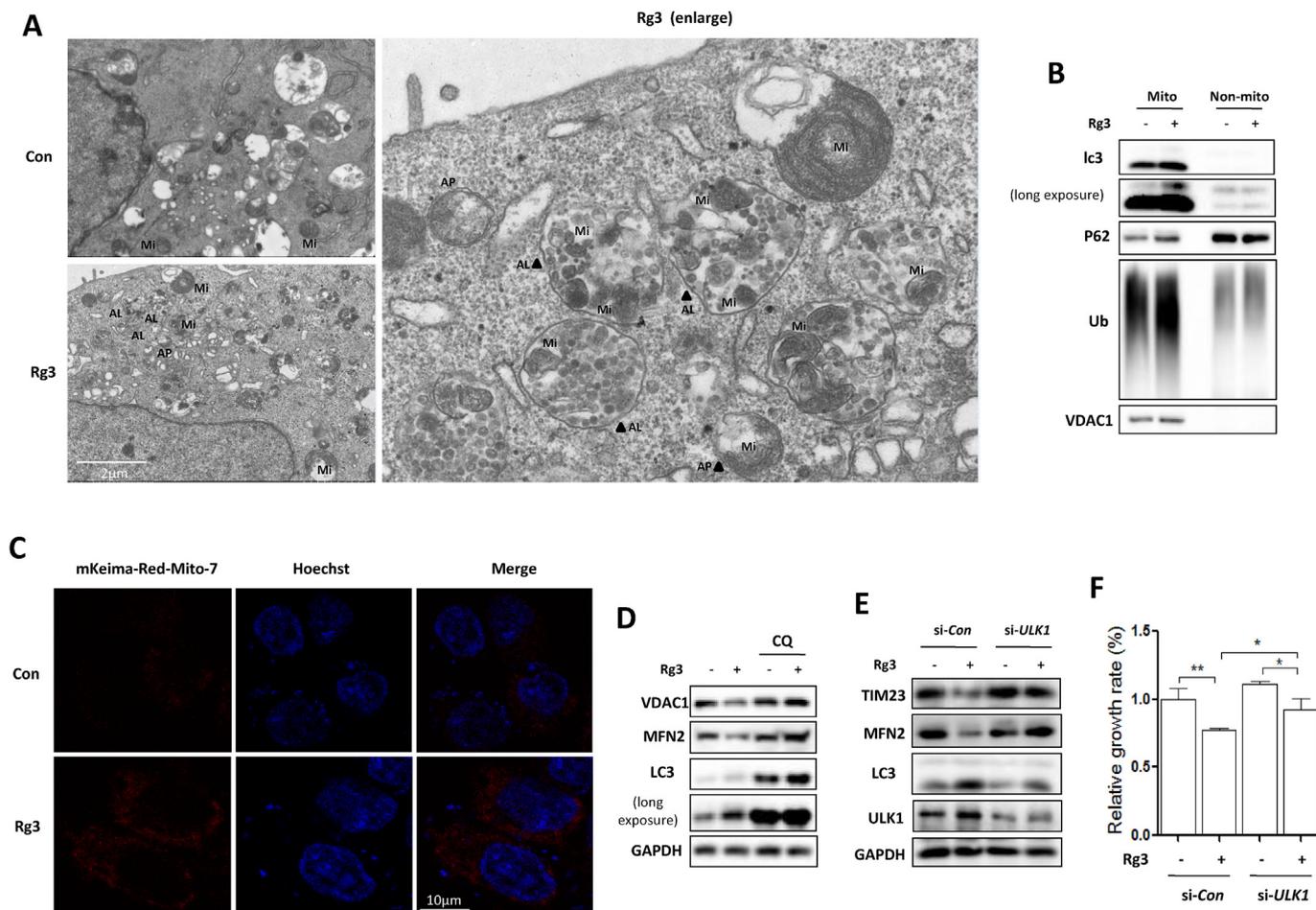


Fig. 1. Rg3 treatment induces mitophagy in human colon cancer cells. (A) TEM images of the ultrastructure of HCT116 cells under Rg3 treatment (20 μM, 12 h). Scale bar 2 μm. ▲ refer to double-membrane autophagosomes (AP) or autolysosomes (AL). (B) HCT116 cells were treated with Rg3 (20 μM, 12 h) and then mitochondrial fractions were prepared and subjected to western blotting analysis. VDACC1 was used as a representative of mitochondrial fraction. (C) HCT116 cells were transfected with the mKeima-Red-Mito-7 plasmid, followed by treatment with Rg3. The fluorescence was detected by confocal microscope. Scale bar 10 μm. (D) HCT116 cells were treated with Rg3 (20 μM) with or without CQ (10 μM) for 12 h. Then mitochondrial protein levels were detected by western blotting with GAPDH was used as loading control. (E) HCT116 cells were first transiently transfected with a nonspecific siRNA or the *ULK1*-specific siRNA and then cells were subsequently treated with Rg3 (20 μM, 12 h). Western blotting was used to determine the mitochondrial protein levels. (F) as in (E), CCK-8 (cell counting kit-8) solution was added into each well of the plate and the absorbance was measured at 450 nm using a microplate reader. **p* < 0.05 ***p* < 0.01.

performed the immunoprecipitation assay to examine the interaction of PINK1 and Parkin proteins. As shown in Fig. 2F, Rg3 treatment markedly enhanced their interaction, indicating the activation of PINK1-Parkin signaling pathway in Rg3-induced mitophagy.

2.2. Identification of Parkin targets by quantitative proteomics

To reveal the molecular mechanism of Rg3-induced mitophagy, quantitative proteomics was performed to identify the targets of Parkin. HEK293 cells were first transfected with GFP-Parkin and then treated with Rg3 before lysis. The lysates were then followed by affinity enrichment using GFP-fusion beads. The beads were thoroughly washed and digested by trypsin. The derived peptides were then pooled together and LC-MS/MS was performed to identify and quantify the target proteins. In the study, we identified 1563 proteins as the candidate targets of Parkin under Rg3 (Table S1). To differentiate specific targets, we set a highly stringent cut-off threshold. Statistical analysis was also applied to each identified protein and only identified proteins with *p*-values less than 0.05 were considered statistically reliable hits.

Subsequently, we performed GO (gene ontology) analysis of the Parkin targets under Rg3 treatment. The distribution of the enrichment ratio of these proteins is presented as a pie chart in Fig. 3A. These targets were broadly distributed in different parts of the cell, and especially enriched in the cytosol, nucleus, mitochondria and ER (endoplasmic reticulum). Among them, mitochondrial components were extracted and further analysed and their distribution was presented as a histogram (Fig. 3B). As shown in Fig. 3C, GO analysis also showed that the Parkin targets exert various molecular functions, including cell cycle, amino acid metabolism, mRNA stability, ubiquitin-dependent protein degradation, mitochondrion organization, apoptosis, mitochondrial transport etc. Most of them was associated with the molecular functions of mitochondria. Furthermore, we analysed the molecular functions of mitochondrial proteins of the Parkin targets and found that most of them belonged to metabolic enzyme and was involved in cellular energy metabolism (Fig. 3D), including glucose, amino acid, lipid etc. Especially in glucose metabolism, the key oxidoreductase GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was identified as a novel target of Parkin protein. GAPDH is an important enzyme in the cytoplasm for energy metabolism and

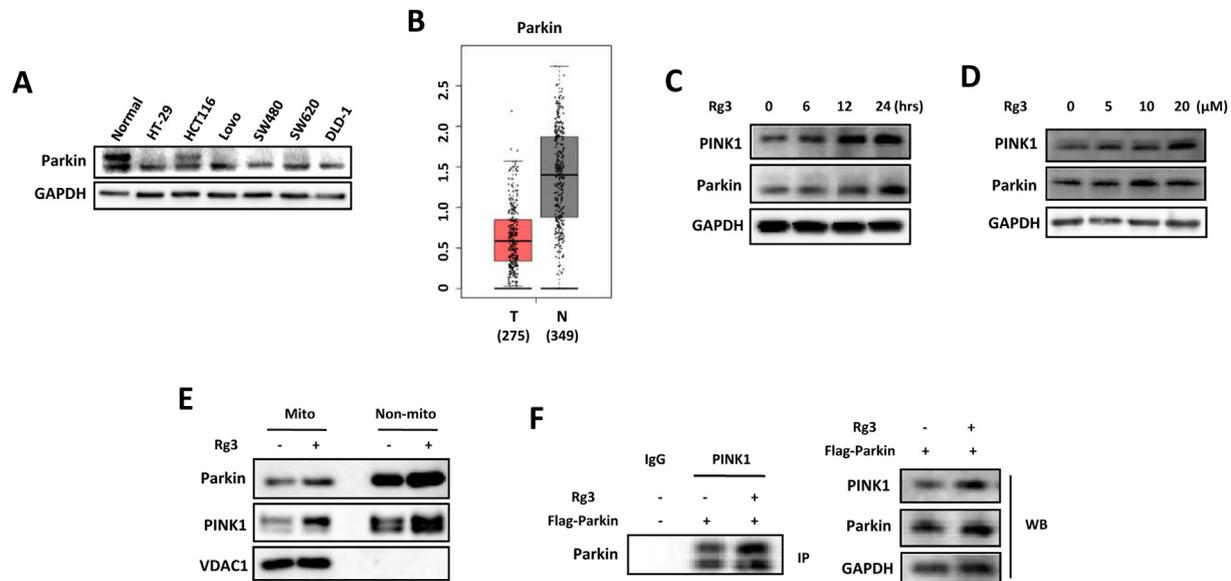


Fig. 2. Rg3 treatment activates the PINK1-Parkin signaling pathway. (A) The expression level of Parkin was measured using western blotting in various human colon cancer cells and normal colon cells. GAPDH served as a loading control. (B) The mRNA level of *PARK2* was shown in colon tumor tissues (T = 275) and nontumor tissues (N = 349) from GEPIA (gene expression profiling interactive analysis). (C) HCT116 cells were treated with Rg3 (20 μ M) for different time. Cell lysates were prepared and western blotting was performed to determine the expression levels of PINK1 and Parkin. (D) as in (C), HCT116 cells were treated with Rg3 (5, 10 or 20 μ M) for 12 h. Western blotting was used to measure the protein levels of PINK1 and Parkin. (E) HCT116 cells were treated with Rg3 (20 μ M, 12 h) and mitochondrial fractions were then prepared and subjected to western blotting analysis. VDAC1 was used as a representative of mitochondrial fraction. (F) HEK293 cells were first transfected with Flag-Parkin plasmid and then treated with Rg3 (20 μ M, 12 h). Cell lysates were prepared and subjected to immunoprecipitation using anti-PINK1 antibody. The associated Parkin protein was detected using immunoblotting.

participates in anaerobic glycolysis to produce ATP (adenosine triphosphate) and pyruvate [27]. Other cellular processes also involve this enzyme, such as apoptosis, membrane trafficking, iron metabolism, and nuclear translocation [28].

2.3. Parkin interacts with GAPDH and causes its ubiquitination

Catalytically inactive GAPDH has been shown to associate with damaged mitochondria, resulting in their engulfment into the lysosome [29,30]. It implied the role of GAPDH in the process of mitophagy. To clarify the biological function of GAPDH in Parkin-dependent mitophagy, we first determined the regulatory effect of Parkin on GAPDH. As shown in Fig. 4A, mass spectrometry analysis showed that GAPDH was a potential Parkin-interacting protein. TIMER (tumor immune estimation resource) analysis demonstrated a negative correlation of Parkin with GAPDH in human colon cancer (Fig. 4B). The interaction between Parkin and GAPDH was further verified by immunoprecipitation, followed by western blotting analysis in HEK293 cells with ectopic expression of Flag-Parkin (Fig. 4C). Consistently, the colocalization of Parkin with GAPDH was also displayed in confocal imaging (Fig. 4D). These results collectively demonstrate that GAPDH interacts directly with Parkin in cells. In addition, we examined the localization of GAPDH under Rg3 treatment. We prepared mitochondrial fractions of HEK293 cells with GFP-LC3 overexpression and performed immunoprecipitation using GFP-Trap Agarose. As shown in Fig. 4E, western blotting results showed that GAPDH was involved in the formation of mitophagosome, together with other damaged mitochondrial proteins, such as MFN2, TOM20 (translocase of outer membrane 20), VDAC1, HSP60 (heat shock protein 60) etc.

As an E3 ubiquitin ligase, Parkin promotes ubiquitination and degradation of different protein substrates [31,32]. In mitophagic process, ubiquitination of mitochondrial proteins is required for the recognition of damaged mitochondria by autophagy-related proteins [33,34]. Given that Parkin interacts with GAPDH, we

determined whether Parkin regulates GAPDH protein levels in cells. We first examined the effect of Parkin on GAPDH expression levels by knocking down *PARK2* in HCT116 cells. As shown in Fig. 4F, knockdown of *PARK2* decreased the protein ubiquitination level but did not influence GAPDH protein level. Further, overexpression of Parkin increased the protein ubiquitination level but GAPDH protein level did not change in HCT116 cells (Fig. 4F), indicating a non-degradative ubiquitination of GAPDH. Moreover, we measured the effect of Parkin on GAPDH activity, which was determined in a coupled enzyme reaction in which GAP (glyceraldehyde-3-phosphate) was converted to BPG (1,3-bisphosphate glycerate) by GAPDH. As shown in Fig. 4G, in HCT116 cells, *PARK2* knockdown significantly reduced GAPDH activity while Parkin overexpression significantly enhanced GAPDH activity, suggesting a positive regulation of Parkin on the substrate GAPDH.

To directly address the question of whether Parkin regulates GAPDH ubiquitination, *in vivo* ubiquitination assay was performed in HEK293 cells with ectopic expression of Flag-GAPDH, HA-ubiquitin and GFP-Parkin. Immunoprecipitation results showed that expression of Parkin markedly increased the ubiquitination level of GAPDH in cells (Fig. 4H), demonstrating that Parkin regulates GAPDH activity through ubiquitination. Under Rg3 treatment, the ubiquitination level of GAPDH was further enhanced (Fig. 4H). In addition, confocal imaging of GAPDH and ubiquitin showed that their colocalization was markedly increased in Rg3-treated HCT116 cells (Fig. 4I). In mitophagy process, Parkin-mediated ubiquitination of mitochondrial proteins are recognized by autophagy adaptor and participate in the formation of mitophagosome [35]. It gives an implication that GAPDH ubiquitination may be associated with Rg3-induced mitophagy.

2.4. GAPDH is required for Rg3-induced mitophagy

The association of Parkin, GAPDH and ubiquitin in the mitochondria may be a key step initiating the clearance of dysfunctional

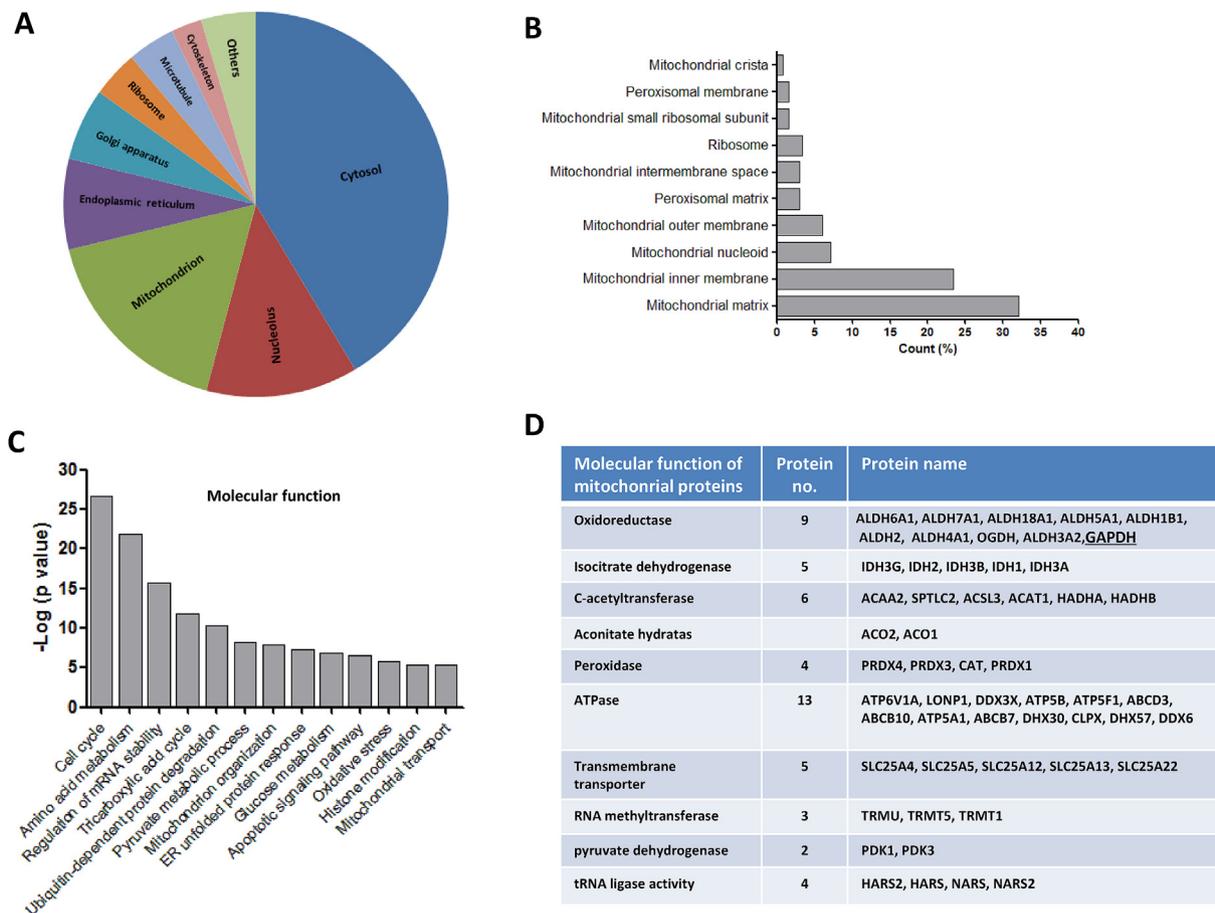


Fig. 3. Quantitative proteomics reveals Parkin-specific target proteins and involved pathways. Rg3-treated HEK293 cells with GFP-Parkin overexpression were lysed and subjected to immunoprecipitation using GFP-Trap Agarose. Total 1563 proteins were profiled as target proteins of Parkin using mass spectrometry analysis. (A) GO analysis of CC (cellular component) distribution of the Parkin targets. (B) GO analysis of the mitochondrial organelle of the Parkin targets. (C) Top molecular and cellular functional classes to which the Parkin-targeted proteins are associated. (D) List of the Parkin-targeted mitochondrial proteins and their molecular functions.

mitochondria under Rg3 treatment. We then investigated the role of GAPDH in the degradation of mitochondria. As shown in Fig. 5A, under Rg3 treatment, Parkin-dependent mitophagy occurred, resulting in the clearance of mitochondrial proteins VDAC1 and HSP60. But knockdown of GAPDH decreased the autophagy level and attenuated the degradation of damaged mitochondria in Rg3-treated cells. Conversely, GAPDH overexpression increased the autophagy level and promoted mitochondrial clearance under Rg3 treatment (Fig. 5B), as illustrated by the significant lower level of mitochondrial proteins TIM23 and HSP60. It demonstrated the participation of GAPDH in Rg3-caused mitophagy. Consistently, in mitochondrial fraction, Rg3 treatment enhanced the accumulation of autophagy proteins LC3, P62 and ubiquitin protein, but GAPDH knockdown diminished their translocation into mitochondria (Fig. 5C). On the contrary, when GAPDH overexpression, the recruitment of autophagy and ubiquitin proteins was increased and a more significant increase was detected under Rg3 treatment (Fig. 5D). Moreover, more Parkin was found to accumulate in mitochondria in cells overexpressing GAPDH, indicating that GAPDH regulates Parkin localization (Fig. 5c–d). Under Rg3 treatment, GAPDH was also required for Parkin translocation to damaged mitochondria, demonstrating the role of GAPDH in vesicular transport [36,37].

Furthermore, we performed confocal imaging to determine the formation of mitophagosome. The autophagy adaptor p62/SQSTM1 targets those ubiquitinated mitochondrial proteins and binds them

to form the mitophagosome [35]. We found that in GAPDH knock-down HCT116 cells, the colocalization of autophagy protein P62 and clustered mitochondrial protein COX IV (cytochrome c oxidase IV) was decreased (Fig. 5E), indicating the impaired formation of mitophagosome. Conversely, overexpression of GAPDH promoted their colocalization and enhanced the formation of mitophagosome (Fig. 5F). In addition, we also examined the translocation of mitochondria to the lysosome using mito-Keima. As shown in Fig. 5G, Rg3 treatment significantly enhanced the formation of red spots in the cytoplasm, but when GAPDH knockdown, the appearance of red spots was markedly weakened in Rg3-treated HCT116 cells. On the contrary, in GAPDH overexpressing cells, confocal imaging displayed more red spots and Rg3 treatment further promoted their formation (Fig. 5H). The above results demonstrated that the Parkin substrate GAPDH is required for the occurrence of mitophagy by Rg3.

2.5. Rg3 exerts the anticancer effect through regulating the nonglycolytic activity of GAPDH

Oxidant stress and redox signaling have been implicated in carcinogenesis [38]. The phenotypic behavior of cancer cells can be affected by ROS level, including their response to various therapeutic interventions. As a key redox-sensitive protein, the activity of GAPDH is also largely influenced by covalent modifications of oxidants [39]. For example, high levels of ROS often results in the

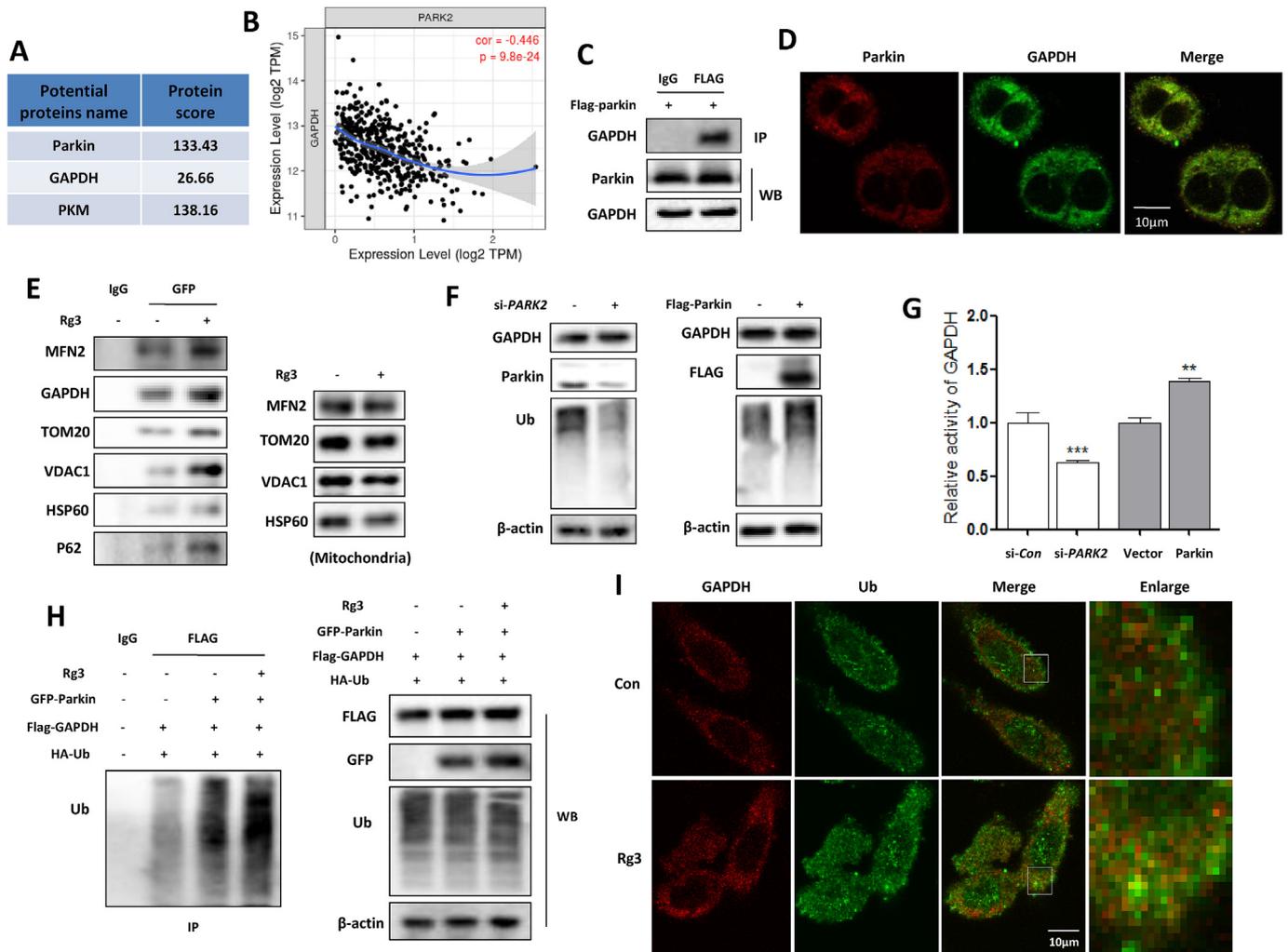


Fig. 4. Parkin interacts with GAPDH and causes its ubiquitination. (A) Protein score of GAPDH in GFP pulldown of cell lysates with GFP-Parkin overexpression. (B) Correlation analysis of Parkin with GAPDH in human cervical cancer through TIMER (tumor immune estimation resource). (C) HEK293 cells with Flag-Parkin overexpression were lysed and subjected to immunoprecipitation using anti-FLAG M2 affinity gel. (D) Confocal imaging of Parkin (red) and GAPDH (green) in HCT116 cells. Scale bar 10 µm. (E) HEK293 cells with GFP-LC3 overexpression were treated with Rg3 (20 µM, 12 h). Cells were harvested and mitochondrial fractions were prepared to immunoprecipitation using GFP-Trap Agarose. (F) HCT116 cells were transfected with the *PARK2*-specific siRNA (left panel) or with Flag-Parkin (right panel), respectively. Cell lysates were prepared for western blotting to determine the protein level of GAPDH. β-actin was used as a loading control. (G) as in (F), cells were harvested to measure the activity of GAPDH. **p < 0.01 ****p < 0.001 (H) *In vivo* ubiquitination assay analysis of the effect of Parkin on ubiquitination of Flag-GAPDH in HEK293 cells with or without Rg3 treatment. (I) HCT116 cells were treated with Rg3 (20 µM, 12 h) and confocal imaging of GAPDH (red) and ubiquitin (green) were performed. Scale bar 10 µm.

depletion of ATP (adenosine triphosphate), nuclear translocation of GAPDH and a decrease in glycolysis of cancer cells [40]. But whether GAPDH has a counter regulation of ROS production is unknown. Here, we knocked down *GAPDH* expression in HCT116 cells and detected lower levels of ROS (Fig. 6A). Meanwhile, there was an increase in cellular antioxidant glutathione (GSH) with *GAPDH* knockdown. On the contrary, overexpression of *GAPDH* resulted in higher levels of ROS (Fig. 6A), which could induce a variety of apoptotic signals in the absence of any external apoptotic stimuli. Thus, *GAPDH* is also a functional enzyme involved in the regulation of oxidative stress. It also implies that *GAPDH*-mediated mitophagy may be associated with ROS generation, which is an effective inducer of mitophagy.

In addition, *GAPDH* has also been described to participate in cell death [41]. When cell death induction, the levels of *GAPDH* in mitochondria are found to apparently increase [39,42], where it binds to VDAC and causes the release of proapoptotic proteins. Here, we also determined the functional role of *GAPDH* in tumor suppressive effect of Rg3. As shown in Fig. 6B, Rg3 treatment significantly inhibited colon cancer cell growth but knockdown of

GAPDH attenuated its growth inhibitory effect. Conversely, *GAPDH* overexpression led to more cancer cell growth inhibition (Fig. 6B). Thus, we speculated that *GAPDH*-mediated mitophagy serves as cell death under Rg3 treatment. Clinically, the expression level of *GAPDH* was closely associated with colon cancer patients' surviving time and patients with high levels of *GAPDH* had prolonged survival (Fig. 6C), indicating the role of *GAPDH* in the prognosis of colon cancer.

In summary, our results strongly suggest that the ubiquitination of *GAPDH* by Parkin is a crucial mechanism for Parkin-dependent mitophagy, which constitutes an important mechanism underlying the tumor-suppressive function of Rg3. Low levels of *GAPDH* in colon cancer mitigates oxidative stress and lead to less cell death and promote tumorigenesis (Fig. 6D).

3. Discussion

Ginsenoside Rg3 is a single compound isolated from both American ginseng and Asian ginseng [43]. Ginseng has been used as herbal medicine for multiple therapeutic purposes for thousands of

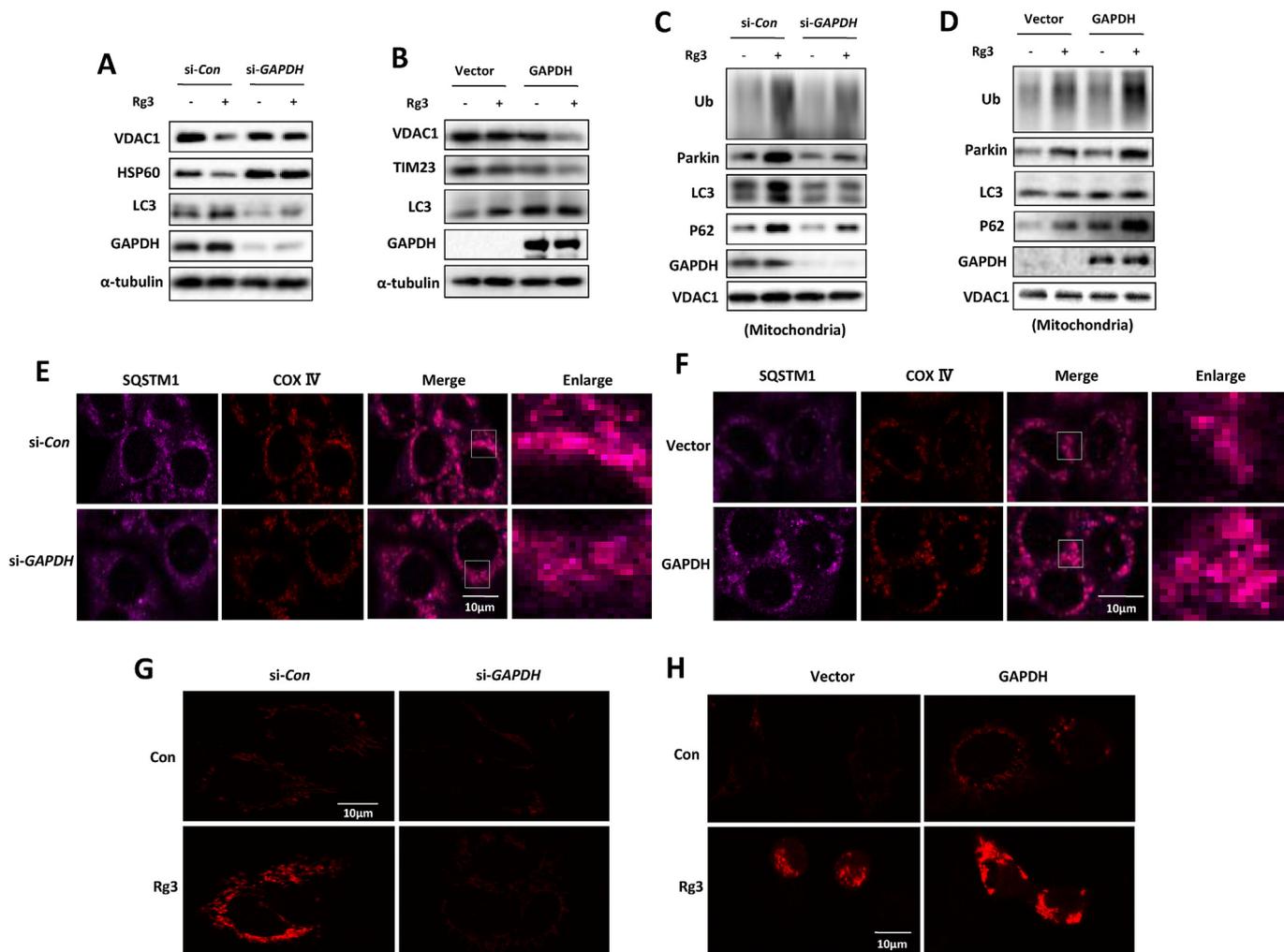


Fig. 5. GAPDH is required for mitophagy induction. (A) HCT116 cells were first transfected with the *PARK2*-specific siRNA and then treated with Rg3 (20 μM, 12 h). Cells were harvested and lysed for western blotting to determine the mitochondrial protein levels. α-tubulin served as a loading control. (B) HCT116 cells were transfected with empty vector or Flag-Parkin and then treated with Rg3. Cell lysates were prepared for western blotting to determine the mitochondrial protein levels. (C) as in (A), after indicated treatment, cells were harvested and prepared for mitochondrial fractions. Western blotting was performed to determine the protein level of Parkin. VDAC1 was used as a representative of mitochondrial fraction. (D) as in (B), mitochondrial fractions were prepared from Rg3-treated cells with or without Parkin overexpression and subjected to western blotting. (E) as in (A), in *PARK2* knockdown cells, confocal imaging of SQSTM1 (pink) and COX IV (red) was performed, respectively. Scale bar 10 μm. (F) as in (B), cells with or without Parkin overexpression were prepared for confocal imaging of SQSTM1 (pink) and COX IV (red). (G) as in (A), HCT116 cells expressing the mKeima-Red-Mito-7 were transfected with the *PARK2*-specific siRNA and then treated with Rg3. The fluorescence was detected by confocal microscope. Scale bar 10 μm. (H) as in (B), in Parkin overexpressing cells, the fluorescence of the mKeima-Red-Mito-7 was examined by confocal microscope.

years in China [8,44]. It is generally believed that the anti-tumor activities of ginseng are attributed to ginseng's immunomodulation [45,46]. Here, we reveal a novel mechanism of tumor suppression of Rg3 through activating Parkin-dependent mitophagy (Figs. 1–2), demonstrating its great potential for use as a safe and effective adjuvant to cancer therapy in the future.

Parkin belongs to an E3 ubiquitin ligase and is regarded as a tumor suppressor. Here, we also detect the low level of Parkin either in human colon cancer cells or tissues (Fig. 2A and B). Currently, the mechanism of the tumor-suppressive function of Parkin is poorly defined. The ubiquitination activity of Parkin has been proved to be crucial for the tumor suppression of Parkin, in which many oncogenic substrates are ubiquitinated and degraded [47,48]. In our study, we identify GAPDH as one novel substrate of Parkin, which is ubiquitinated but not degraded by Parkin (Fig. 4). Conversely, GAPDH regulates the cellular transport of Parkin and is indispensable for Parkin translocalization to mitochondria (Fig. 5C and D). In the future study, we will identify the

ubiquitination sites of GAPDH and further determine the function of GAPDH ubiquitination in mitophagy and tumor suppression. GAPDH is revealed to inhibit cancer cell growth (Fig. 6B) and it may be the tumor suppression mechanism of Parkin. GAPDH has long been recognized as an important metabolism enzyme for cellular energy production through anaerobic glycolysis. Recent studies [28,41] have shown that GAPDH has multiple nonglycolytic functions. An association of GAPDH with apoptosis has been described in many studies, although enhanced GAPDH expression and enzymatic function are reported in tumorigenesis [40,49]. So far, the mechanism underlying the inhibitory effect of GAPDH on cellular proliferation remains unclear. In our study, we present the ubiquitination and regulation of GAPDH by Parkin, which further enhances the activity of GAPDH and GAPDH-mediated mitophagy; conversely, GAPDH regulates the cellular distribution of Parkin and is required for Parkin-dependent mitophagy (Figs. 4G and 5). Whether GAPDH regulates cell death through mitophagy? As is known, mitophagy defects have been associated with cancer

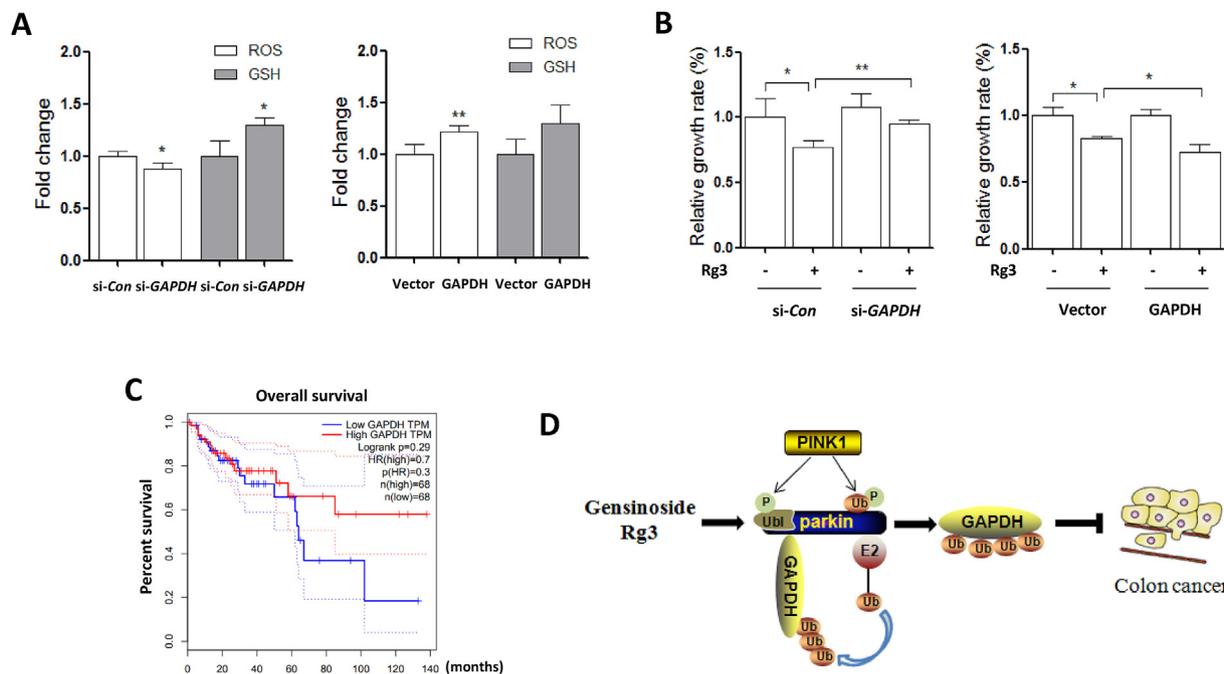


Fig. 6. GAPDH controls cancer cell growth through regulating cellular oxidative stress. (A) HCT116 cells were transfected with the GAPDH-specific siRNA (left panel) or with Flag-GAPDH (right panel), respectively. And then cells were labeled with MitoSOX™ Red or ThiolTracker™ Violet and cell fluorescence was measured using flow cytometry. * $p < 0.05$ ** $p < 0.01$ (B) as in a, Rg3 (20 μM) was applied to the GAPDH knockdown or overexpression cells for 24 h. CCK-8 solution was added into each well of the plate and the absorbance was measured at 450 nm using a microplate reader. * $p < 0.05$ ** $p < 0.01$ (C) The association of Parkin expression level with colon cancer patients' overall survival was analysed from GEPIA. (D) A graph model of anticancer activity by Rg3 through activating Parkin-dependent mitophagy.

development [50,51], in which the accumulation of dysfunctional mitochondria results in the carcinogenesis and tumor progression. Thus, mitophagy tends to be a tumor suppression mechanism. In mitophagy defect cells, we indeed detect a weakened inhibitory effect of Rg3 on cancer cell growth (Fig. 1F). Therefore, GAPDH-mediated mitophagy may be revealed as a novel mechanism of GAPDH as a cell death regulator. In future, we will identify the ubiquitination sites of GAPDH and further confirm their effects on mitophagy and cancer development.

In carcinogenesis, oncogenic transformation often results in the activation of proliferative reprogramming pathways to promote ROS generation and increase cellular oxidative stress [52]. Generally, these stresses have anti-proliferative effect and threaten cancer cell survival. Thus, many therapeutic interventions are developed through manipulating cellular oxidative stress [38]. As the main active component of ginseng, Rg3 has been reported to be able to reduce intracellular ROS level and have an anti-oxidant effect [53,54]. But under some circumstances, it can also increase ROS level, activate oxidative stress and mitochondria-dependent apoptosis [19,20,55]. Pretreatment with ROS scavenger N-acetyl-L-cysteine attenuates the inhibitory effect of Rg3 on cancer cells. Here, in colon cancer, our results show that Rg3 exerts anti-cancer activity in a GAPDH-dependent manner, in which GAPDH possesses an ability to activate oxidative stress (Fig. 6A and B), although higher levels of antioxidant GSH are also detected with ectopic expression of GAPDH. It implies that the growth inhibition of GAPDH may be associated with a large amount of ROS production, but much work still needs to be done.

In this study, we provide evidence that the representative ginsenoside Rg3 has an inhibitory effect on human colon cancer by inducing Parkin-dependent mitophagy. These findings also provide a significant basis to understand the mechanism by which the tumor suppressive effect of Parkin is functionally related to the nonglycolytic activity of GAPDH. However, the possibility that Rg3

is acting through other nonspecific mechanisms can not be ruled out and need further investigation. Prospectively, this line of investigation supports the development of a novel cancer therapy in which GAPDH ubiquitination is used as an effective adjuvant in the clinical treatment of colon cancer.

Declaration of competing interest

No potential conflicts of interest were disclosed.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jgr.2021.06.009>.

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