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ARID1A down-regulation reduces autophagic flux in colorectal cancer: a potential candidate in cancer therapy

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ABSTRACT

Aim: This study planned to examine the latent impacts of *ARID1A* in modulating autophagy in colorectal cancer (CRC), thereby providing new insights into the potential regulatory role of *ARID1A* in CRC.

Background: *ARID1A*, an essential component of the SWI/SNF complex, is considered a recognized tumor suppressor, which is frequently inactivated and down-regulated in various human cancers, including CRC. Nevertheless, its potential role in regulating cell autophagy, a crucial cellular homeostatic mechanism that plays a pro-death or pro-survival role in cancer, remained unknown in CRC.

Methods: The expression of *ARID1A* was evaluated using real-time quantitative PCR (qPCR) and western blotting in the HCT116 cell line. After the shRNA-mediated knockdown of *ARID1A*, cellular proliferation and autophagy flux were evaluated using the MTT assay, P62 and LC3Western blotting, and the LysoTracker method, respectively.

Results: Transduction of *ARID1A* shRNA lentivirus resulted in approximately 86 % downregulation of *ARID1A* mRNA expression in HCT116 cells. The MTT assay of HCT116 cells revealed approximately 1.5-fold significant increase in cell proliferation following the *ARID1A* down-regulation. Based on the western blot results, aggregation of p62 and LC3-II revealed autophagy flux inhibition after *ARID1A* down-regulation. In confirmation of this result, the LysoTracker staining showed lysosomal alkalinization, autophagosome aggregation, and inhibition of autophagic flux following *ARID1A* down-regulation. Therefore, enhanced autophagosome presence and impaired autophagic flux induced by *ARID1A* down-regulation may be at least in part as a result of lysosomal environment imbalance.

Conclusion: This study reports autophagy inactivation as the possible mechanism by which *ARID1A* down-regulation promotes CRC. Therefore, autophagy inducers may represent promising therapeutic targets for *ARID1A*-deficient tumors.

Keywords: Colorectal neoplasms, ARID1A, ShRNA knockdown, Autophagy, Antineoplastic therapy.

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Introduction

Colorectal cancer (CRC) is the second most diagnosed cancer in women and the third most diagnosed in men. This disease is a predominant leading cause of cancer mortality, resulting in roughly 10% of all tumor-associated deaths worldwide each year (1).

Promotion of programmed cell death and prevention of the cell cycle in cancer cells are effective therapeutic approaches in cancer. Programmed cell death is divided into different types, including apoptosis and autophagic cell death (2).

Autophagy is a multistep and catabolic process that involves autophagosome formation that captures intracellular organelles or proteins, fusion with

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lysosomes to produce autolysosomes, and eventually, degradation and reutilization of damaged intracellular elements to preserve cellular homeostasis. Autophagic flux is defined as a measure of autophagic degradation activity rather than autophagosome formation (3, 4). Autophagic flux is tightly regulated through multiple energy sensor pathways, such as the mechanistic target of rapamycin (mTOR), AMP-activated kinase (AMPK), SIRT1, and PARPs (5). The MAPK-ERK pathway and the PI3K-AKT pathway activate mTOR, thus resulting in autophagy inhibition. In contrast, the P53 and AMPK pathways inhibit mTOR and induce autophagy. Increasing evidence reports a dual role of autophagy in cancer progression, functioning as both oncogenic and tumor-suppressive (6, 7). Some evidence suggests that autophagy may promote cancer cell survival and tumorigenesis by inhibiting cell death (7, 8), while accumulating data support the idea that autophagy is recognized as a tumor-suppressive mechanism and, like a double-edged sword, excessive autophagy could also lead to autophagic apoptosis (6, 9). Therefore, studying autophagy-related signaling pathways is vitally important. Several cytotoxic agents kill tumor cells by causing extensive cell injury that triggers autophagy-mediated cell death (8, 9).

ARID1A, an essential component of the SWI/SNF chromatin-remodeling complex, which contains an AT-rich interactive domain, functions as a recognized tumor suppressor gene in various kinds of human cancers. The SWI/SNF protein complex regulates the transcription of genes associated with numerous crucial cellular processes, including DNA damage repair, differentiation, proliferation, and development, by changing the accessibility of the promoter to transcriptional factors (10, 11). *ARID1A*, as a DNA-binding subunit of the SWI/SNF protein complex, is crucial for targeting this complex to enhancers and preserving their availability. Loss of *ARID1A* leads to aberrant targeting of SWI/SNF to enhancers, impairs differentiation and development programs, and causes extensive gene expression dysregulation, contributing to tumorigenesis (12). For example, in colon cancers lacking *ARID1A*, a broad depletion of SWI/SNF occurs at enhancer locations, leading to significant alterations in gene expression (13). *ARID1A* is generally considered a tumor suppressor gene that can suppress a series of biological characteristics such as invasion, proliferation, and apoptosis in CRC cells. It's down-regulation has led to a remarkable enhancement of cell proliferation in vitro (14). In CRC cells, *ARID1A* silencing may contribute to enhancing invasive and migratory abilities (15). In mice, *ARID1A* knockout per se is sufficient to drive the formation of invasive colon tumors, indicating that its main role is that of a tumor suppressor (13). Notably, *ARID1A* is involved in regulating the PI3K/AKT/mTOR signal transduction pathway, which is a distinguished autophagy-inhibiting pathway (16). It is hypothesized that *ARID1A* inhibits the activation of the EGFR/PI3K/Akt/mTOR pathway through its ability to reposition nucleosomes that condense the chromatin around these genes. Silencing of *ARID1A* in various cancer cells leads to stimulation of the downstream PI3K/AKT/mTOR axis, thereby inhibiting autophagy, which leads to sensitizing *ARID1A*-deficient cancer cells to mTOR, PI3K, and AKT pathway inhibitors (14, 16, 17).

ARID1A is frequently mutated in different types of human malignancies. (18). In human CRC, *ARID1A* ranks as the third most frequently mutated gene, with a mutation frequency between 10 and 40%, following APC and P53. (13, 19, 20). In addition to inactivating mutations, abnormal DNA methylation and histone modifications have been identified as key mechanisms contributing to *ARID1A* down-regulation in CRC. (21, 22).

Nevertheless, there is no evidence to date on the potential impact of ARID1A on regulating autophagy in CRC; there is no knownCRC. Given the important effect of *ARID1A* in CRC cell viability suppression and the emerging role of autophagy as a context-dependent regulator of cancer cell survival and death, we intend to test the hypothesis as to whether the impact of *ARID1A* down-regulation on the CRC promotion may, at least in part, be attributed to the dysregulation of the cell autophagy pathway. To test this hypothesis, we investigated the effects of *ARID1A* downregulation on autophagic flux, lysosomal function, and cell viability in the human colorectal carcinoma cell line HCT116.

Methods

The cellular culture

The National Cell Bank of Iran (Tehran, Pasteur Institute) was the source of the HCT116 colorectal cancer (CRC) and HEK293T cell lines. HEK293T cells were grown in DMEM medium, while HCT116 cells

were cultured in RPMI 1640 culture medium. Each medium was enriched with antibiotics (1% penicillin, 1% streptomycin), glutamine (2 mM), and 10% fetal bovine serum (FBS). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Lentivirus production

The shRNA-expressing lentiviral plasmid (pLKO.1-puro) was purchased from Addgene. The *ARID1A* shRNA sequence (oligo ID: TRCN0000059090) cloned into this vector is shown in Table 1. The empty pLKO.1-puro vector was applied as the control. The lentivirus encoding *ARID1A* shRNA and empty lentivirus were synthesized through transfecting HEK293T packaging cells using the 2nd-generation lentiviral system. In brief, HEK293T cells at approximately 80% confluence were co-transfected using the standard protocol involving polyethylenimine (PEI) transfection with 0.5 µg of either the pLKO.1 empty vector (negative control) or the pLKO.1 lentiviral vector encoding ARID1A shRNA, along with 375 ng of the packaging plasmid (pSPAX2) and 125 ng of the plasmid encoding the envelope protein (pMD2.G) (23). Then, 48 h after transfection, the harvested viral supernatants were subjected to centrifugation at 2,000 × g for 5 min at 4˚C, then aliquoted into small volumes for storage at −80°C.

Development of HCT116 cells with stable knockdown of *ARID1A* via shRNA

HCT116 cells were dispensed into 6-well plates and, following 24 hours, transduced with lentiviral supernatant carrying either an empty control vector or *ARID1A*-targeting shRNA at a defined multiplicity of infection (MOI) of 5–50, supplemented with 8 µg/ml polybrene. Following the 24 hours of transduction, cells infected with the lentivirus were cultured in a selection medium enriched with puromycin (1 µg/ml, Santa Cruz Biotechnology) for 3 weeks. The efficiency of *ARID1A* knockdown was evaluated by Real-Time RT-PCR and Western blot techniques.

Isolation of RNA and quantitative real-time PCR

Total cellular RNA was isolated using the TriPure RNA isolation kit (Roche Applied Science, Germany). Then, RNA was reverse-transcribed into cDNA with the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. It was utilized to perform real-time quantitative PCR (qPCR). The expression level of *ARID1A* was assessed by RT-qPCR using the ABI 7500 Sequence Detection System (Applied Biosystems, USA) and a master mix of SYBR Green (ABI, UK). Each RT-qPCR assay was conducted in triplicate, and relative quantifications were performed using the 2^−ΔΔCt method, normalizing gene expression to GAPDH levels. Table 2 lists the sequence of primers applied in the current study.

Western blot assay

After cellular lysis in RIPA buffer containing the phosphatase and protease inhibitor cocktails (Roche Applied Science), protein concentration was detected using a BCA protein assay kit (Thermo Scientific Pierce). Protein band separation was performed via 12% Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and proteins were subsequently transferred to nitrocellulose membranes. Then, membrane blocking was performed with skim milk (5%) in PBS (1 h at room temperature) and exposed to primary antibodies overnight at 4˚C: LC3B (L7543; Sigma-Aldrich), *ARID1A* (sc-373784; Santa Cruz Biotechnology), P62 (88588S; Cell Signaling),

Table 1. The sequence of *ARID1A* shRNA

shRNA	sequence
Sense oligo	5'-CCGGCCTCTCTTATACACAGCAGATCTCGAGATCTGCTGTGTATAAGAGAGGTTTTTG -3'
Antisense oligo	5'- AATTCAAAAACCGTTGATGAACTCATTGGTTCTCGAGAACCAATGAGTTCATCAACGG -3'

Table 2. The sequences of primers applied for Real-Time RT-PCR

Primer name	Primer sequence
ARID1A	F: CAGTACCTGCCTCGCACATA
	R: GCCAGGAGACCAGACTTGAG
GAPDH	F: CGACCACTTTGTCAAGCTCA
	R: AGGGGTCTACATGGCAACTG

and GAPDH internal control (ab181602; Abcam). Membranes were next exposed to the corresponding HRP-conjugated secondary antibody for 1 hour at room temperature. Then, the bands were visualized by applying an enhanced chemiluminescence system (ECL). The band density measurements were performed by Image Lab software from Bio Rad.

MTT cell survival assay

Proliferation was quantified by the MTT assay. Cells were cultured in 96-well plates at 2000 cells per well and maintained in RPMI-1640 medium enriched with fetal bovine serum (FBS, 10%). After 24 hours, cells were maintained at 37 °C for 4 h in 100 µl fresh media with 0.5 mg/mL of MTT dye (3-4,5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide) (Sigma, St. Louis, MO, USA). The medium of each well was removed, and 100 µl of DMSO was used to solubilize the reduced formazan dye in each well. Then, the absorbance measurement was conducted at 490 nm using a microplate reader. All experiments were conducted in triplicate.

Detection of acidic organelles by LysoTracker

The quantification of acidic organelles was performed by treating the HCT116 cell lines transduced with either *ARID1A* shRNA virus or control virus with LysoTracker Red DND-99 (Life Technologies NZ, L7528).

The LysoTracker Red was added to each well at a final concentration of 50 nM. After 30 min incubation in the dark and removing the LysoTracker Red solution, cells were rinsed in PBS pH 7.4 three times. Acidic organelles were imaged immediately under inverted fluorescence microscopy (inverted fluorescence microscopy LX81, Olympus, Japan) equipped with a 577 nm excitation filter. The fluorescence intensity of images was measured using ImageJ.

Statistical evaluation

The SPSS software, version 23.0, was used for statistical data analysis. Data are expressed as the mean ± SD from three separate experiments. One-way ANOVA with Tukey's post hoc test was used to analyze the data. Statistical significance was defined as $P \leq 0.05$. The graphs were generated by Prism version 6.00 (GraphPad Software).

Results

The effectiveness of *ARID1A* knockdown via shRNA in HCT116 cells

In our recent study, we demonstrated variable mRNA expression levels of *ARID1A* in six distinct CRC cell lines, with high expression observed in HT 29 and HCT116 cells, and almost undetectable levels in SW48 cells. (21). Therefore, to investigate the function

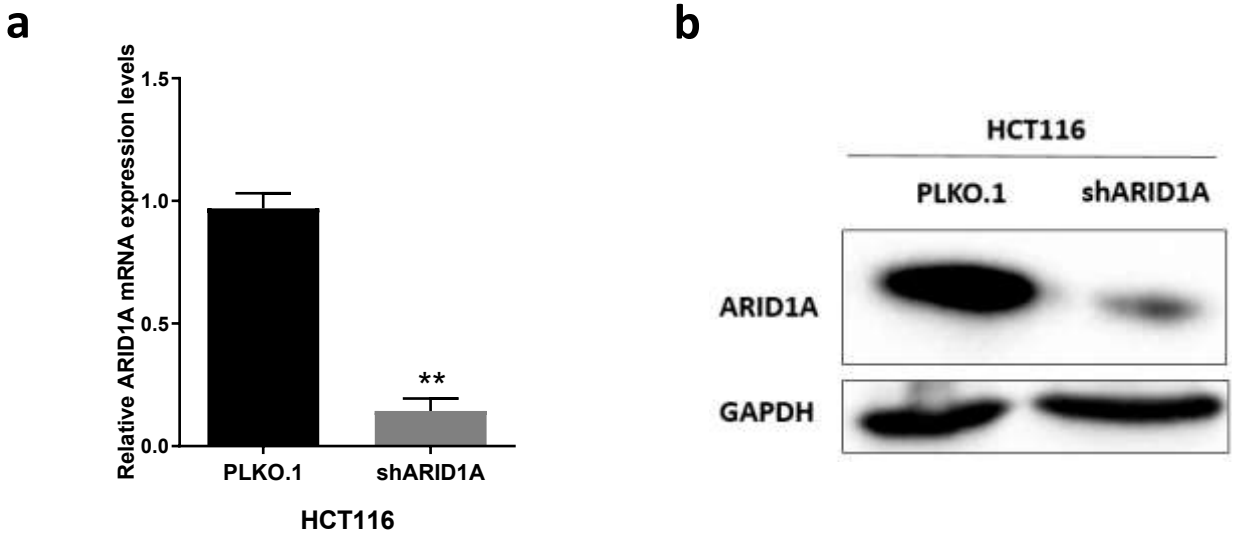

Figure 1. The effectiveness of ARID1A knockdown via shRNA in HCT116 cells. Analyses through qPCR (a) and western blotting (b) demonstrated a significant decrease in ARID1A expression in HCT116 cells transduced with ARID1A shRNA lentivirus compared to those transduced with control virus. ARID1A expression data were standardized to GAPDH. Western blot images represent three independent experiments performed in triplicate. Quantification data are presented as the mean ± SD obtained from three separate experiments. (**$p < 0.01$).

of *ARID1A* in cancer, the HCT116 cell line with the highest expression of *ARID1A* across six CRC cell lines was selected for subsequent knockdown. Western blot and qPCR analysis consistently confirmed that transduction of *ARID1A* shRNA lentivirus resulted in stable knockdown of *ARID1A* in HCT116 cells (Figure 1a and b). As shown in Figure 1a, the mRNA expression levels of *ARID1A* were significantly downregulated by approximately 86 % in HCT116 cells transduced with *ARID1A* shRNA lentivirus compared to those transduced with control virus ($p < 0.01$).

MTT proliferation assay showed that *ARID1A* down-regulation enhances cancer cell proliferation

According to the results of the MTT proliferation assay, we found that *ARID1A* down-regulation by *ARID1A* shRNA led to an approximately 1.5-fold increase in the proliferation rate in comparison to control virus–transduced cells ($p < 0.05$) (Figure 2). Thus, *ARID1A* seems to be a determining factor for controlling the rate of cell division. Collectively, the MTT proliferation assay suggested that *ARID1A* loss alone is sufficient to drive tumor development. Therefore, additional molecular alterations are not required to collaborate with *ARID1A* loss to drive tumorigenesis.

ARID1A down-regulation impairs "autophagic flux" in the HCT116 cell line

Immunoblot analysis of the relative levels of LC3-II compared to LC3-I has been widely used to reflect the number of autophagosomes and to monitor autophagic activity (24). Therefore, in the present study, to identify the impact of *ARID1A* expression on autophagic activity in colorectal cancer, the ratio of LC3II/I was evaluated by Western blotting in the HCT116 cell line after *ARID1A* down-regulation. The Western blotting results (Figures 3a, 3c) showed that after *ARID1A* down-regulation, the ratio of LC3II/I was significantly increased (approximately 2.2-fold) ($p < 0.01$).

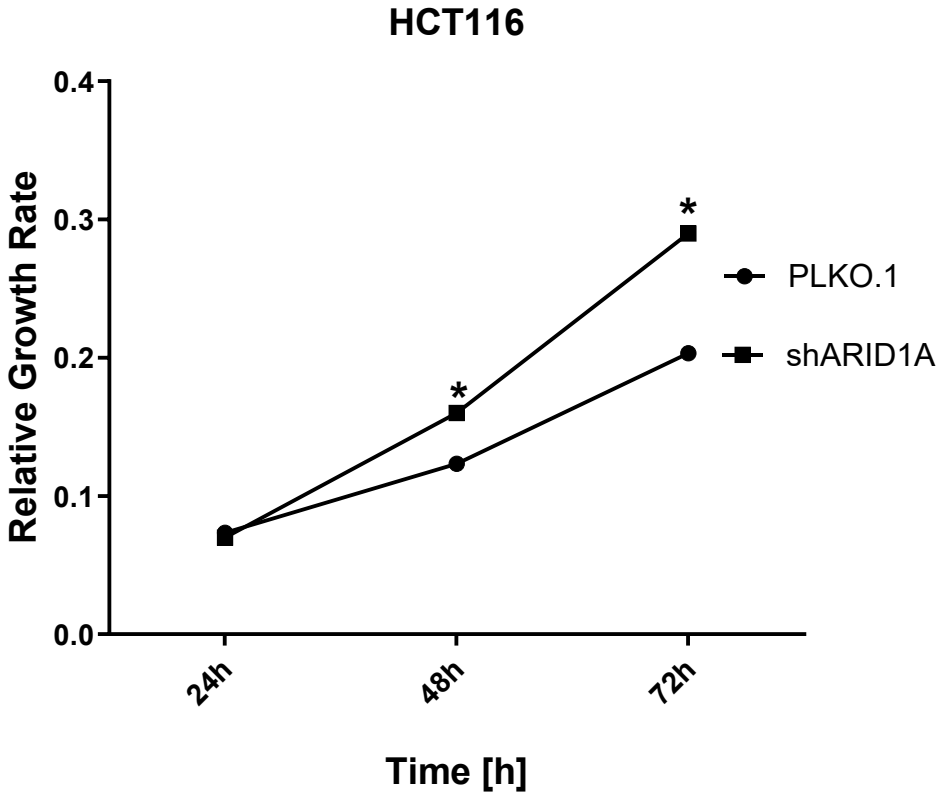

Figure 2. *ARID1A* inhibits cell proliferation in vitro. MTT proliferation assay showed ARID1A down-regulation (shARID1A) in HCT116 cells caused an increase in cell proliferation. Data are the mean ± SD obtained from three separate experiments. (*$p < 0.05$).

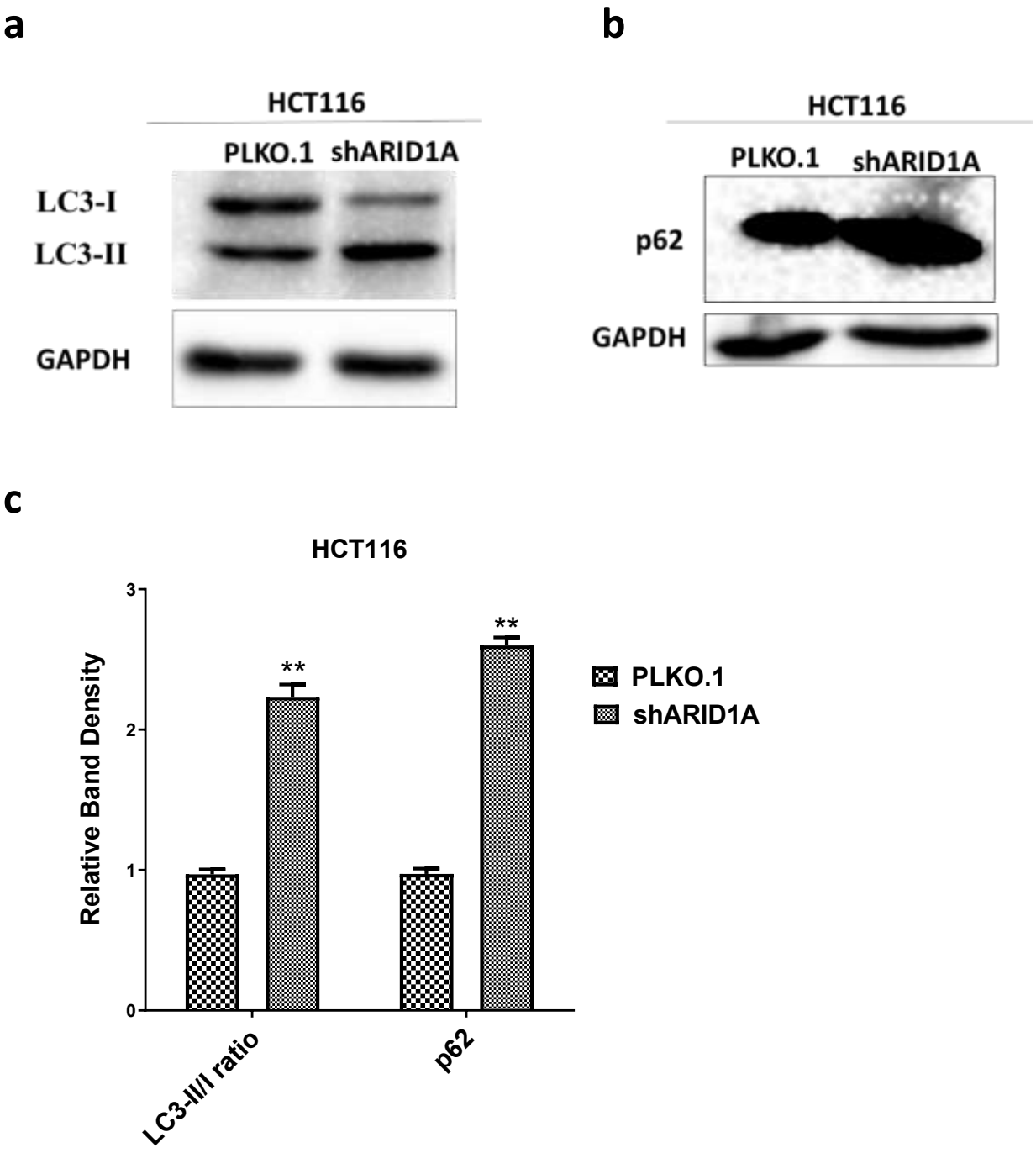

Figure 3. Autophagic flux was inhibited after *ARID1A* down-regulation in the HCT116 cell line. HCT116 cells were transduced with either lentivirus expressing *ARID1A* shRNA (shARID1A) or control lentivirus (PLKO.1); subsequently harvested and subjected to western blot analysis for LC3 (a) and p62 (b) protein levels. Western blot images represent three independent experiments performed in triplicate. The LC3-II/I ratio and p62 levels were measured quantitatively, using *GAPDH* as a reference protein (c). Quantification data are presented as the mean ± SD obtained from three separate experiments. (**$p < 0.01$).

Although the LC3-II amounts correlate with the number of autophagosomes, increased autophagosome number can be associated either with autophagy induction or the blockage of the autophagic flux. "Autophagic flux" denotes the total autophagic degradation process—i.e., cargo transport to the lysosome—rather than just the creation of autophagosomes. The autophagy strength is represented

not only by autophagy initiation but also by normal autophagic flux. Therefore, LC3-II accumulation that was observed in the current study after *ARID1A* down-regulation in the HCT116 cell line may arise from either activation of autophagy or inhibition of autophagic flux. Thus, the protein expression level of another widely used autophagy marker, p62, was evaluated to distinguish the induction of autophagy or impairment of autophagic flux in the HCT116 cell line. Considering the p62 degradation through autophagy, its elevated levels suggested a disruption in the autophagic flux pathway (25). Western blotting results (Figures 3b, 3c) revealed that in comparison with control virus–transduced cells, the expression level of the p62 protein was significantly upregulated following *ARID1A* down-regulation in the HCT116 cells ($p < 0.01$). As shown in Figures 3b, 3c, the changes in p62 protein levels paralleled the rise in LC3-II during *ARID1A* down-regulation in the HCT116 cell line, indicating that the increased LC3-II after *ARID1A* down-regulation was caused by a blockage in autophagosome degradation rather than increased autophagosome generation, and autophagic flux was impaired in the HCT116 cell line.

ARID1A down-regulation caused lysosomal alkalinization in the HCT116 cell line

Evidence indicated that acidity has a key role in the activation of lysosomal proteases in the autophagic degradation process. To assess whether the impaired autophagy flux induced by *ARID1A* down-regulation was due to the lysosomal function inhibition, we used a sensitive lysosomotropic pH probe, Lysotracker Red DND-99, to qualitatively investigate the impact of *ARID1A* down-regulation on lysosomal pH in the HCT116 cell line (Figure 4). Lyso-Tracker Red DND-99 emits red fluorescence in acidic compartments, including lysosomes, in a pH-sensitive manner; intensified staining indicates a drop in lysosomal pH. As shown in Figure 4, we observed that after *ARID1A* down-regulation in the HCT116 cell line, red fluorescence significantly decreased by approximately 60% compared with the control cells ($p < 0.01$), indicating an increase in lysosomal PH. Collectively, Figure 4 suggests that the acidic condition of the lysosome was compromised after *ARID1A* down-regulation and determines the possibility that autophagosome accumulation and blockage of autophagic flux induced by *ARID1A* down-regulation are at least in part due to disturbance of the lysosomal internal environment.

Discussion

Many reports suggest that *ARID1A* functions as a recognized tumor suppressor and is critically involved in the progression of multiple cancer types (12). Despite the frequent occurrence of *ARID1A* loss and autophagy dysfunction in colorectal cancer (26), the impact of *ARID1A* on the autophagy process remains unexplored in colorectal cancer. In this study, we discovered that *ARID1A* down-regulation is highly effective in inhibiting autophagy flux and increasing proliferation in HCT116 colorectal cancer cells. Thus, it is likely that *ARID1A* down-regulation may promote CRC by inhibiting autophagy. This shows a remarkable clinical importance, as loss of *ARID1A* expression may predict a favorable response to therapies involving autophagy inducers.

Autophagy, an essential pathway for cell homeostasis, differentiation, and survival, has a dual role in cancer (27). Depending on the cancer context and cell type, autophagy can either trigger cell death or promote cell survival. When autophagy is primarily induced through signaling pathways involving activation of AMP-activated protein kinase (AMPK) and inhibition of PI3K/Akt/mTOR, the protein expression of LC3II, as an autophagosome membrane component, is induced to generate the autophagosome. Then, the autophagosome integrates into the lysosome to degrade and recycle damaged intracellular components by hydrolytic enzymes (2, 28). Blocking autophagy was suggested as a promising approach to reduce cancer cell growth. However, recent multiple cell-based and animal model studies have demonstrated that inhibiting autophagy can lead to tumor cell hyperproliferation, and autophagy induction by drugs in tumor cells is regarded as a novel therapeutic approach against cancer (7). Autophagy deficiency has been linked to the development of tumors in mouse models with mutations or epigenetic alterations affecting autophagy-related genes in multiple human cancer types (29, 30). Knockdown of RAB21 and ATG5 in LoVo and SW480 CRC cells in chicken chorioallantoic membranes (CAM) models resulted in increased tumor growth (7). The Rab3IP upregulation

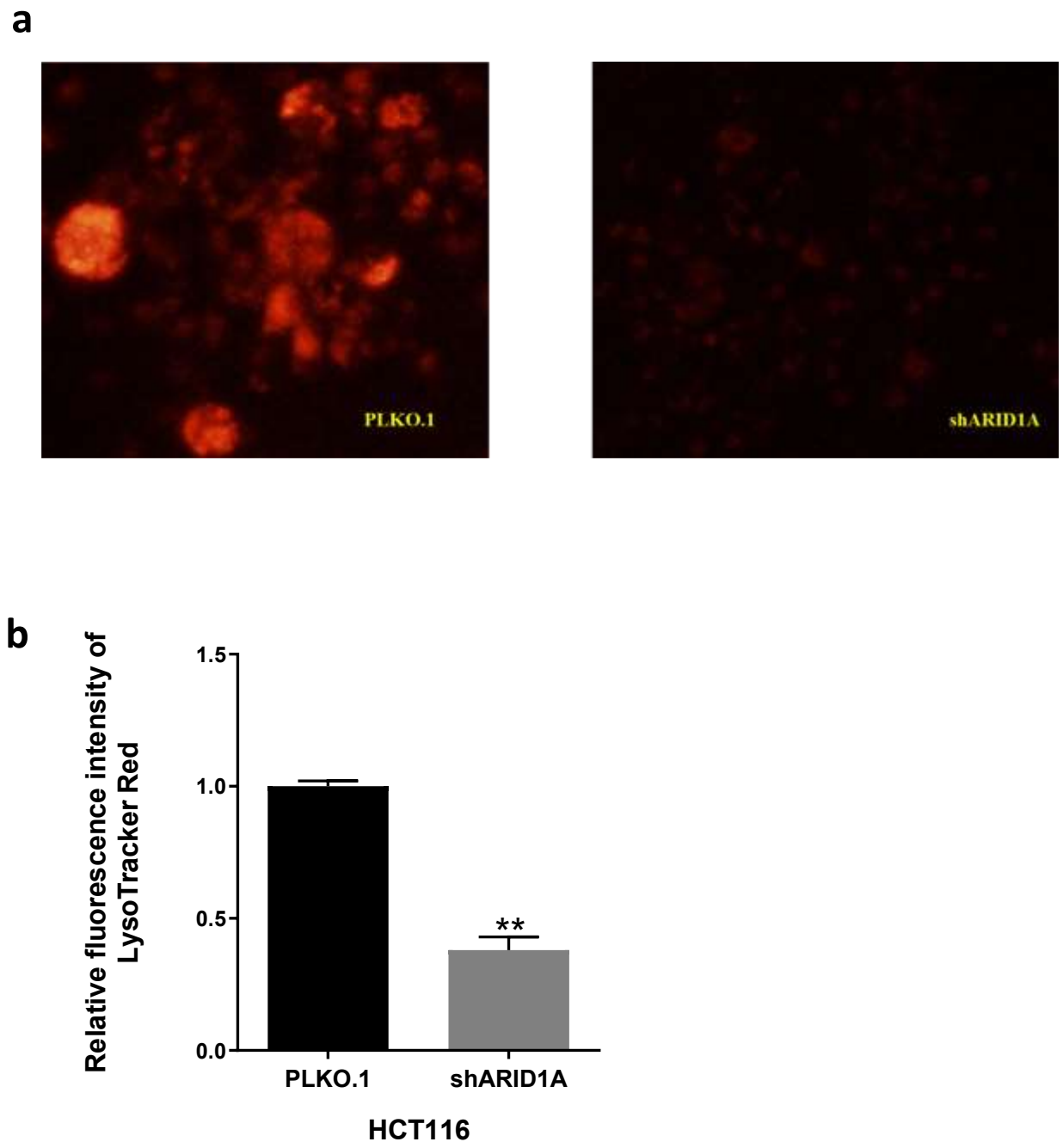

Figure 4. Lysosomal acidic environment was impaired after *ARID1A* down-regulation in the HCT116 cell line. Lyso-Tracker Red staining was applied to recognize the acidic organelles and lysosome acidity in HCT116 cells transduced with either *ARID1A* shRNA-expressing lentivirus (Lenti shARID1A) or control lentivirus (Lenti PLKO.1). As shown by Lyso-Tracker Red staining, red fluorescence dramatically decreased after *ARID1A* down-regulation. (a) Representative images were photographed under a fluorescent microscope. Images represent three independent experiments. (b) The quantitative analysis of fluorescence images. The relative fluorescence intensity of LysoTracker Red was detected using ImageJ software. Quantification data are presented as the mean ± SD obtained from three separate experiments. (**$p < 0.01$).

increases gastric cancercells' proliferation through autophagy inhibition (31). Autophagy has been demonstrated to suppress cancer cell proliferation, such as in breast and bladder cancer cells (32). Heterozygous knockout of Beclin 1, a gene involved in the initiation of autophagy, leads to enhanced spontaneous tumorigenesis (29). The reduced expression of Beclin-1 has been detected in human breast cancer (33). Therefore,

autophagy has been considered a protective mechanism against cancerous transformation (34). Breast cancer-associated autophagy induced by autophagy prevents pulmonary metastasis. Recently, increased evidence indicates that autophagy can augment the effects of anticancer drugs to enhance tumor shrinkage (35). O'Donovan et al. showed that autophagy induction by lithium could enhance chemosensitivity in apoptosis-deficient colorectal and esophageal cancer cells, leading to the induction of an autophagy-related cell death (36). Inducing autophagy by rapamycin, a classic autophagy inducer, enhances responsiveness of colon cancer cells to chemotherapy drugs through the autophagy/p62/Nrf2 pathway (37). In addition, miR-125b prevents the resistance of hepatocellular carcinoma cells to oxaliplatin through a mechanism involving the down-regulation of EVA1A-mediated autophagy (38, 39).

Previous studies observed that dysregulated PI3K/Akt signaling in human CRC is associated with the differentiation, motility, proliferation, and growth pattern of cancer cells (40), while the PI3K/Akt pathway is a well-known autophagy-inhibiting pathway (41, 42). Growing evidence suggests that the *loss of ARID1A expression* leads to PI3K/AKT pathway activation, and thereby increased sensitivity to treatment with PI3K/AKT inhibitors in *ARID1A*-deficient cells (14, 16). In ovarian, gastric, glioma, and CRC cells, silencing of *ARID1A* has been shown to trigger activation of the AKT and PI3K phosphorylation, indicating a connection between *ARID1A* expression loss and activation of the PI3K/AKT pathway (16). This is consistent with our results. Indeed, one possible explanation for this autophagy inhibition by *ARID1A* down-regulation could be attributed to the induction of the PI3K/AKT signaling cascade, which requires further assessment in future studies.

Our results are consistent with the only available experimental study of *ARID1A* effect on autophagy (43), which demonstrated that knockdown of *ARID1A* inhibits autophagy through activation of the PI3K/Akt/mTOR pathway, which reverses the therapeutic efficacy of anti-PD(L)1 agents in lung adenocarcinoma.

Using a sensitive lysosomotropic pH probe, Lysotracker Red DND-99, we found that *ARID1A* down-regulation caused lysosomal alkalinization, which determined the possibility that autophagosome accumulation and blockage of autophagic flux induced by *ARID1A* down-regulation is at least in part due to disturbance of the lysosomal internal environment.

Our results support the findings of multiple studies that inhibition of autophagy promoted the growth and survival of HCT116 colorectal cancer cells. Wu et al. showed that autophagy flux inhibitors chloroquine (CQ, a lysosomal acidification blocker and autophagosome degradation inhibitor) and Bafilomycin A1 (Baf-A1, a lysosomal inhibitor) promoted HCT116 cells' proliferation, migration, and EMT through inhibition of autophagy-dependent CTNNB1 degradation that had been induced by TRAF6 (44). Kuang et al. demonstrated that in CRC, FOXS1 facilitated TGFBI degradation via the autophagy–lysosome pathway instead of the ubiquitin–proteasome system, and autophagy flux inhibition by Bafilomycin A1 was observed to markedly restore TGFBI expression and promote survival and proliferation of CRC cells (45). Zhang et al. found that upon blockade of autophagy with 3-MA, a classic autophagy inhibitor, apoptosis of both CRC cell lines HCT116 and HT29 was reduced (39). The autophagy inducer, rapamycin, inhibits growth and triggers cell death by apoptosis in HCT-116 CRC cells. Furthermore, silencing of circHIPK3 sensitizes the resistant HCT116 cells to oxaliplatin through the induction of autophagy (39). Han et al. demonstrated that through autophagy activation, betulin induced cell cycle arrest of HCT116 cells (2). Considering that autophagy flux inhibition led to HCT116 cells hyperproliferation, it is likely that the impact of *ARID1A* on HCT116 cell proliferation may partly be mediated through the regulation of autophagy.

The present study has some limitations. First, we have used a CRC cell line in our study. Thus, it may be necessary to examine other cell lines in future studies to show that the model in our study may be generally applicable to CRC. Second, although, as mentioned above, multiple studies have shown that inhibition of autophagy promotes the proliferation of HCT116 cells, it remains unclear whether the enhanced proliferation observed following *ARID1A* down-regulation is directly attributable to suppressed autophagic activity rather than parallel *ARID1A*-dependent pathways. Therefore, the use of pharmacological modulators of autophagy, such as the activator rapamycin or the inhibitor bafilomycin A1, may be required to elucidate this relationship. Third, the

mechanism *ARID1A* regulates the autophagy pathway requires further elucidation, including investigation of the PI3K/Akt/mTOR pathway, which will be our goal for future studies. In addition, more studies on the animal model of CRC and patient-derived samples are essential to uncover the mechanisms linking *ARID1A* to autophagy.

While the data suggest that *ARID1A* loss may predict responsiveness to autophagy-inducing therapies, this hypothesis remains speculative, as to date, direct clinical evidence evaluating the responsiveness of *ARID1A*-deficient CRC patients to autophagy-modulating therapies remains unclear. Most clinical studies assessing autophagy modulators—such as chloroquine or hydroxychloroquine—have not stratified CRC patients based on *ARID1A* status. Consequently, it is unclear whether *ARID1A* deficiency predicts enhanced sensitivity or resistance to autophagy inhibition in the clinical setting. Therefore, Prospective studies incorporating *ARID1A* mutational or expression status are warranted to determine whether autophagy modulators could provide a therapeutic benefit specifically for this molecularly defined CRC subgroup.

Conclusion

Collectively, our in vitro study is the first report to suggest that *ARID1A* expression may regulate autophagy flux in colorectal cancer cells, and suppression of autophagy flux may be at least in part a mechanism of CRC cell proliferation promotion in *ARID1A*-deficient CRC tumors. This discovery will present a novel approach for predicting effective therapeutic responses to inducers of the autophagy pathway in *ARID1A*-deficient CRC tumors, which might further improve treatment outcomes.

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Conflict of interests

There is no conflict of interest for the authors of this article.

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