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Running title: BATF2 reverse MDR in GC

BATF2 reverses multidrug resistance of gastric cancer cells and centrosome clustering by suppressing ATM phosphorylation

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Chromosome instability (CIN) is a major contributor to drug resistance and recurrence. As a crucial mechanism of CIN, centrosome clustering has emerged as a promising therapeutic strategy. However, the roles and regulatory mechanisms of centrosome clustering in gastric cancer remain unclear. BATF2 was previously identified as a key modulator of multidrug resistance (MDR) in gastric cancer (GC). To examine the involvement of centrosome clustering in the mechanism by which BATF2 reverses MDR in GC, adriamycin (ADR)- and vincristine (VCR)-resistant cell lines, NCI-N87/ADR and NCI-N87/VCR, were used for investigations. Expression of BATF2 was downregulated in both drug-resistant cells, particularly in NCI-N87/ADR cells. Cells with BATF2 knockdown exhibited higher cell viability and lower apoptosis rates, and such changes were reversed by BATF2 overexpression. The enhanced centrosome clustering in cells transfected with sh-BATF2 was accompanied by increased KIFC1 expression, which was inhibited after BATF2 overexpression. BATF2 reversed MDR and inhibited centrosome clustering by inhibiting ATM phosphorylation, which was evidenced by ATM overexpression. Meanwhile, KU-60019, a specific inhibitor of ATM, could markedly reverse the pro-tumor effects of BATF2 knockdown. In conclusion, BATF2 is a potential target for reversing MDR in GC, and targeting KIFC1-related centrosome clustering by suppressing ATM phosphorylation is proposed as a key mechanism.

Key words: gastric cancer; multidrug resistance; centrosome clustering; BATF2

Gastric cancer (GC) is a malignancy originating from gastric mucosal epithelium [1]. Global Cancer Statistics estimate that GC is the fifth leading cause of both cancer incidence and cancer-related deaths worldwide, with 968,350 newly diagnosed GC cases (4.9% of all new cases) and 659,853

GC-caused deaths (6.8% of all cancer-related deaths) in 2022 [2]. Treatment options for GC depend primarily on the stage of the disease at the time of presentation, but most patients are seen at an advanced stage and require pharmacotherapy, mainly chemotherapy [3]. Conventional chemotherapeutics comprises of cisplatin, epirubicin, paclitaxel and 5-fluorouracil. Nevertheless, the 5-year overall survival rate for GC remains below 30% because of limited curative effect of chemotherapy [4]. Although chemotherapy drugs have continued to advance over the past few years, improving survival outcomes for patients with advanced GC, progress has reached a bottleneck [5, 6]. The main reason is that GC cells develop multidrug resistance (MDR), which leads to reduced sensitivity to chemotherapy [1, 7, 8]. Therefore, identifying methods to reverse MDR clinically and improve chemotherapy efficacy has become a focal point and a challenge in current research.

Mounting evidence suggests that the MDR mechanism of GC is closely related to chromosomal instability (CIN) abnormalities [9, 10]. CIN is widely recognized as a hallmark of tumors [11], including GC, and is positively associated with treatment resistance, poor clinical outcomes, immune evasion, and a high risk of recurrence and metastasis [12, 13]. Whereas CIN usually exhibits centrosome amplification [14, 15], and to avoid multipolar spindles and cell death, cancer cells achieve bipolar division by inducing supernumerary centrosome clustering, an adaptive survival strategy that allows cancer cells to divide successfully despite bearing supernumerary centrosomes [15, 16]. As a crucial mechanism of CIN, centrosome clustering has been proposed as a promising target for therapeutic intervention of human cancers [17, 18]. Nevertheless, the regulatory mechanisms of centrosome clustering and functions of centrosome clustering remain largely unclear.

Kinesin Family Member C1 (KIFC1) is a novel driver protein and centrosome clustering regulator participating in extra centrosome clustering in tumor cells [16, 19]. Elevated expression of KIFC1 has been observed in GC tissues [20], and its high expression is linked to a worse clinical outcome [21, 22]. KIFC1 knockdown could repress the sphere formation of GC cells, indicating the close involvement of KIFC1 in GC stem cells [20]. In addition, KIFC1 mediates the resistance to drugs, such as docetaxel [23] and cisplatin [24] in other cancers. However, whether KIFC1 mediates MDR of GC cells, and the associations between its functions and centrosome clustering in GC, have not been reported in GC.

Basic Leucine Zipper ATF-Like Transcription Factor 2 (BATF2), also termed SARI, has been

recognized as a novel tumor suppressor in human tumors [25-27]. In GC, Xie et al. demonstrate that reduced BATF2 expression in GC tissues is linked to peritoneal recurrence post-curative gastrectomy, and that GC growth and metastasis can be restrained by enhancing BATF2 expression [28]. Our previous studies have demonstrated the important role of BATF2 in reversing MDR and inhibiting chemotherapy resistance in GC [29-31]. However, whether BATF2 regulates centrosome clustering and thus affects the mechanism of MDR in GC remains unknown. To address this issue, we conducted the current study to elucidate the possible linkages among BATF2, centrosome clustering and MDR in GC based on two MDR cell models. This study may provide novel insights to illustrate the mechanism of MDR in GC.

Materials and Methods

Establishment of drug-resistant cells. GC cell line NCI-N87 from Wuhan Procell Life Technology Co., LTD. (#CL-0169, China) was maintained in RPMI-1640 medium bearing an additional adding of 10% FBS (#16140071, Gibco) and 1% penicillin-streptomycin under the conditions of 5% CO_2 at 37 °C. Adriamycin (ADR, #A864033, Macklin, Shanghai, China) and Vincristine (VCR, #V861369, Macklin)-resistant cells, NCI-N87/ADR and NCI-N87/VCR, were acquired via enhancing drug dose gradually as previously described [32-34]. For the culture of drug-resistant cells, a final concentration of 0.5 mg/mL ADR and 1 mg/mL VCR was used to maintain the drug-resistant phenotype.

Cell transfection. Lentiviral vector system was employed to overexpress BATF2 and ataxia-telangiectasia mutated (ATM) or to knockdown BATF2. Based on DSIR website (http://biodev.cea.fr/DSIR/), we designed short hairpin RNA (shRNA) against BATF2: 5'-ACACTATGTTAGTATCTAA-3' (sense), and 5'- ACACTATGTTAGTATCTAA-3' (antisense). From NCBI database, we retrieved sequences of mature BATF2 and ATM. Subsequently, we transfected vectors bearing sh-BATF2, BATF2, ATM as well as matching (negative control) NC sequences into 293T cells, along with the packaging plasmids (pMDLg/pRRE: pVSV-G: pRSV-Rev=5:3:2) with the aids of HighGene reagent, followed by collecting and concentrating lentivirus particles 48h after transfection. For lentiviral infection, NCI-N87/ADR and NCI-N87/VCR cells (1×10^5/ml) were inoculated into 6-well plates, and lentivirus (1×10^8 TU/ml) was added to the medium at 70-90% confluency. Following 48 h post-infection, 2.5 μg/ml

puromycin was added to select stably transfected cells.

qRT-PCR. Total RNAs isolation from cells were conducted with the aids of TRIzol reagent (#15596018, Invitrogen). The isolated RNAs were reversely transcribed into cDNA using FastKing RT SuperMix (#KR118-02, TIANGEN), followed by PCR amplification with the aids of SYBR Green PCR Master Mix (#A4004M, Lifeint). The used primers are: BATF2-F: 5'-GCTCCTGTGGGCAAGAGAAT-3'; BATF2-R: 5'-AGAGAGCAGGTTTGTGCTCC-3'; GAPDH-F: 5'- CCATGGGGAAGGTGAAGGTC-3'; GAPDH-R: 5'-AGTGATGGCATGGACTGTGG-3'.

Western blot. After cell lysis with RIPA, concentration of cell proteins was determined with the aids of BCA assay kit. Proteins were separated, transferred onto PVDF membranes, followed by incubation with antibodies BATF2 (#PA5-103647, Thermo), anti-KIFC1 (#12313, CST), anti-ATM (#2873, CST) and anti-phosphorylated ATM (p-ATM, #5883, CST) overnight at 4 °C. Secondary antibodies incubation was then carried out for 60 min at dark. The blotting bands were washed by ECL reagent and were packaged using a cling film, and were transferred to cassette for film exposure.

Immunofluorescence. Immunofluorescence was performed according to the methods in previous studies. In brief, cells were re-suspended into a 12-well plate containing cell sliver, and were treated with ADR or VCR for 24 h. After cell fixation, permeabilization and blocking, anti-α-tubulin and anti-centrin-2 were added to incubate with cells overnight at 4 °C. Afterwards, IgG H&L (Alexa Fluor® 647) secondary antibody incubation and DAPI staining were carried out. Finally, sections were canned on a laser scanning confocal microscope after sealed with fluorescent quenching sealant.

CCK-8 assay. Cells were placed in 96-well plates (2,000 cells/well), and were treated with ADR or VCR. After 24 h of treatment, 10 μl CCK-8 was added to each well for 2 h to calculate the cell counts on the basis of the optical density at 450 nm.

Flow cytometry (FCM). Apoptosis was determined using an Annexin V-FITC apoptosis kit (#C1062S, Beyotime). Briefly, after digested with 0.25% EDTA-free Tyrisin, cells were collected by centrifugation. Then, cells were washed by pre-cooled PBS and were resuspended in 300 μl binding Buffer, followed by incubation with 5 μl Annexin V-FITC and 10 μl of propidium iodide in dark. The cells were analyzed by an CytoFLEX S FCM (Beckman Coulter Life Sciences, USA),

and were analyzed using CELL Quest software.

Co-Immunoprecipitation (Co-IP). To explore the interaction between BATF2 and ATM, we conducted Co-IP assay according to the manual of the rProtein A/G Magnetic IP/Co-IP Kit (#abs9649, absin). Briefly, cells were collected and washed by PBS, and then were lysed in 1x Lysis/Wash Buffer (Enhanced) on ice. After centrifugation at 14,000 × g, 4 °C for 10 min, the supernatant was collected, and was precleared with protein A/G beads for 2 h before incubation with primary antibodies: anti-ATM (#16592-1-AP, Proteintech) and anti-BATF2 (#16592-1-AP, Proteintech). Isotype immunoglobulin G (IgG) antibody was used as negative control. Afterwards, Protein A/G MagPoly Beads were added to the immunoprecipitation mixture and rotated for 30 min. Afterwards, the immunoprecipitated protein complexes were washed with Elution buffer, and the resulting supernatants were collected, and Neutralization Buffer was added. Finally, the collected supernatants were detected by Western blot.

Statistical analysis. All experiments were conducted with three repetitions. Data presenting in the form of mean±standard deviation was employed for statistics. Data for changes on cell viability along with the drug dosages was compared by two-way RM ANOVA. ANOVA coupled a Tukey's post hoc test was employed for statistics of other data. $P < 0.05$ donates statistical difference.

Results

BATF2 overexpression enhances susceptibility of NCI-N87 cells to ADR and VCR. Expression of BATF2 was detected in drug-resistant cells: NCI-N87/ADR and NCI-N87/VCR. Compared with that in NCI-N87 cells, significant decrease in its protein expression was observed in these two drug-resistant cells, particularly in NCI-N87/ADR cells (Figure 1A). To elucidate the exact roles of BATF2 expression in MDR, we first established BATF2 overexpression or knockdown model in NCI-N87/ADR and NCI-N87/VCR cells (Figure 1B). Subsequently, cell viability was determined using the CCK-8 assay, and we found that BATF2 overexpression exhibited enhanced chemosensitivity (low cell viability) to ADR and VCR than matched NC group. Cells with BATF2 knockdown showed highest cell viability, indicating that BATF2 knockdown decreased susceptibility to ADR and VCR (Figure 1C). Consistently, FCM analysis indicated that in two drug-resistant cells, BATF2 overexpression exhibited highest apoptosis rate, whereas BATF2 knockdown exhibited lowest apoptosis rate (Figures 1D, 1E). All these results suggest that BATF2

inhibition promotes MDR, while BATF2 overexpression sensitizes NCI-N87 cells to chemotherapy.

BATF2 overexpression inhibits KIFC1 expression and centrosome clustering. KIFC1 is a centrosome clustering molecule that prevents cancer cells from undergoing centrosome-amplification-induced apoptosis [35]. We found that expression of KIFC1 was markedly reduced in NCI-N87/ADR and NCI-N87/VCR cells with BATF2 overexpression, whereas KIFC1 expression was observably enhanced in NCI-N87/ADR and NCI-N87/VCR cells with BATF2 knockdown (Figure 2A). Centrosome clustering was further observed by immunofluorescence staining. As shown in (Figure 2B), ratio of centrosome clustering was high in cells with BATF2 knockdown in comparison with corresponding NC. After BATF2 overexpression, the ratio of centrosome clustering significant decreased. Similar results were also observed in NCI-N87/VCR cells (Figure 2B).

BATF2 overexpression inhibits the elevated p-ATM in drug-resistant cells. Expression of ATM and p-ATM was determined, and we found that compared with NCI-N87 cells, the drug-resistant NCI-N87/ADR and NCI-N87/VCR cells showed markedly elevated level of p-ATM, particularly in NCI-N87/ADR cells (Figure 3A). To investigate the possible interaction between BATF2 and ATM, we conducted Co-IP assay. The results indicated that BATF2 and ATM were co-precipitated with each other (Figure 3B), suggesting that BATF2 could directly interact with ATM. Besides, in both NCI-N87/ADR and NCI-N87/VCR cells, BATF2 overexpression could significantly reduce the level of p-ATM, while BATF2 knockdown could elevate p-ATM level in drug-resistant cells (Figure 3C). These results suggested that p-ATM level was increased in multidrug-resistant NCI-N87 cells, and such increased p-ATM level could be inhibited by BATF2 overexpression.

BATF2 mediates chemosensitivity and centrosome clustering by inhibiting p-ATM. To validation whether ATM involving in the actions of BATF2-mediated chemosensitivity, we further overexpressed ATM in BATF2-overexpressed multidrug-resistant NCI-N87 cells. ATM overexpression showed no influences on the expression of BATF2 (Figure 4A). BATF2 overexpression could significantly reduce the level of p-ATM/ATM, while such decrease could be partly reversed following ATM overexpression (Figure 4A). BATF2 overexpression exhibited enhanced chemosensitivity (lower cell viability and higher cells apoptosis) to ADR and VCR than matched NC group, and such susceptibility was partly reversed following ATM overexpression (Figures 4B, 4C). Moreover, overexpression of ATM elevated the reduced KIFC1 and reduced ratio

of centrosome clustering caused by BATF2 overexpression in both NCI-N87/ADR and NCI-N87/VCR cells (Figures 5A, 5B). Meanwhile, 3 μM KU-60019 (#Y242876, Beyotime), a specific inhibitor of ATM, was used to inhibit ATM in cells with BATF2 knockdown. As expected, KU-60019 treatment markedly reduced the increased level of p-ATM/ATM caused by BATF2 knockdown in both two cells (Figure 6A). In addition, BATF2 knockdown observably promoted cells viability (Figure 6B) and inhibited cell apoptosis (Figure 6C), while such effects of BATF2 knockdown were markedly reversed after KU-60019 treatment (Figures 6B, 6C). Altogether, these findings suggested that BATF2 could mediate chemosensitivity and centrosome clustering by inhibiting p-ATM.

Discussion

Although chemotherapy can prolong the survival of patients with advanced GC, the overall survival rate is still not ideal. MDR is the primary factor that limits the therapeutic effect of chemotherapy and leads to the death of patients [36, 37]. CIN is one of the main features of solid tumors, including GC, and has been demonstrated to contribute to the therapeutic resistance in cancer treatment [38, 39]. Although CIN is a feature of GC [40], centrosome clustering, a contributor to CIN, has not been reported in GC, especially its relationship with MDR in GC.

The centrosome is the primary microtubule-organizing center, which ensures the symmetry and bipolarity of cell division by establishing bipolar spindle; this function is essential for accurate cell mitosis [18]. Unlike normal cells, cancer cells often exhibit supernumerary centrosomes, called centrosome amplification, which is a hallmark of cancer and is strongly associated with tumor progression, poor clinical outcomes, and multiple carcinogenic phenotypes, such as aneuploidy [41]. For cancer cells with supernumerary centrosomes, to avoid the emergence of multiple spindles that can trigger cell death, cancer cells usually achieve pseudo-bipolar division by centrosome clustering to form pseudo-bipolar spindles, thus ensuring cell survival, which is an adaptive survival mechanism of tumor cells [42, 43]. Therefore, targeting centrosome clustering may be a promising therapeutic strategy, and understanding the processes that control centrosome clustering is crucial for this strategy. In this study, we found that overexpression of BATF2 not only inhibited the supernumerary centrosomes clustering, but also reduced cell viability and increased apoptosis of both NCI-N87/ADR and NCI-N87/VCR cells, possibly due to the induction of multipolar division.

The results of this study emphasize the involvement of centrosome clustering in MDR, and targeting centrosomes clustering may enhance the sensitivity to chemotherapeutics.

KIFC1 has recently been recognized as centrosome clustering molecule, which is fundamental for viability of tumor cells bearing supernumerary centrosome [16, 44]. Specifically, KIFC1 allows tumor cells bearing multiple centrosomes to survive cell division, which is not necessary for normal somatic cells, but is necessary for normal division in tumor cells bearing supernumerary centrosomes [45, 46]. The loss of KIFC1 could cause death of tumor cells by inducing deadly multipolar division, but no influence was observed for the division of normal cells possessing two centrosomes [47]. Expression of KIFC1 was found to be up-regulated in GC tissues [21], and its enhanced expression was strongly linked to lymph node/distant metastasis, depth of invasion, TNM stage, tumor size and a worse survival in GC [22]. Hence, KIFC1 suppression is proposed as an available strategy to for GC treatment. In this study, we found that expression of KIFC1 was elevated in both ADR and VCR-resistant GC cell lines, and such elevated expression could be markedly reduced after BATF2 overexpression, accompanied by a decrease in the number of cells with centrosome clustering. The cooccurrence of KIFC1 dysregulation with centrosome clustering suggests that targeting KIFC1 might be an available strategy to modulate centrosome clustering.

BATF2, a transcription factor of bZIP family, has been reported to participate in the progression of a wide variety of human cancers. Particularly, BATF2 exerts as a tumor suppressor, and its high level is frequently associated with an inhibition of tumor growth and metastasis [25, 48-52]. In GC, BATF2 expression was reduced in GC tissues [28]. Such reduced BATF2 expression in GC showed correlations with a worse survival outcome and stem cell-like properties, and enhanced BATF2 level was linked to an increased responsiveness to 5-Fluorouracil and an improved outcomes of GC patients who received postoperative chemotherapy [53]. In terms of mechanism, BATF2 was found to inhibit drug transporter ABCG2 and to inhibit AKT phosphorylation while promote PTEN stability [53]. In previous studies [29-31], we investigated the functional roles and the underlying mechanisms of BATF2 in GC MDR cells, and found that it can reverse MDR through decreasing P-glycoprotein (P-gp, an ABC transporter) mediated drug efflux via inhibiting AP-1 [29] or Wnt/β-catenin signaling [30], or through inhibiting Drp1-dependent mitochondrial fission via p53/ERK pathway in GC [31]. In this study, we found that BATF2 could reverse MDR and inhibit KIFC1-mediated centrosome clustering by inhibiting p-ATM *in vitro*. Consistently, a previous study

also revealed the regulatory roles of ATM kinases on centrosome clustering [46]. ATM kinase is a key protein that is frequently recruited and activated in DNA damage response (DDR) pathway to trigger cell cycle arrest and promote DNA repair [54]. DDR defect is an established driver and hallmark of cancers, and the activation of ATM in DDR pathway is found to be benefits for abnormal cell survival [54]. Through the autophosphorylation at Ser1981, ATM modulates the cellular responses to DNA double-strand breaks [55]. p-ATM was observed to be elevated in drug-resistant NCI-N87/ADR and NCI-N87/VCR cells, and such enhanced p-ATM level could be inhibited after BATF2 overexpression. The possible mechanism by which overexpression of BATF2 reverses MDR in GC cells has been summarized in Figure 7. These results indicated that BATF2 could target KIFC1-related centrosome clustering by suppressing p-ATM, thus enhancing the chemosensitivity of GC cells to VCR and ADR. This study broadens our understanding of the mechanism of BATF2 in MDR in GC, and contributes to the translational feasibility of BATF2 in GC.

Limitations and prospects: Although this study demonstrated the role of BATF2 in reversing MDR of GC cells to VCR and ADR by inhibiting KIFC1-mediated centrosome clustering, whether KIFC1-mediated centrosome clustering is involved in resistance of GC cells to other chemotherapeutics is largely unclear, and this should be investigated in future studies. In addition, our results suggest that ATM positively regulates KIFC1 as a downstream of BATF2, and ATM can reverse the inhibitory effects of BATF2 on KIFC1. However, it is not yet clear whether ATM regulates KIFC1 at the transcriptional level or the protein level, and this will be investigated in our subsequent studies. Besides, a previous study indicated that expression of BATF2 showed close linkage with infiltration of activated CD4+ memory T cells, and the signature based on CD36-BATF2/MYB could be used as an indicator for predicting the response to anti-PD-1 immunotherapy [56]. Li et al. [57] observed upregulated expression of PD-L1 in GC MKN-45 cells with BATF2 knockdown, indicating the potential of BATF2 as a biomarker for predicting the efficacy of PD-L1 blockade therapy in GC. Therefore, whether BATF2 mediates the sensitivity of GC cells to immune checkpoint blockade therapy should also be investigated in future studies. Finally, *in vivo* validation should be conducted in our future investigations to strengthen the reliability and translational value of this study.

In summary, this study demonstrated that centrosome clustering existed in MDR GC cells. BATF2

overexpression could inhibit cell viability, centrosome clustering and KIFC1 expression, while induce apoptosis in NCI-N87/ADR and NCI-N87/VCR cells. We proposed that BATF2 could reverse MDR in GC cells and inhibit KIFC1-mediated centrosome clustering via inhibiting p-ATM. This study provided a new perspective on the mechanism by which BATF2 reverses MDR in GC.

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Figure Legends

Figure 1. BATF2 mediates chemosensitivity of NCI-N87 cells to ADR and VCR. A) protein expression of BATF2 in different cells determined by Western blot; B) mRNA expression of BATF2 after overexpression or inhibition in two drug-resistant cells; C) CCK-8 assay for determining the cell viability after BATF2 overexpression or inhibition; D) representative images of flow cytometry for analyzing cell apoptosis after BATF2 overexpression or inhibition; E) Quantification of cells apoptosis determined by flow cytometry. $*p < 0.05$, $**p < 0.01$

Figure 2. BATF2 regulates KIFC1 expression and centrosome clustering. A) protein bands (upper) and quantification (lower) of KIFC1 in two drug-resistant cells after BATF2 overexpression or inhibition; B) representative immunofluorescence images (left) and corresponding quantification (right) of the proportion of cells with centrosome cluster after BATF2 overexpression or inhibition. $*p < 0.05$, $**p < 0.01$

Figure 3. BATF2 mediates ATM phosphorylation in drug-resistant cells. A) level of p-ATM/ATM in NCI-N87 cells and drug-resistant NCI-N87 cells determined by Western blot; B) Western blot bands

of Co-IP for investigating the interactions between BATF2 and ATM; C) levels of p-ATM/ATM after BATF2 overexpression or inhibition. *p < 0.05, **p < 0.01

Figure 4. BATF2 promotes chemosensitivity by inhibiting ATM phosphorylation. A) protein bands and quantification of BATF2, ATM and p-ATM expression in each group; B) cell viability of cells in each treatment group determined by CCK-8 assay; C, representative images (left) and corresponding quantification (right) of flow cytometry for analyzing cell apoptosis in each treatment group. *p < 0.05, **p < 0.01

Figure 5. BATF2 restrains centrosome clustering by inhibiting ATM phosphorylation. A) protein bands (upper) and quantification (lower) of KIFC1 expression in two drug-resistant cells in each group; B) representative immunofluorescence images (left) and corresponding quantification (right) of the proportion of cells with centrosome cluster in each group. *p < 0.05, **p < 0.01

Figure 6. ATM inhibitor reverses the effects of BATF2 knockdown on cells viability and apoptosis. A) protein bands and quantification of ATM and p-ATM expression in each group; B) cell viability of cells in each treatment group determined by CCK-8 assay; C) representative images (left) and corresponding quantification (right) of flow cytometry for analyzing cell apoptosis in each treatment group. *p < 0.05, **p < 0.01

Figure 7. Overview of the mechanism under BATF2-mediated MDR in GC.

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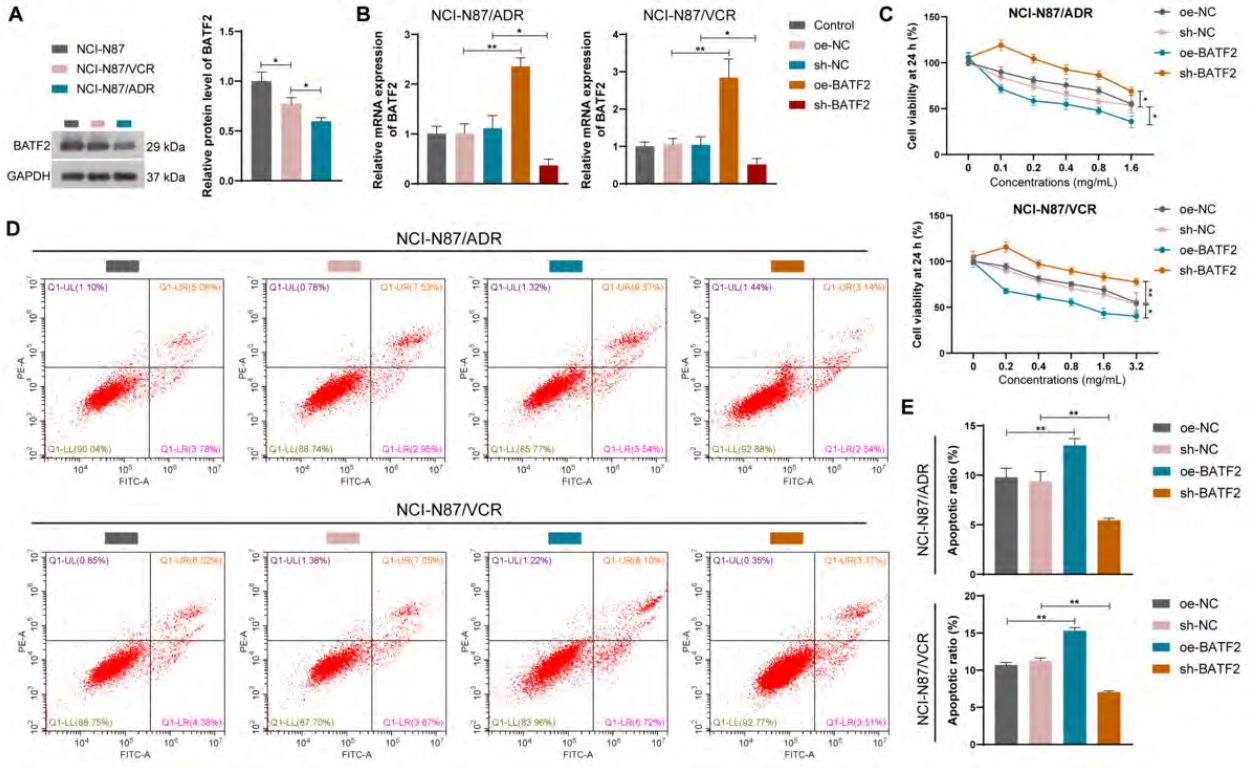

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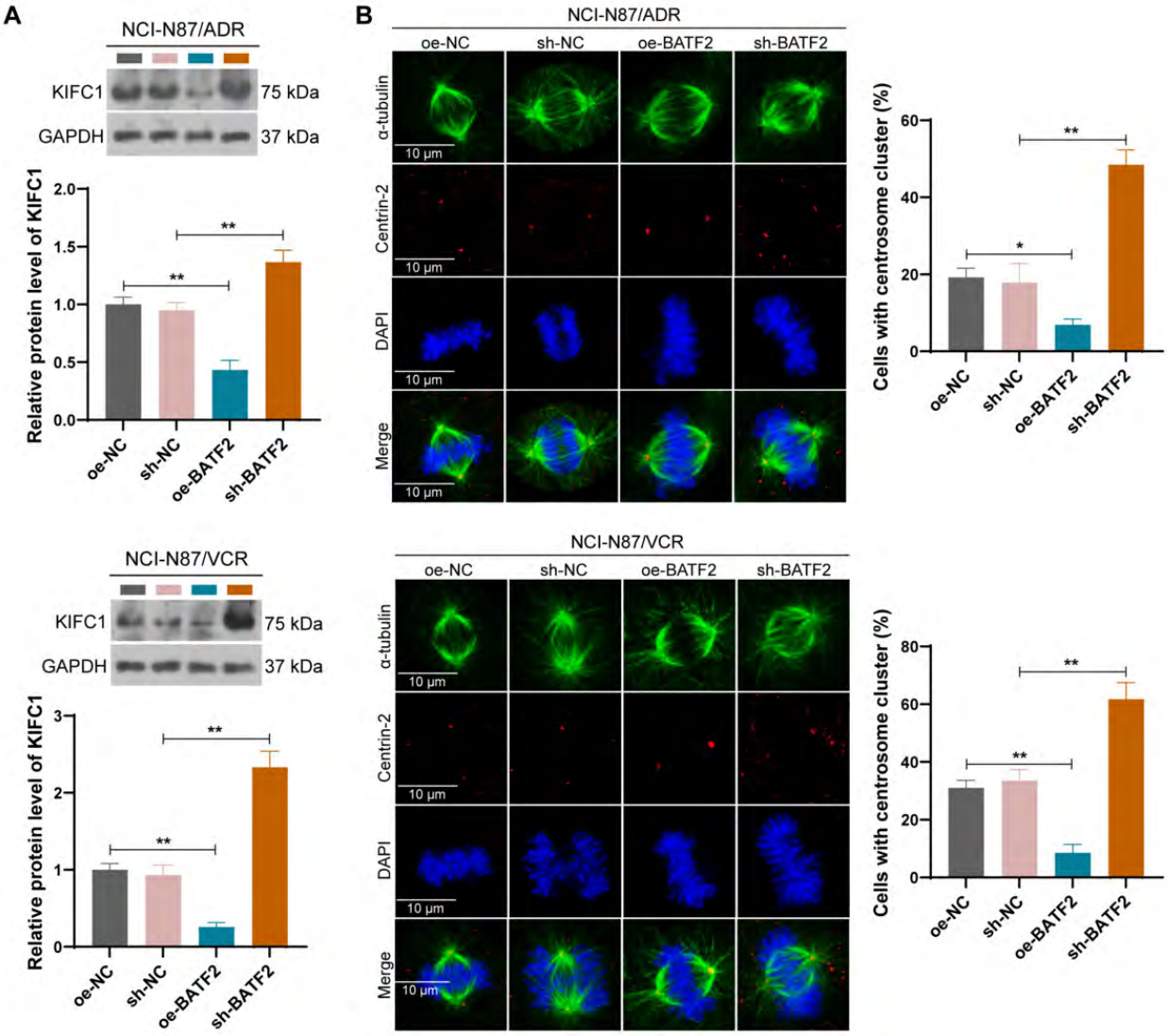

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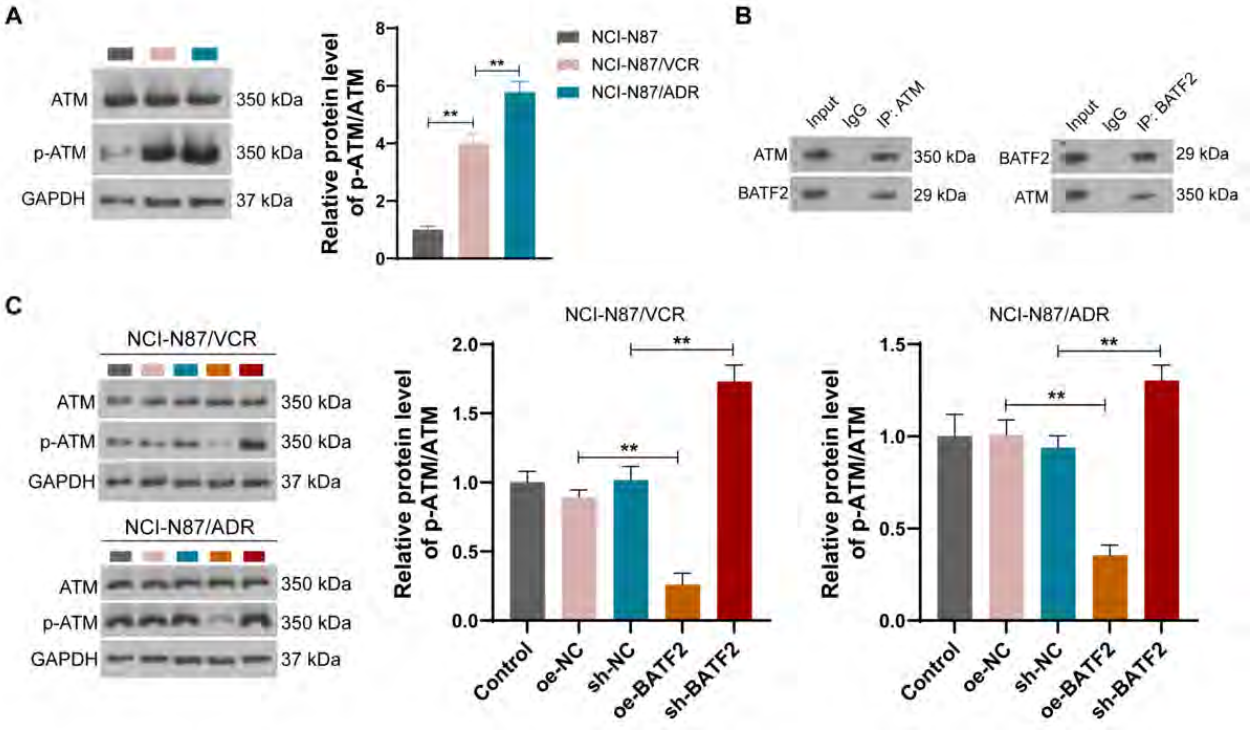

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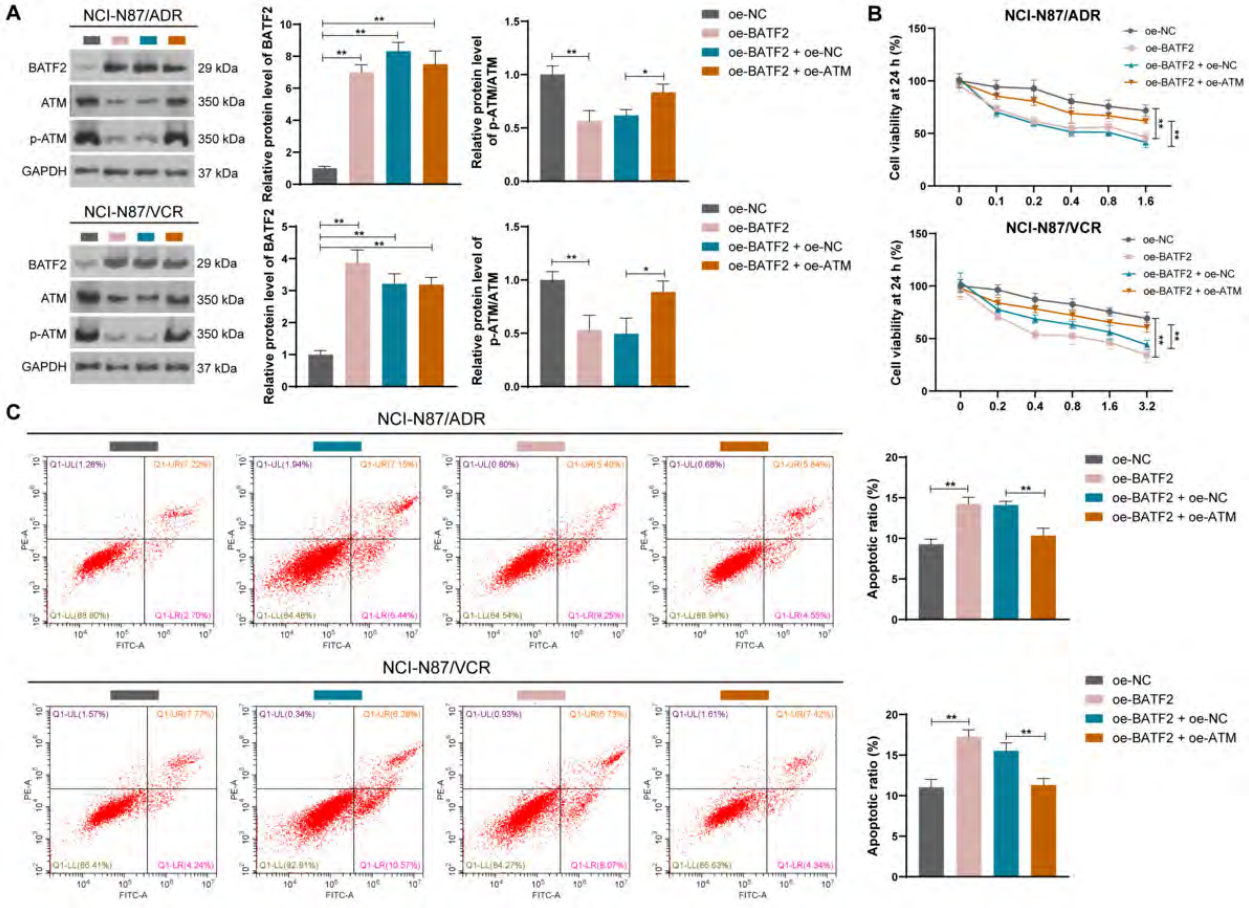

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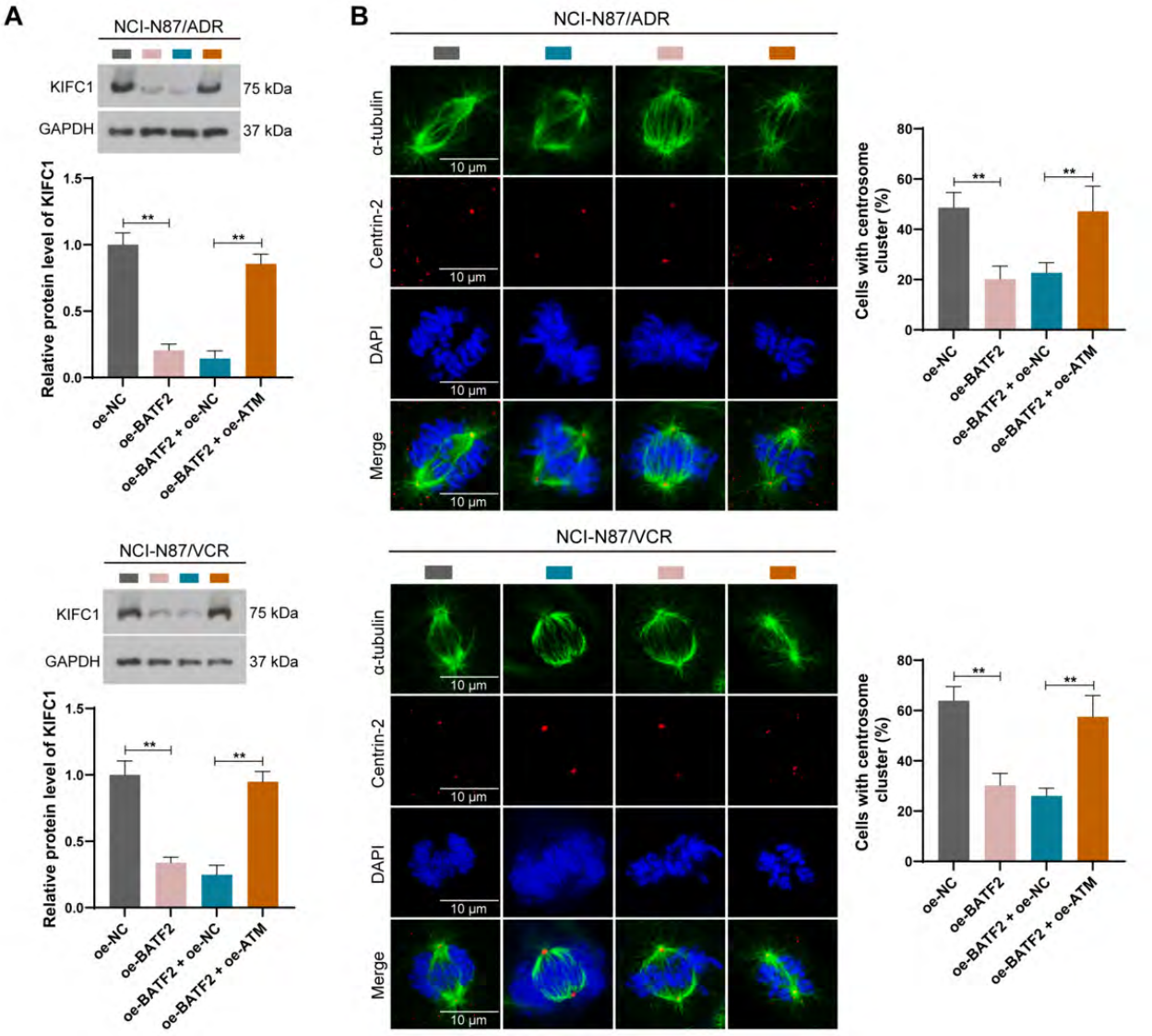

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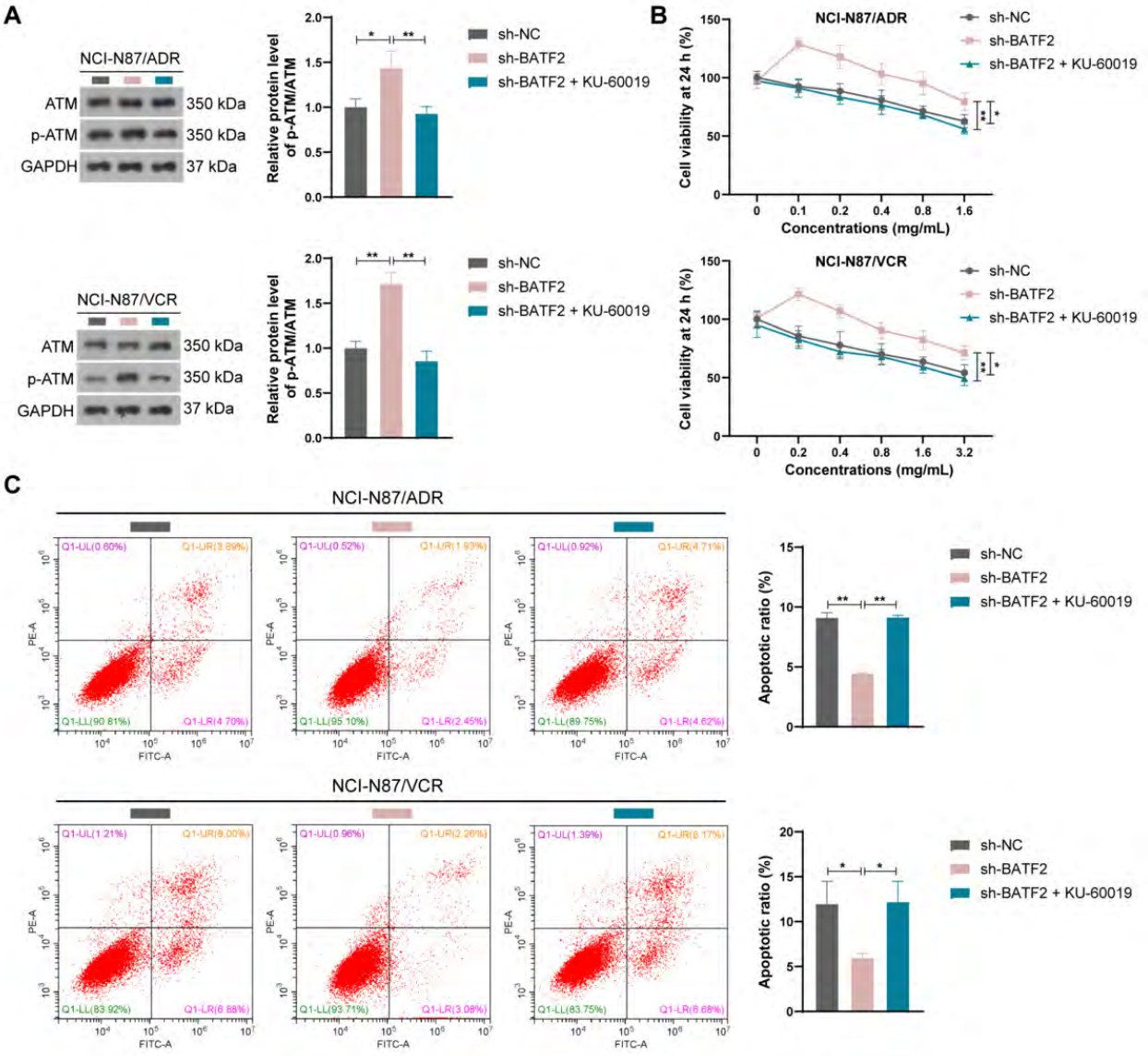

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