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DNA damage regulates ARID1A stability via SCF ubiquitin ligase in gastric cancer cells

Z.-H. JIANG[1], X.-W. DONG[2], Y.-C. SHEN[1], H.-L. QIAN[1], M. YAN[1], Z.-H. YU[1], H.-B. HE[1], C.-D. LU[1], F. QIU[1]

[1]Department of Gastrointestinal Surgery, and [2]Department of Gastroenterology, Ningbo Medical Center, Li Huili Hospital, Ningbo, Zhejiang, China

Abstract. – OBJECTIVE: The gene product of the AT-rich interactive domain 1A (SWI-like) gene (ARID1A) is a member of the SWI/SNF adenosine triphosphate-dependent chromatin-remodeling complexes, which plays an essential role in controlling gene expression and is also involved in cancer development. ARID1A is frequently mutated in a wild variety of cancers and function as a tumor suppressor in several kinds of cancers. ARID1A was down-regulated in gastric cancer, and associated poor patient prognosis. However, how ARID1A protein is regulated in gastric cancer remains largely unknown.

MATERIALS AND METHODS: Here, we show that ARID1A protein is rapidly ubiquitinated and degradated in gastric cancer cells in response to DNA damage treatment.

RESULTS: Using genetic and pharmacologic Cullin inactivation coupled with in vitro ubiquitination assay, we demonstrate that ARID1A is a substrate of the Cullin-SKP1-F-box protein (SCF) complexes. Moreover, gastric cancer cells with forced expression of ARID1A showed an increased sensitivity to DNA damage reagents. Thus, our data uncovered a previous unknown posttranscriptional regulation of ARID1A by SCF E3 ligase in gastric cancer cells in DNA damage response.

CONCLUSIONS: These findings suggest ARID1A might be a promising drug target in gastric cancer treatment.

Key Words:
ARID1A, SCF E3 ligase, Gastric cancer, Ubiquitination.

Introduction

Gastric cancer (GC) is the second leading cause of cancer death and the fourth most common cancer worldwide, and significant effort has been focused on clarifying the pathology of gastric cancer[1]. Like other malignant tumors, gastric cancer is known to arise through multiple genetic and epigenetic alterations, and these molecular changes could eventually result in the aberrant regulation of many cancer-associated genes, such as oncogenes and tumor-suppressor genes. Etiological studies have provided evidence that two distinct environmental infectious agents, *Helicobacter pylori* (*H. pylori*) and Epstein-Barr virus (EBV), play key roles in gastric carcinogenesis[2-5]. *H. pylori* infection could induce gastric epithelium chronic inflammation to cause aberrant polyclonal methylation[6]. Recent studies showed that aberrant DNA methylation in gene promoter regions had a crucial role in gastric carcinogenesis[7,8]. Those studies found a link between aberrant DNA methylation and infection with these two unique pathogens in gastric cancer.

BAF250A, the gene product of the AT-rich interactive domain 1A (SWI-like) gene (ARID1A), is a member of the SWI/SNF adenosine triphosphate-dependent chromatin-remodeling complexes. The SWI/SNF chromatin-remodeling complex has been shown to play an essential role in controlling gene expression and is also involved in cancer development[9]. Several components of this complex were inactivation of in certain type of cancer. ARID1A is located at Ch1p36.11, which is frequently deleted in human cancers. Recently, using Genome-wide association study (GWAS) and RNA-seq, about 43%-57% of ovarian clear cell carcinomas, 30% of ovarian endometrioid carcinomas and 8%-27% of gastric carcinoma were found mutations of ARID1A[10-12]. The majority of ARID1A mutations are frame shift or nonsense mutations, which caused loss of function of ARID1A, suggesting it should be a tumor suppressor[13]. Recently, ARID1A was shown to be down-regulated in gastric cancer, and were associated poor patient prognosis[14,15]. However, how ARID1A protein is regulated in gastric cancer remains largely unknown.

Corresponding Author: Zhouhua Jiang, MD; e-mail: Jiang-zhouhua@hotmail.com
Feng Qiu, MD; e-mail: mouseloujun@163.com

Here, we showed that ARID1A was rapidly ubiquitinated and degradated in response to DNA damage response. Inhibition of SCF E3 ligase by genetic and pharmacologic Cullin1 inactivation prevented the degradation of ARID1A. Over-expressing of ARID1A in gastric cancer cells inhibited DNA damage reagents induced apoptosis. Our data might shed new insight on the post-translational regulation of ARID1A and in gastric cancer cells in DNA damage response.

Materials and Methods

Cell Culture

Gastric cancer cells line NCI-N87 and AGS cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Gibco BRL, Gaithersburg, MD, USA). All these cells were cultured in a 5% CO_2/95% air at 37°C. DMSO, proteasome inhibitor MG132, epoxomicin and calpain-specific inhibitor calpeptin were purchased from Sigma (St. Louis, MO, USA).

Plasmids and Transfection

Flag-ARID1A plasmid was purchased from Addgene (Cambridge, MA, USA). All the transient transfections were used with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Isolation and Real-time PCR Analysis

Total RNA was isolated from cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. In order to quantify the transcripts of the interest genes, Real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Dalin, China) on Light-Cycler 480 (Roche, Basel, Switzerland). The primers used were available upon request.

Western Blotting

Protein extracts were loaded on 10% SDS-PAGE, electrophoresed, and transferred to nitrocellulose membrane. After blocking with 5% nonfat milk in PBS, the membranes were incubated with the primary antibodies and followed by horseradish peroxidase (HRP)-linked secondary antibodies. The signals were detected by chemiluminescence phototope-HRP kit WBKLS0100 (Millipore, Boston, MA, USA) according to manufacturer's instructions. Antibodies were obtained from the following sources: anti-ARID1A (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-cleaved caspase-3 (Cell Signaling, Beverly, MA, USA), anti-poly-ADP ribose polymerase (PARP) (Santa Cruz Biotech, Santa Cruz, CA, USA), anti-Flag M2 (Sigma, USA) and anti-GAPDH (Cell Signaling, Beverly, MA, USA), anti-Cullin1 and SKP1 (Santa Cruz Biotech, Santa Cruz, CA, USA).

Immunoprecipitation (IP)

Cells were lysed in 4 ml of lysis buffer (150 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Nonidet P40, and 50 mM PMSF) for 20 min at 4°C. Lysates were cleared using centrifugation (13,000 rpm, 20 min), the supernatant was then subjected to IP with 15 μl anti-mouse IgG or FLAG M2 antibody with 50 μl protein G beads (Sigma, St. Louis, MO, USA) overnight at 4°C with gentle rotation. Beads containing immune complexes were washed with lysis buffer 6 times. Precipitates were denatured in 2X SDS buffer at 99°C for 5 min.

Apoptosis Assay

Cells were collected, rinsed and fixed overnight in 70% cold ethanol at -20°C. Then, cells were treated with Tris-HCl buffer supplemented with 1% RNaseA and stained with 25 mg/ml propidium iodide (PI, Sigma, St. Louis, MO, USA). The samples were read on a Coulter Elite Flow Cytometer using Elite software program 4.0 for two-color detector (Beckman Coulter, Fullerton, CA, USA). The percentage of cells in the apoptotic sub-G1 phage was calculated using multicycle software (Phoenix Flow Systems, San Diego, CA, USA).

Statistical Analysis

Values were shown as mean ± SD. Statistical differences were determined by a Student's *t*-test. Statistical significance is displayed as * ($p < 0.05$), ** ($p < 0.01$) or *** ($p < 0.001$).

Results

ARID1A was Down-Regulated in Response to DNA Damage in Gastric Cancer Cell Lines

Previous studies suggested a critical role of ARID1A in gastric cancer development[16]. To further investigate the function of ARID1A in gastric cancer cells, we first checked the endogenous

protein level of ARID1A during DNA damage response. Gastric cancer cells line NCI-N87 and AGS cells were treated with etoposide (VP16), which is a well-known DNA damage response (DSB) inducing reagent. We found that ARID1A was markedly decreased with VP16 treatment in a dose dependent manner (Figure.1A-1B). To rule out whether this effect is VP16-specific, NCI-N87 and AGS cells were treated with other DNA damage reagents including thapsigargin (TG), paclitaxel (PTX) as well as doxorubincin (Dox). All of these treatments led to decrease of ARID1A (Figure. 1C-1D). Together, our data demonstrated that the decrease of ARID1A protein is a common event during DSB of gastric cancer cells.

ARID1A was Rapidly Ubiquitinated and Degradated in Response to DNA Damage Response

To investigate how ARID1A was reduced during DSB, we next checked the mRNA level of ARID1A during DSB of NCI-N87 cells. However, we found the ARID1A mRNA level in NCI-N87 cells was almost unchanged during DSB (Figure 2A). We then asked whether ARID1A was regulated at the post-transcriptional level. To this end, NCI-N87 cells were treated with 30 μg/ml protein synthesis inhibitor cycloheximide (CHX) with or without VP16 for the indicated time points. As depicted in Figure 2B, the half-life of ARID1A protein was dramatically decreased in VP16-treated cells than untreated NCI-N87 cells, suggesting that the stability of ARID1A was decreased upon VP16 treatment. To clarify which pathway is responsible for ARID1A degradation, NCI-N87 cells were treated with VP16 in the presence of proteasome inhibitor MG132 or epoxomicin or calpain-specific inhibitor calpeptin. We found that both MG-132 and epoxomicin, but not calpeptin, could efficiently prevent VP16-induced ARID1A degradation (Figure 2C). Moreover, in the presence of MG132, ubiquitinated ARID1A protein was appeared in response to VP16 treatment (Figure 2D). Taken together, these data suggested that ARID1A was rapidly ubiquitinated and degradated in response to DNA damage by proteasome.

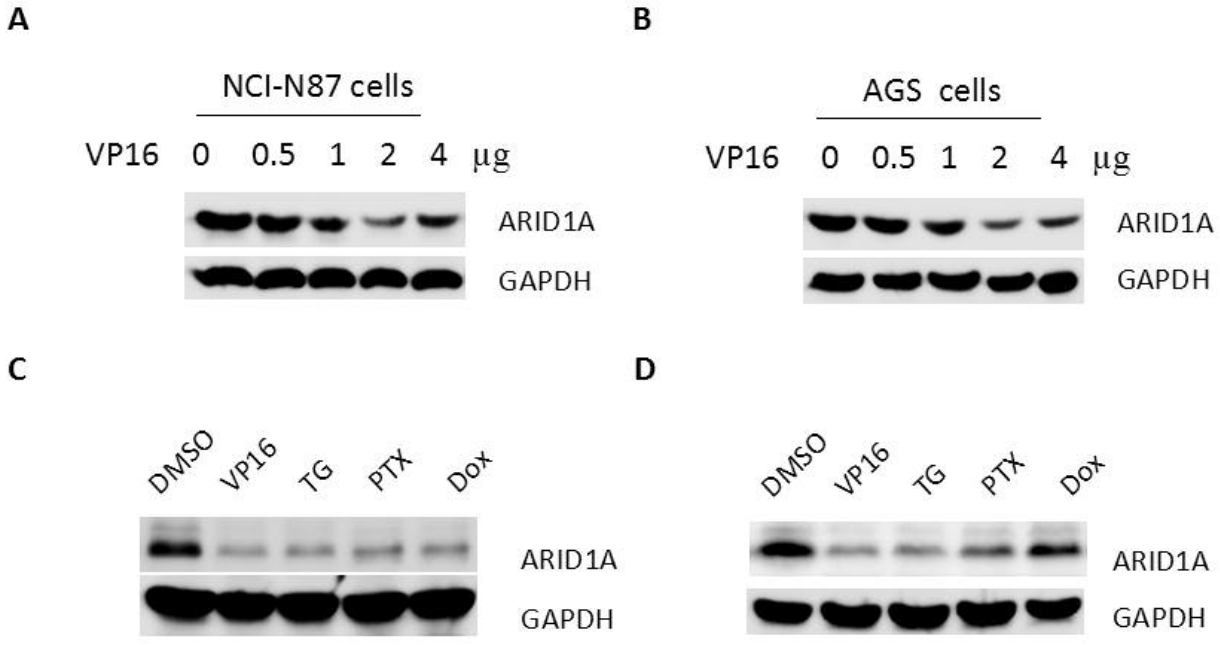

Figure 1. ARID1A was down-regulated in response to DNA damage in gastric cancer cell lines. ***A,*** NCI-N87 cells were treated with indicated doses of VP16 for 24h and the ARID1A protein expression was detected by Western blot. GAPDH was used as loading control. ***B,*** AGS cells were treated with indicated doses of VP16 for 24h and the ARID1A protein expression was detected by Western blot. GAPDH was used as loading control. ***C,*** NCI-N87 cells were treated with DMSO, VP16, TG, PTX or dox for 24h and the ARID1A protein expression was detected by Western blot. ***D,*** AGS cells were treated with DMSO, VP16, TG, PTX or dox for 24h and the ARID1A protein expression was detected by Western blot.

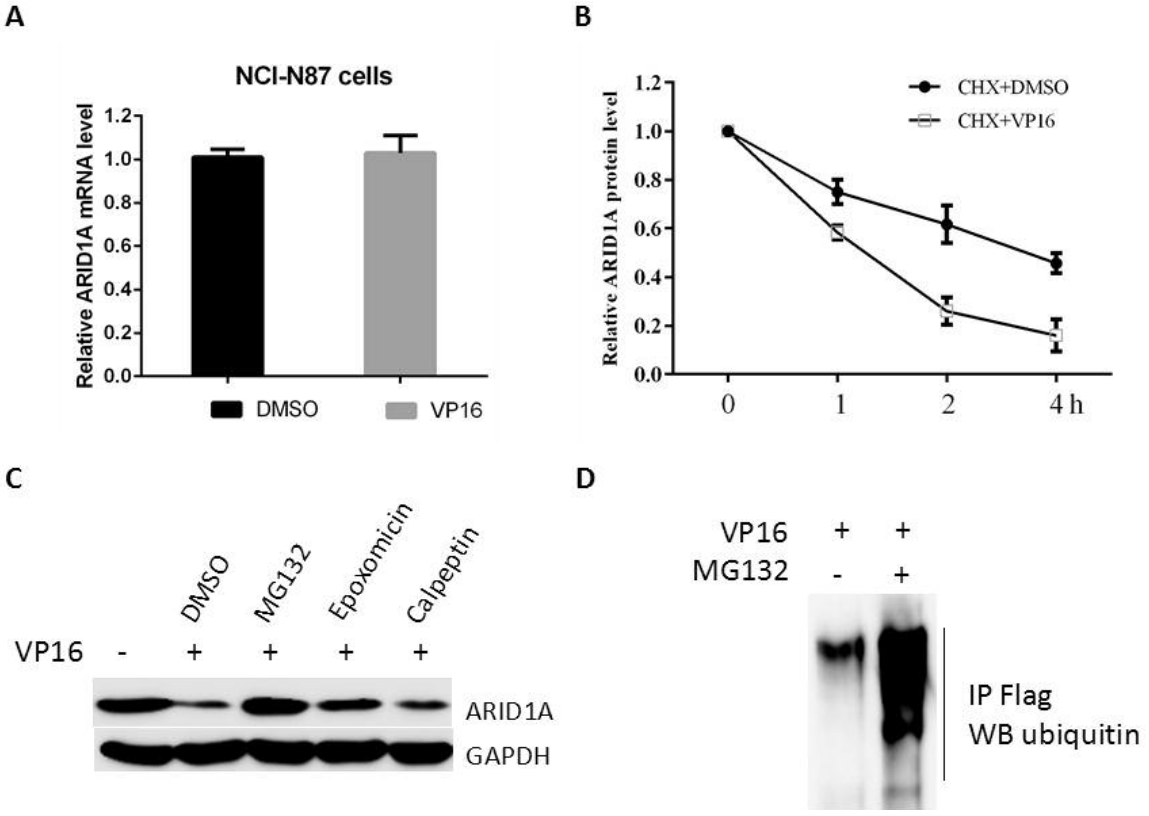

Figure 2. ARID1A was rapidly ubiquitinated and degradated in response to DNA damage response. A, NCI-N87 cells were treated with or without VP16 (2 μg/ml) for 24h and the mRNA level of ARID1A was measured by Real-time RT-PCR. B, NCI-N87 cells were treated with or without VP16 (2 μg/ml) for 20h, 20 μg/ml CHX was added for the indicated times and the protein level of ARID1A was examined by Western blot with GAPDH as loading control. Folds of decrease of ARID1A/GAPDH ratios against untreated cells are shown as means ± SD of three independent experiments. C, NCI-N87 cells were treated with VP16 (2 μg/ml) in the presence or absence of DMSO, MG132 (20 μM), epoxomicin (10 μM), or calpeptin (50 μM) for 8 h, and then the protein level of ARID1A was examined by Western blot. D, NCI-N87 cells were transfected with Flag-ARID1A for 24h and then treated with VP16 (2μg/ml) in the presence or absence of MG132 (20 μM) for 8h. Exogenous ARID1A was immunoprecipitated with Flag M2 antibody. The ubiquitinated ARID1A was detect by Western blot with ubiquitin antibody.

Cullin-SKP1-F-box E3 Ligase was Required for the Efficient Degradation of ARID1A During DSB.

As the Cullin-based ubiquitin ligases have well-known roles to target many key proteins for ubiquitination and destruction during DNA damage response[17,18], we then asked whether one of those Cullin-based ubiquitin ligases is required for the degradation of ARID1A. NCI-N87 cells were treated with VP16 in the presence of MLN4924, a potent and selective inhibitor of NEDD8-activating enzyme (NAE), which is an essential component of the NEDD8 conjugation pathway that controls the activity of the cullin-RING subtype of ubiquitin ligases[19]. We found that MLN4924 could efficiently block VP16-induced ARID1A degradation, suggesting that ARID1A is a target of Cullin-based ubiquitin ligases (Figure 3A). Then, we asked which Cullin is required for the degradation of ARID1A. Five dominant negative (DN) Cullin members, including DN-Cullin1, DN-Cullin2, DN-Cullin3, DN-Cullin4A and DN-Cullin4B, were over-expressed into NCI-N87 cells, respectively. Then VP16 was added to trigger DSB. As shown in Figure 3B, only DN-Cullin1 could significantly stabilize ARID1A (Figure 3B), suggesting that it might be a substrate of a SCF E3 ligase complex. To further test this possibility, we then asked whether ARID1A interacts with the components of the SCF complex. 293T cells were transfected with pcDNA3-Flag-ARID1A for 48 hour. Flag-ARID1A was purified by Flag M2 beads. We found that both endogenous Cullin1 and SKP1

were detected in the precipitated Flag-ARID1A complex (Figure 3C). Taken together, these results indicated that Cullin-SKP1-F-box E3 ligase was required for the efficient degradation of ARID1A during DSB.

Over-expression of ARID1A Promoted VP16-induced Apoptosis in Gastric Cancer Cells.

To investigate the biological function of ARID1A, Flag-ARID1A was over-expressed into gastric cancer cells NCI-N87 and AGS cells. These cells were then treated with VP16. We found that VP16 treatment also reduced the exogenous expressed ARID1A (Figure 4A-B), which further supported that ARID1A is regulated at the post-transcriptional level during DSB. Then, we tested whether ARID1A contributes to the sensitivity of gastric cancer cells to DNA damage reagents. Upon VP16 treatment, we could observe dramatically increase of apoptosis in NCI-N87 cells overexpressing of ARID1A compared with empty vector transfection cells (Figure 4C). In agree with this, NCI-N87 cells overexpressing of ARID1A showed increased levels of the cleaved caspase 3 and PARP1 (Figure 4D). Thus, these data indicated that gastric cancer cells with forced expression of ARID1A showed an increased sensitivity to DNA damage reagents.

Discussion

Recent studies showed that the prevalence of ARID1A mutations appeared to vary among tumor types, and revealed the frequent presence of mutations in gastric cancer[13]. Moreover, the clinicopathological significance of ARID1A inactivations has also been examined and found a significant relationship between ARID1A mutations and mismatch repair deficiency in gastric cancers, suggesting ARID1A might have critical roles in DNA damage response[20,21]. Defects in the DNA mismatch repair and DNA damage response have been reported to be involved in the development of gastric cancers[22,23].

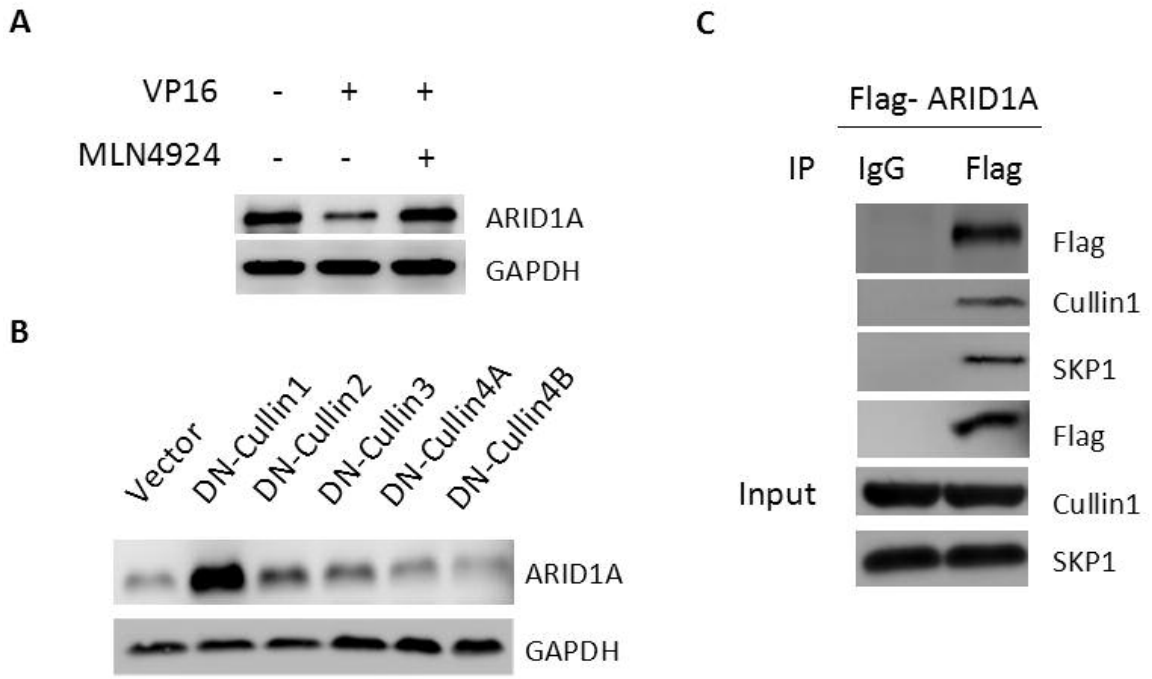

Figure 3. Cullin-SKP1-F-box E3 ligase was required for the efficient degradation of ARID1A during DSB. ***A,*** NCI-N87 cells were treated with VP16 (2 μg/ml) in the presence or absence of MLN4924 (1 μg/ml) for 4h. The protein level of ARID1A was examined by Western blot. ***B,*** Five Dominant Cullins were transfected into NCI-N87 cells for 24h, VP16 was then added for 14h. The protein level of ARID1A was examined by Western blot. ***C,*** NCI-N87 cells were transfected with Flag-ARID1A for 24h. Lysates of NCI-N87 cells were subjected to immunoprecipitation with Mouse IgG or Flag antibodies followed by Western blot with indicated antibodies.

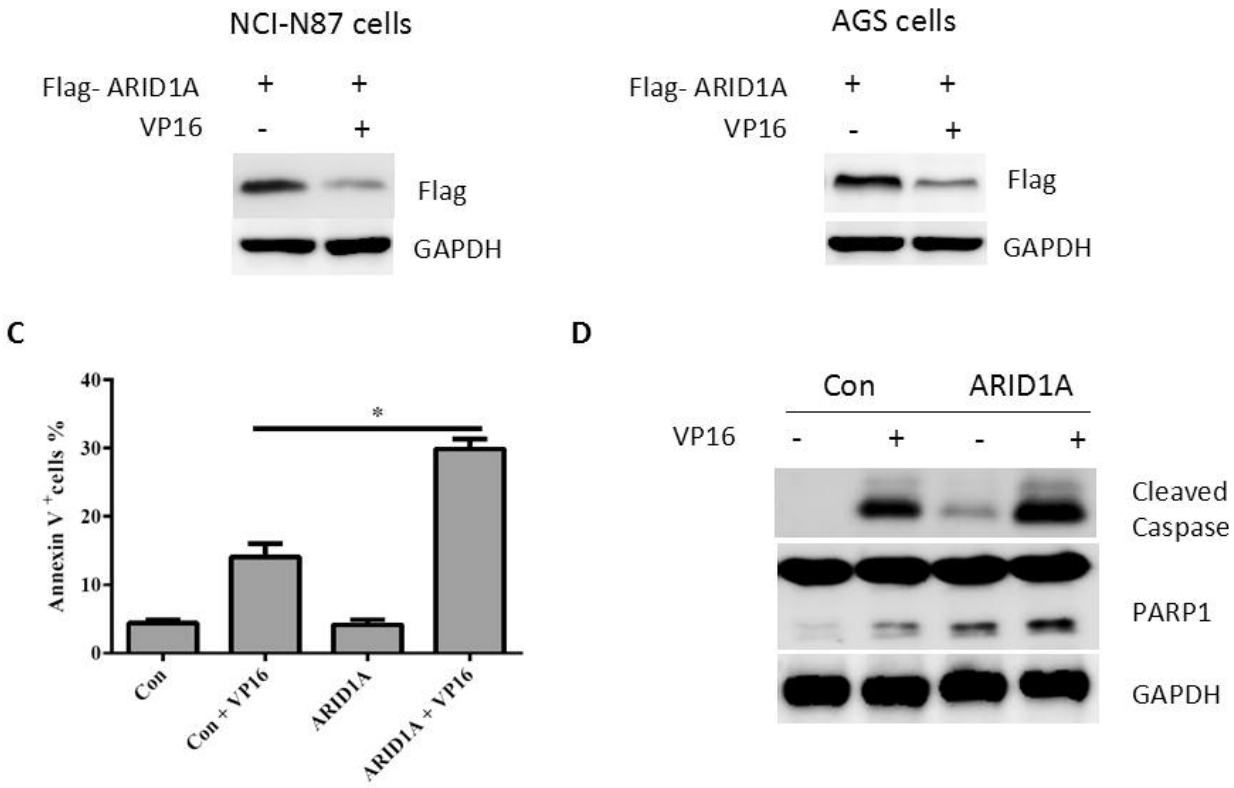

Figure 4. Over-expression of ARID1A prevented VP16-induced DSB in gastric cancer cells. A, NCI-N87 cells were transfected with Flag-ARID1A for 24h and treated with or without VP16 (2 μg/ml) for 20h. The exogenous expression of ARID1A was examined by Western blot with Flag antibody. B, AGS cells were transfected with Flag-ARID1A for 24h and treated with or without VP16 (2 μg/ml) for 20h. The exogenous expression of ARID1A was examined by Western blot with Flag antibody. C, NCI-N87 cells transfected with or without Flag-ARID1A were either untreated or treated with VP16 (2 μg/ml) for 24h. Apoptotic cells were analyzed by a sub-G1 DNA content assay. * $p<0.05$ versus mock transfected cells treated with VP16. D, NCI-N87 cells transfected with or without Flag-ARID1A were either untreated or treated with VP16 (2 μg/ml) for 24h. Then the indicated proteins were examined by Western blot.

In the present study, our data revealed a post-transcriptional regulation of ARID1A and a novel role ARID1A in DNA damage response. We showed that ARID1A protein was rapidly ubiquitinated and degradated in gastric cancer cells in response to DNA damage. Using genetic and pharmacologic Cullin inactivation coupled with in vitro ubiquitination assay, we demonstrated that ARID1A is a substrate of the Cullin-SKP1-F-box protein (SCF) complexes. Our biochemical data revealed that Cullin1 and SKP1 interacted with and targeted ARID1A for ubiquitination and degradation. Moreover, we identified that ARID1A contributes to the sensitivity of gastric cancer cells to DNA damage reagents. Upon VP16 treatment, we observed dramatically increase of apoptosis in cells overexpressing of ARID1A with increased levels of the cleaved caspase 3 and PARP1.

Thus, our results uncovered a previous unknown posttranscriptional regulation of ARID1A by SCF E3 ligase in gastric cancer cells in DNA damage response. Together, our data suggest that ARID1A might regulate DNA damage response, implicating ARID1A as a novel, promising therapeutic target for gastric cancer therapy.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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