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The role of Beclin 1 in IR-induced crosstalk between autophagy and G2/M cell cycle arrest

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ABSTRACT

Objectives: Beclin 1 is a well-established core mammalian autophagy protein. Autophagy has been demonstrated to play roles in cellular responses to DNA damage, such as cell cycle regulation and apoptosis. In the present study, we investigated the exact mechanism by which Beclin 1 acts as a bridge between autophagy and cell cycle, when cells are exposed to ionizing radiation (IR).

Materials and methods: Western blotting and coimmunoprecipitation were performed to investigate protein expression levels and interactions. Immunofluorescence was used to monitor the localization and distribution of the indicated proteins. The levels of apoptosis and cell cycle changes were evaluated by flow cytometry. Double thymidine deoxyribonucleoside (TdR) blocking was conducted to differentiate G2 from mitotic delay. In vitro kinase assays using ATM kinase were performed to elucidate the specific phosphorylation site in Beclin 1.

Results: In this study, we show that Beclin 1 knockdown reduces IR-induced autophagy. IR enhanced Beclin 1/PIK3CIII complex activity as demonstrated by the results of coimmunoprecipitation and immunofluorescence assays. An investigation to assess the possible relationship between autophagy and G2/M arrest showed that, similar to the autophagy inhibitor 3MA, Beclin 1 knockdown delayed IR-induced G2/M arrest. Furthermore, the interactions between Beclin 1 and several G2/M checkpoint-related proteins, namely, PLK1 and CDC25C, were observed to increase. In addition, we observed that both 3MA and Beclin 1 inhibition decreased IR-induced apoptosis. Regarding the potential mechanism associated with this phenomenon, we showed that IR induced the interaction between Beclin 1 and Tip60 as well as their redistribution. Furthermore, we demonstrated that Beclin 1 T57 may be a targeted phosphorylation site for ATM.

Conclusions: In the present study, we demonstrate the crucial and intricate roles of Beclin 1 in IR-induced autophagy, G2/M cell cycle arrest, and apoptosis. Additionally, Tip60 and ATM were identified as important molecular regulators of Beclin 1. Our findings show the precise mechanism of crosstalk between IR-induced autophagy and G2/M cell cycle arrest.

1. Introduction

Autophagy is an evolutionarily conserved catabolic process that protects cells from a wide variety of stresses, such as nutrient starvation, organelle damage, and protein aggregation [1–3]. Autophagy involves the sequestration of cytoplasmic “cargo”, such as intracellular proteins, lipids, and organelles, into a phagophore and their subsequent delivery to the lysosomal compartment. The resulting breakdown products, such as amino acids, nucleosides, carbohydrates, and fatty acids, provide substrates for subsequent recycling, thus maintaining cellular metabolism [4]. Autophagy is an important cellular housekeeping mechanism and is crucial for maintaining cell homeostasis. Accordingly, many cancer cell types upregulate or downregulate autophagy [5], which functions to support tumorigenesis, metabolism, and resistance to therapy.

Recently, autophagy was reported to be an important player in the DNA damage response to genotoxic stress. Several DNA damage response (DDR) proteins, such as ATM, DNA-PKcs, p53, PARP-1, and Tat-

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interactive protein 60-kDa (Tip60) have been suggested to play significant roles in DNA damage-induced autophagy [6]. Autophagy was also demonstrated to play roles in cellular responses to DNA damage, such as cell cycle regulation and apoptosis. The cell cycle checkpoint is one of the fundamental mechanisms governing genome integrity. Upon DNA damage, cells activate checkpoints to initiate cell cycle arrest, allowing time for DNA repair. Successful repair leads to the subsequent release of cells from cell cycle arrest after the recovery of genome integrity. However, if DNA damage cannot be repaired, cells will either be permanently arrested until death or undergo cycling with genome instability. Recently, crosstalk between autophagy and the DDR has emerged as a topic of interest, particularly with regard to the underlying molecular mechanism and the roles of crosstalk in cell fate and the determination of other endpoints. Although a series of crucial players in the DDR have been identified as functioning significantly in autophagy, the detailed mechanism of this phenomenon remains largely unclear.

Beclin 1 was one of the first mammalian autophagy-related proteins to be identified and is thought to function during autophagy initiation [7]. Beclin 1 contains three structural domains: a BH3 domain at the N-terminus that primarily binds to BCL2, a central coiled-coil domain that binds to ATG14 and UVRAG, and an evolutionarily conserved domain that binds to PIK3C3 [8]. Beclin 1 is a component of the PtdIns3K complexes that participate in phagophore nucleation and elongation [9]. In addition, Beclin 1 recruits the class III phosphatidylinositol (PI) 3-kinase VPS34, which is one of the elements in a complex with VPS15/ATG14L, to generate phosphatidylinositol 3-phosphate (PI(3)P) by phosphorylating the 3-position of PI [10]. PI(3)P recruits proteins with PI(3)P-binding domains to modulate intracellular trafficking and autophagosome formation [11]. Intriguingly, studies have shown that Beclin 1 plays a role in mitosis [12] and that it bridges autophagy, apoptosis, and cellular differentiation [13]. However, the autophagic roles of Beclin 1 and its role in the DDR remain a major research focus, and the exact mechanism by which it acts as a bridge between autophagy, the cell cycle, and apoptosis has yet to be fully elucidated. In this study, we describe a distinct bridging role for Beclin 1 in the crosstalk between ionizing radiation (IR)-induced autophagy and cell cycle arrest.

2. Materials and methods

2.1. Antibodies and reagents

All antibodies were commercial products. Anti-Myc (9E10), anti-actin (H-6), anti-HA, anti-Beclin 1(G-11), anti-GAPDH (9B3), anti-pan14-3-3 (B-8), and anti-Bcl2 (4C11) antibodies were purchased from Santa Cruz. Anti-LC3, anti-SQSTM1/p62, and anti-pChk2 (Thr68) antibodies were obtained from Cell Signaling. Anti-Flag (F3165) and 3-MA (M9281) antibodies were purchased from Sigma. The secondary antibodies, namely, HRP-conjugated anti-rabbit IgG (H + L) and HRP-conjugated anti-mouse IgG (H + L), were purchased from Zhongshan Golden Bridge Biotechnology. The protein A/G beads (17-0963-020) were purchased from GE Health Care, and the NP40 solution (85124) was purchased from Thermo.

2.2. DNA constructs

Wild-type Beclin 1 and the T57A mutant were obtained by PCR amplification and subcloning into GST vectors. The recombinant Beclin 1 proteins (WT and T57A) were expressed and purified as described in our previous study [6]. Monomeric RFP (mRFP)-Beclin 1 was obtained by PCR amplification and was subcloned into the vector pEGFP. The Beclin 1, PIK3R4, PIK3C3, ATG14L, PLK1, CDC25C, CDC25A, AURKB, CCNB1, and Tip60 constructs were all generated by PCR amplification and subcloning into the vector pCMV. ShRNA against Beclin 1 (sense: gatccTGA GGATGACAGTGAACAGcttcctgtcagaCTGTTCACTGTCATCCTCAttttg; antisense: aattcaaaaaTGAGGATGACAGTGAACAGtctgacaggaagCTGTTCACT GTCATCCTCAg) was cloned into the pGreenPuro™ shRNA Cloning and Expression Lentivector (Cat.# SI505A-1). All of the constructs were confirmed by sequencing.

2.3. Cell culture, transfection, and ionizing radiation

HEK293T and HeLa cells were cultured in high-glucose (25 mM) Dulbecco's modified Eagle's medium (Gibco 12800-017) supplemented with 10% fetal bovine serum (HyClone, SN30087.02). The cells were maintained in a humidified incubator with 5% CO_2 at 37 °C. The Shcontrol and ShBeclin 1 cells were generated from HeLa cells via stable transfection with the constructed and confirmed shRNA vectors. Colonies that were resistant to puromycin (2 μg/ml) were isolated and analyzed by western blotting. For the transient expression of proteins, HEK293 cells were transfected with the recombinant DNA plasmids using Lipofectamine 2000 (Invitrogen). The transfected HEK293 cells were harvested and analyzed 36 h after transfection. Cells were irradiated using ^{60}Co γ-rays at a dose rate of 1.98 Gy/min.

2.4. Coimmunoprecipitation

For coimmunoprecipitation (co-IP), 293 T cells were transfected with the above plasmids. The cells were harvested after 36 h of transfection and lysed with lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40 (NP-40), 0.1% SDS, and 1 mM DTT, pH 7.5) supplemented with a protease inhibitor cocktail (Roche) at 4 °C for 1 h. After centrifugation at 13,000 rpm for 10 min, the supernatant was subjected to IP via incubation with the indicated antibodies and was then cleared with Protein A/G-agarose beads for 3 h at 4 °C. The beads were washed with lysis buffer three times, boiled for 5 min and analyzed by western blotting.

2.5. Western blot analysis

Cells were subjected to ^{60}Co γ-ray irradiation, collected at the indicated time points and then lysed in NP-40 supplemented with a protease inhibitor cocktail (Roche). Equal amounts of protein from every sample were subjected to SDS-PAGE and then transferred onto a nitrocellulose membrane. The membranes were blocked with 5% milk powder in TBST (20 mM Tris-HCl, 500 mM NaCl, and 0.1% Tween 20 (*v*/v), pH 7.5) for 1 h at room temperature and then incubated with the indicated antibodies overnight. The membranes were subsequently washed with TBST three times and incubated with the appropriate secondary antibody for 1 h at room temperature. After another three washes with TBST, the proteins were visualized using Imagequant LAS500 (GE).

2.6. Immunofluorescence staining

For immunofluorescence staining, after irradiation for the indicated time points, cells were fixed in cold 4% paraformaldehyde overnight at 4 °C. The fixed cells were then permeabilized in PBS containing 0.25% Trion X-100 for 30 min, washed with PBS 3 times at room temperature, and then blocked with 1% BSA in PBS for 30 min. The cells were incubated with the indicated primary antibodies for 12 h at 4 °C, washed with PBS 3 times, and then were incubated with AlexaFlour 568-goat anti-rabbit IgG and AlexaFlour 488-goat anti-mouse IgG for 1 h at room temperature. Subsequently, the cells were washed with PBS 3 times, stained with DAPI (to visualize the DNA) at room temperature, and observed using an LSM 510 laser scanning confocal microscope (Zeiss).

2.7. In vitro ATM kinase assay

For the ATM kinase assay, HEK293T cells were collected 1 h after ^{60}Co γ-ray treatment and then immunoprecipitated with the ATM antibody. The cells were lysed in TGN buffer (50 mM Tris, 50 mM glycerophosphate, 150 mM NaCl, 10% glycerol, 1% Tween-20, 1 mM NaVO4, 1 mM DTT, and 1× protease cocktail inhibitor, pH 7.5). The immune complexes were washed twice with TGN buffer, twice with LiCl buffer (50 mM Tris-HCl and 0.5 M LiCl, pH 7.5), and twice with ATM kinase buffer (10 mM HEPES, 50 mM glycerophosphate, 50 mM NaCl, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 μM ATP, and 1 mM DTT, pH 7.5). The beads were split into two equal parts and resuspended in 30 μl of kinase buffer. Two micrograms of GST-Beclin 1 WT, GST-Beclin 1 T57A, or GST-P53 and 10 μCi 32P-ATP were mixed together and incubated at 30 °C for 30 min. The reaction was stopped by adding 30 μl of SDS sample buffer and boiling for 5 min. Samples were resolved by SDS-PAGE, and the radioactive signal was detected by autoradiography [14].

2.8. Apoptosis assay

Cells were plated in six-well plates and treated with the indicated doses of γ-ray irradiation. Twenty-four hours later, the cells were collected, washed three times in PBS and stained using an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences, San Diego, CA, USA) following the manufacturer's protocol. The cells were then detected by flow cytometry (BD FACS Canto) and analyzed using FCS Express v2.0.

2.9. Flow cytometric analysis of the cell cycle

Cells were harvested and fixed in 75% ethanol either immediately or at the indicated times after 4 Gy of γ-ray irradiation. The cells were subsequently resuspended in PBS containing 0.1% saponin and 1 μg/ml RNase A (Sigma, St. Louis, MO, USA), incubated for 20 min at 37 °C, and stained with 25 μg/ml propidium iodide (PI) (Sigma). The cell cycle distribution was analyzed via flow cytometry, and > 10,000 cells per sample were counted.

2.10. Double thymidine deoxyribonucleoside (TdR) block

Shcontrol or ShBeclin 1 cells in the logarithmic growth phase were selected for analysis. For the first blocking step, cells were treated with 2 mmol/L TdR for 16 h. After three washes with fresh medium, the cells were incubated in TdR-free medium for 9 h. Subsequently, the cells were treated with 2 mmol/L TdR for another 16 h, which constituted the second blocking step. The double-blocked cells were released by washing twice with fresh medium and were subsequently incubated in TdR-free medium containing 10% fetal bovine serum. The cells were harvested at the indicated time points and detected by flow cytometry.

2.11. Statistical analysis

The results of statistical evaluations are presented as the means ± SD (standard deviation). The data were analyzed using Student's *t*-test, one-way ANOVA, and the X^2 test using SPSS (SPSS Statistics for Windows, Version 19.0. Chicago: SPSS Inc., USA) to assess statistical significance, with $P < .05$ was considered significant.

3. Results

3.1. Beclin 1 inhibition reduces IR-induced autophagy

Although Beclin 1 has been identified as a key regulator of autophagy, the exact mechanism by which it controls IR-induced autophagy remains controversial. To investigate the involvement of Beclin 1 in IR-induced autophagy, we constructed Beclin 1 knockdown HeLa cell lines through shRNA transfection. As shown in Fig. 1A, compared to the Shcontrol cells, the level of Beclin 1 expression in the ShBECN 1–2 cells was reduced by > 95%. Therefore, we selected this cell line (designated ShBECN 1) for further study. To evaluate the dynamic role of Beclin 1 in IR-induced autophagy, we exposed cells to 4 Gy IR and collected them at the indicated time points. Subsequently, the levels of the classic autophagy markers MAPLC3 and SQSTM1/p62 were examined (Fig. 1B). MAPLC3 is the mammalian autophagic protein that localizes to the autophagosome membrane as well as to the cytosol, therefore, detection of a punctuated pattern of cytosolic MAPLC3 indicates involvement of MAPLC3 in autophagosome formation, a phenomenon used to monitor autophagy. The conversion of MAPLC3-I to MAPLC3-II is the marker for the formation of autophagosome. IR increased the LC3-II/LC3-I ratio, which reached its peak level at 4 h and returned to the baseline level at 8 h. Compared with Shcontrol cells, the fold-change of LC3-II/LC3-I ratio was lower in the IR-treated ShBECN 1 cells, especially at 4 h after IR (Fig. 1C). To further investigate if the increase of autophagosomes was accompanied by a change of total protein degradation, endogenous p62/SQSTM1 was examined. As an autophagy-lysosome substrate, p62 protein is associated with protein aggregates in a number of aggregation diseases, and there is a general correlation between the inhibition of the autophagy-lysosomal protein degradation and increased levels of p62. The decrease of p62 was observed from 0.25 h to 4 h after IR and then begun to increase. Compared with Shcontrol group, the level of SQSTM1/p62 was higher in ShBECN 1 cells (Fig. 1D). The lower LC3-II/LC3-I ratio and the higher SQSTM1/p62 level together in ShBECN 1 cells suggested that Beclin 1 plays an important role in IR-induced autophagy.

3.2. IR enhances Beclin 1/ PIK3CIII complex activity

Beclin 1 has been reported to interact with PIK3C3-PIK3R4 to form protein complexes that play a key role in autophagy regulation [15]. Additionally, ATG14L, together with Beclin 1, synergistically promotes the formation of double-membraned organelles and is also important for autophagy activation [16]. To investigate whether IR affected Beclin 1/PIK3CIII complex activity and the association between Beclin 1 and ATG14L, we cotransfected plasmids encoding Beclin 1 with those encoding PIK3R4, PIK3C3, or ATG14L. Subsequently, co-IP experiments were performed at either 15 min or 1 h after 4Gy IR exposure. As anticipated, the results showed that the interaction between Beclin 1 and PIK3R4 was enhanced after IR treatment, especially at 15 min (Fig. 2A). A similar augmented interaction was observed between Beclin 1 and PIK3C3 (Fig. 2B). As for ATG14L, cotransfection with Beclin 1 significantly increased the level of ATG14L expression. In addition, the interaction between Beclin 1 and ATG14L was enhanced at 1 h after IR (Fig. 2C). These results suggest that IR regulates autophagy by enhancing Beclin 1/PIK3CIII complex activity and the association between Beclin 1 and ATG14L.

In addition, we monitored the colocalization between Beclin 1 and GFP-LC3 by immunofluorescence. As shown in sFig 1A, IR induced autophagy. And the colocalization of Beclin 1/GFP-LC3 was observed after IR. 2FYVE, a PtdIns3P-binding protein, is recruited to the endoplasmic reticulum (ER) by PIK3C3-produced PtdIns3P to initiate omegasome formation. GFP-PHOSBP and GFP-PHPLCδ1 were used to monitor the Golgi PtdIns(4)P and PtdIns(4,5)P2 in the plasma membranes of mammalian cells [17]. As shown in sFig 1B-1D, the colocalization of Beclin 1/2FYVE was observed after IR, but no colocalization of Beclin 1 was detected with GFP-PHOSBP or GFP-PHPLCδ1. These results demonstrating that IR induces autophagy also suggest that Beclin 1 plays an important role in PIK3C3 activity.

3.3. IR-induced G2/M arrest is delayed by the autophagy inhibitor 3MA

To elucidate the possible relationship between autophagy and G2/M (mitosis) arrest, we next examined the effects of the autophagy inhibitor 3MA on IR-induced G2/M arrest. Surprisingly, a remarkable delay in G2/M arrest was observed in the 3MA-pretreated cells (Fig. 3A). As shown in Fig. 3B and C, the percentage of G2/M arrested cells observed for the 3MA-treated cells was lower than that observed

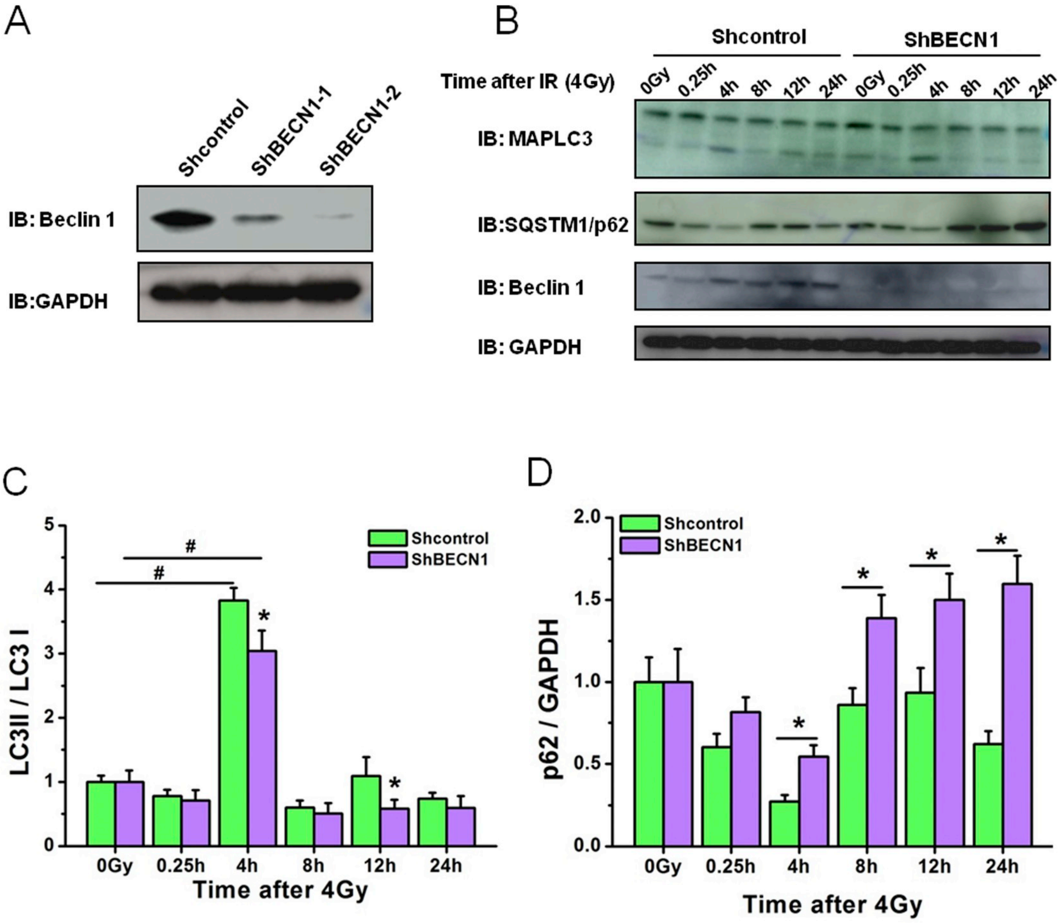

Fig. 1. Role of Beclin 1 in IR-induced autophagy.

A. *HeLa* cells were stably transfected with either ShBECN 1–1 or ShBECN 1–2. Shcontrol was used as negative control. Individual clones were obtained under puromycin selection. The knockdown effects were confirmed by western blotting. **B**. Isogenic cell lines were treated with mock (0 Gy) or IR (4 Gy). Cell lysates were harvested at the indicated time points and subjected to western blotting. The level of LC3B lipidation and SQSTM1 degradationwere detected. GAPDH was used as an internal protein loading control. **C**. The relative levels of LC3-II/LC3-I quantified from (**B**) are shown in the bar graph. The ratio was normalized by each value in mock (0 Gy) group. **D**. Quantification of the immunoblotting results of SQSTM1 degradation from (**B**) are shown in the bar graph, which was represented as means ± SD of SQSTM1/GAPDH. The above data were repeated > 3 times and quantified using ImageJ. $^{*}P < .05$ versus the Shcontrol group, and $^{\#}P < .05$ versus the mock IR (0 Gy) group.

for the control cells at every time point after IR. In addition, the percentage of G2/M arrested cells peaked at 12 h after IR for the control cells but did not peak until 24 h after IR for the 3MA-pretreated cells. This result indicates that there is indeed a close association between autophagy and G2/M arrest, at least under IR exposure.

To confirm whether 3MA delayed G2/M arrest by inhibiting ROS production, we pretreated cells with an ROS scavenger. Neither Edaravone nor NAC delayed G2/M arrest in a manner that was similar

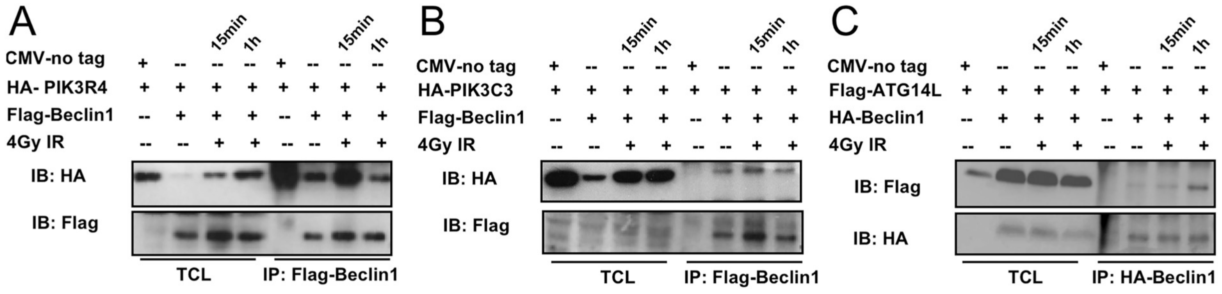

Fig. 2. Beclin 1 regulation of IR-induced autophagy.

A. HEK293T cells were cotransfected with plasmids encoding Flag-Beclin 1 and HA-PIK3R4. CMV-no tag served as the negative control. Thirty-six hours after transfection, the cells were treated with 4 Gy IR and were subsequently harvested at both 15 min and 1 h after IR. Partial of the total cell lysate were collected to detect basic protein expression level. The remained cell lysatewas immunoprecipitated by Flag antibody and then immunoblotted with both HA antibody and Flag antibody. The expression of Flag was used as loading control. IP, immunoprecipitation; TCL, total cell lysate. The similar interactions of Beclin 1 with PIK3C3 (**B**) or ATG14L (**C**) were also detected. The abbreviations includes PIK3R4 (Phosphoinositide-3-Kinase Regulatory Subunit 4), PIK3C3(Phosphatidylinositol 3-Kinase Catalytic Subunit Type 3), and ATG14L (Autophagy Related 14). Fig. 3. Delay of IR-induced G2/M arrest by the autophagy inhibitor 3-MA.

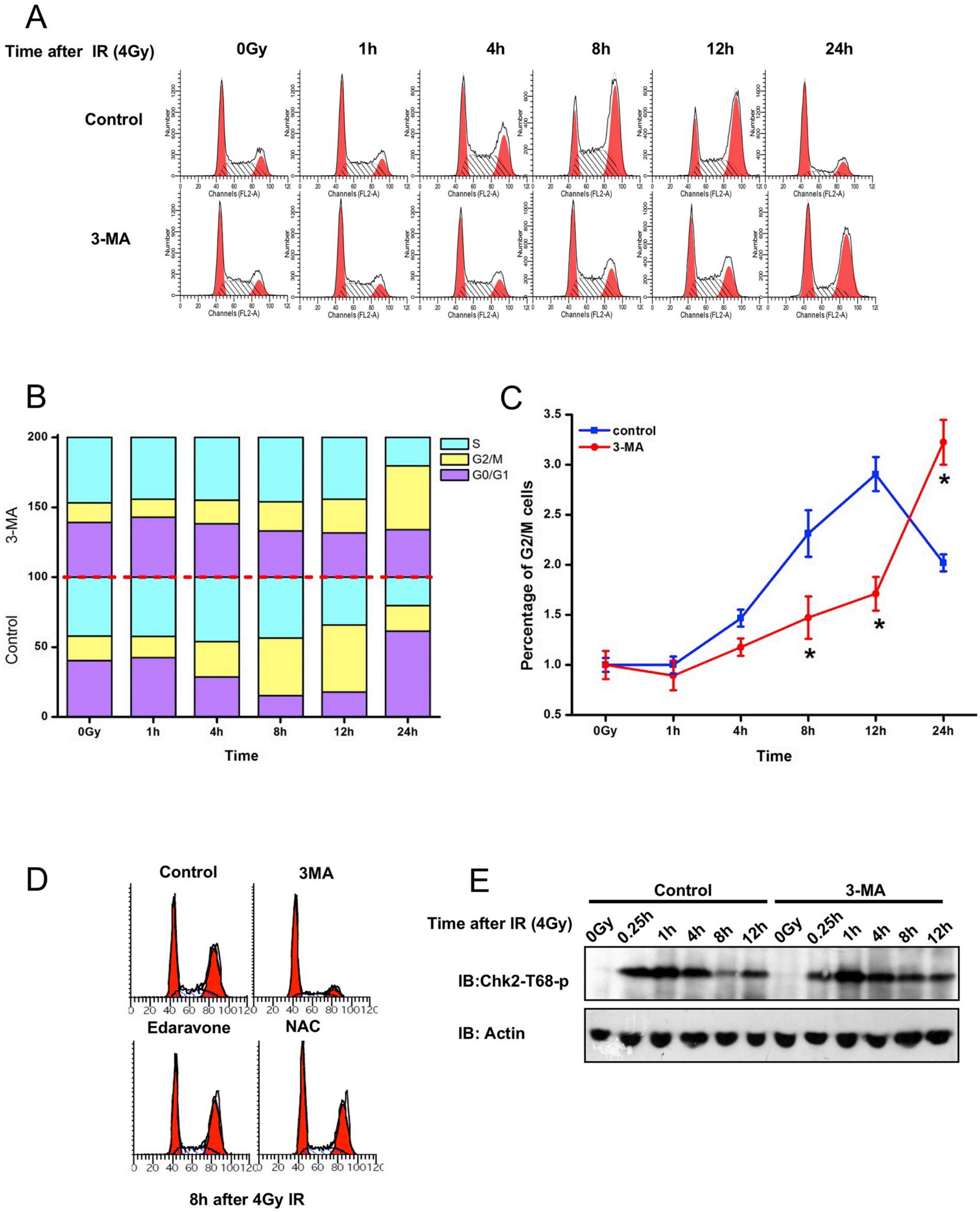

Fig. 3. Delay of IR-induced G2/M arrest by the autophagy inhibitor 3-MA.

A HeLa cells were pretreated with 3-MA (2 mM) for 2 h and subsequently exposed to 4Gy γ-ray irradiation. The cells were harvested at the indicated time points and then analyzed via flow cytometry. Representative histograms are shown. Cells treated with DMSO were used as a negative control. The mock IR group was not exposed to the γ-ray irradiation, labelled as 0 Gy. **B**. Quantitative data of (**A**) shows the proportion of cells in the G2/M, S or G0/G1 phases at different times after irradiation. The bottom set shows the control group while the top set shows the 3-MA group. **C**. The line graph specificly indicates the proportion of cells at the G2/M checkpoint from (**A**). The data are the means ± SD from three independent experiments. $^*P < 0.05$ versus the control group. **D**. HeLa cells were separately pretreated with 3-MA, Edaravone, and NAC. The cells were harvested at 8hafter 4-Gy γ-ray irradiation. Representative histograms are shown. **E**. Cells are pretreated with 3-MA or DMSO. The phosphorylation of Chk2 (T68) was measured at the indicated time points after 4-Gy irradiation. Actin was used as a loading control

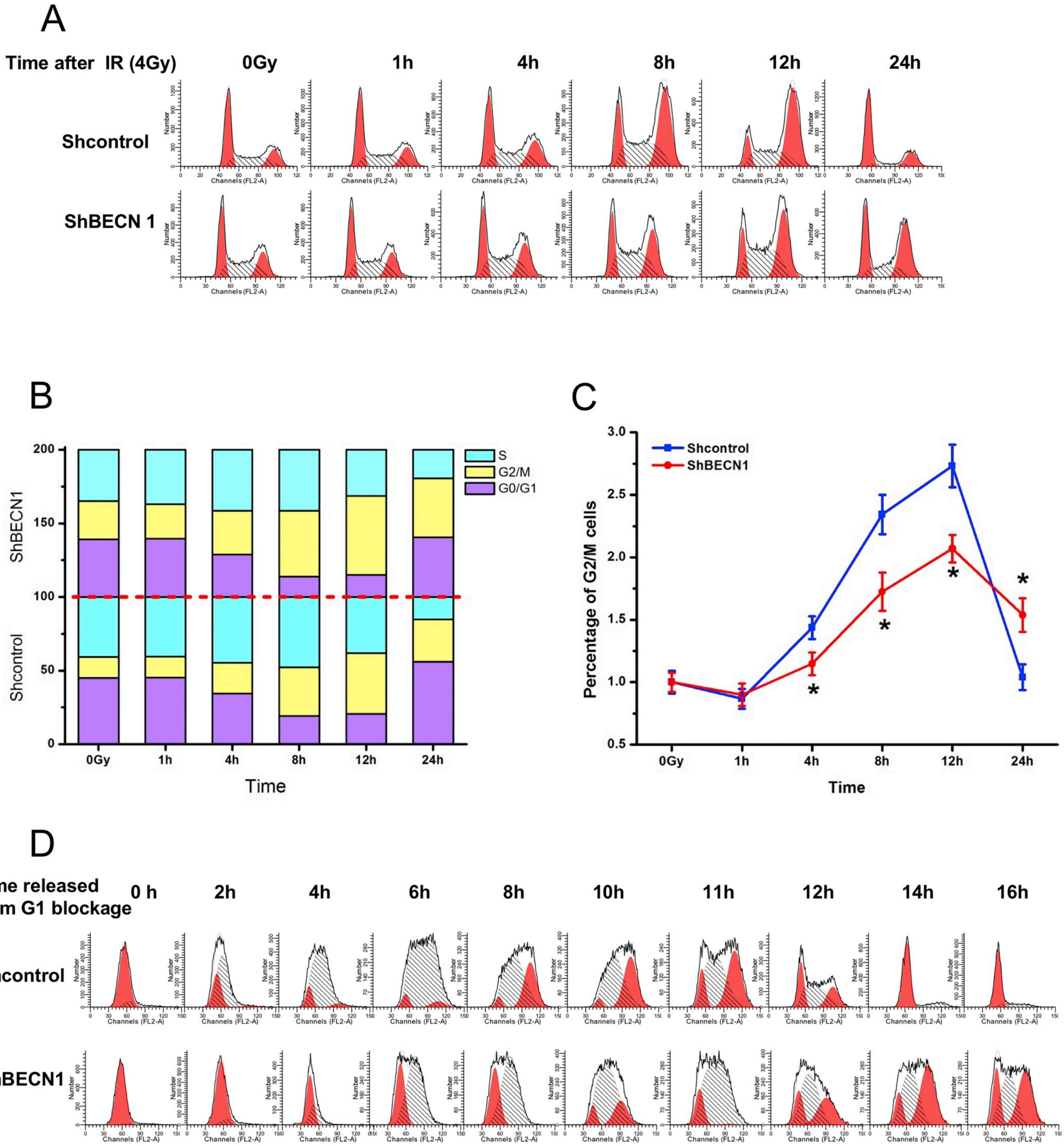

Fig. 4. Knockdown of Beclin 1 delays IR-induced G2/M arrest.

A. Both Shcontrol and ShBECN 1 stable Hela cell lines were exposed to 4Gy γ-ray irradiation. The cells were harvested at the indicated time points and then analyzed via flow cytometry. Representative histograms are shown. The mock IR group (0Gy) was not exposed to the γ-ray irradiation. **B**. Quantitative data of (**A**) shows the proportion of cells in the G2/M, S or G0/G1 phases at different times after irradiation. The bottom set shows the Shcontrol group while the top set shows the ShBECN 1 group. **C**. The line graph specificly shows the proportion of cells at the G2/M checkpointfrom (A). The data are the means ± SD from three independent experiments. $^{*}P < .05$ versus the Shcontrol group. **D**. The Shcontrol and ShBECN 1 Hela cells in the logarithmic growth period were submitted to the 2 mmol / L TdR for 16 h. After twice PBS washing, cells were then changed to be cultured in normal medium for 9 h. After another twice PBS washing, cells were again submitted to the 2 mmol / L TdR for anther 16 h. After twice PBS washing, cells were again cultured in normal medium.The cells were harvested at the indicated time points and detected via flow cytometry. Representative histograms are shown.

to that observed when cells were treated with 3MA (Fig. 3D). ATM is the most crucial kinase in the regulation of cell cycle progression under conditions of DNA damage. To explore whether 3MA delays G2/M arrest by affecting ATM activity, we evaluated the phosphorylation of its substrate, Chk2, at the T68 site. However, no inhibitory effect was observed according to the western blot results (Fig. 3E). Thus, these results indicate that some other mechanisms must contribute to 3MA-mediated G2/M arrest.

3.4. Beclin 1 inhibition delays IR-induced G2/M arrest

As an autophagy inhibitor, 3MA primarily targets the Beclin 1/PIK3CIII complex, which motivated us to determine whether Beclin 1 is the crucial protein that links autophagy and the cell cycle under IR exposure. To examine the involvement of Beclin 1 in cell cycle progression, we measured cell cycle progression in ShBECN 1 and Shcontrol cells. As anticipated, similar to 3MA, Beclin 1 knockdown delayed IR-induced G2/M arrest (Fig. 4A, B, and C). These results suggest that Beclin 1 knockdown impairs the progression through G2/M. To differentiate between G2 and mitotic delay, we analyzed the ability of cells depleted of Beclin 1 to resume mitosis following release from double TdR block. The percentage of G2/M cells for the Shcontrol cells peaked at 11 h after the block, whereas in the Beclin 1 knockdown cells, the percentage of G2/M cells did not peak until 16 h after the block (Fig. 4D). The above results confirmed our hypothesis that Beclin 1 plays a crucial role in IR-induced G2/M arrest.

3.5. Beclin 1 interacts with a group of G2/M checkpoint-related proteins

To gain a better understanding of the specific mechanisms underlying the involvement of Beclin 1 in IR-induced G2/M arrest, we examined the interactions between Beclin 1 and G2/M checkpoint-related proteins. As is commonly known, PLK1 (Polo-Like Kinase 1), CDC25C (Cell Division Cycle 25C), AURKB (Aurora Kinase B), and CCNB1 (Cyclin B1) are essential for the control of the cell cycle at the G2/M transition. The interaction between Beclin 1 and PLK1 was observed to increase after IR, as demonstrated by the observed co-IP of either protein shown in Fig. 5A. At the same time, IR also enhanced the interaction between Beclin 1 and CDC25C when Beclin 1 was immunoprecipitated (Fig. 5B). When CDC25C was immunoprecipitated, the interaction appeared to decrease, but when the different levels of CDC25C were taken into consideration, the level of Beclin 1 that coimmunoprecipitated with CDC25C was still increased (Fig. 5B). CDC25A is required for the progression from the G1 to the S phase of

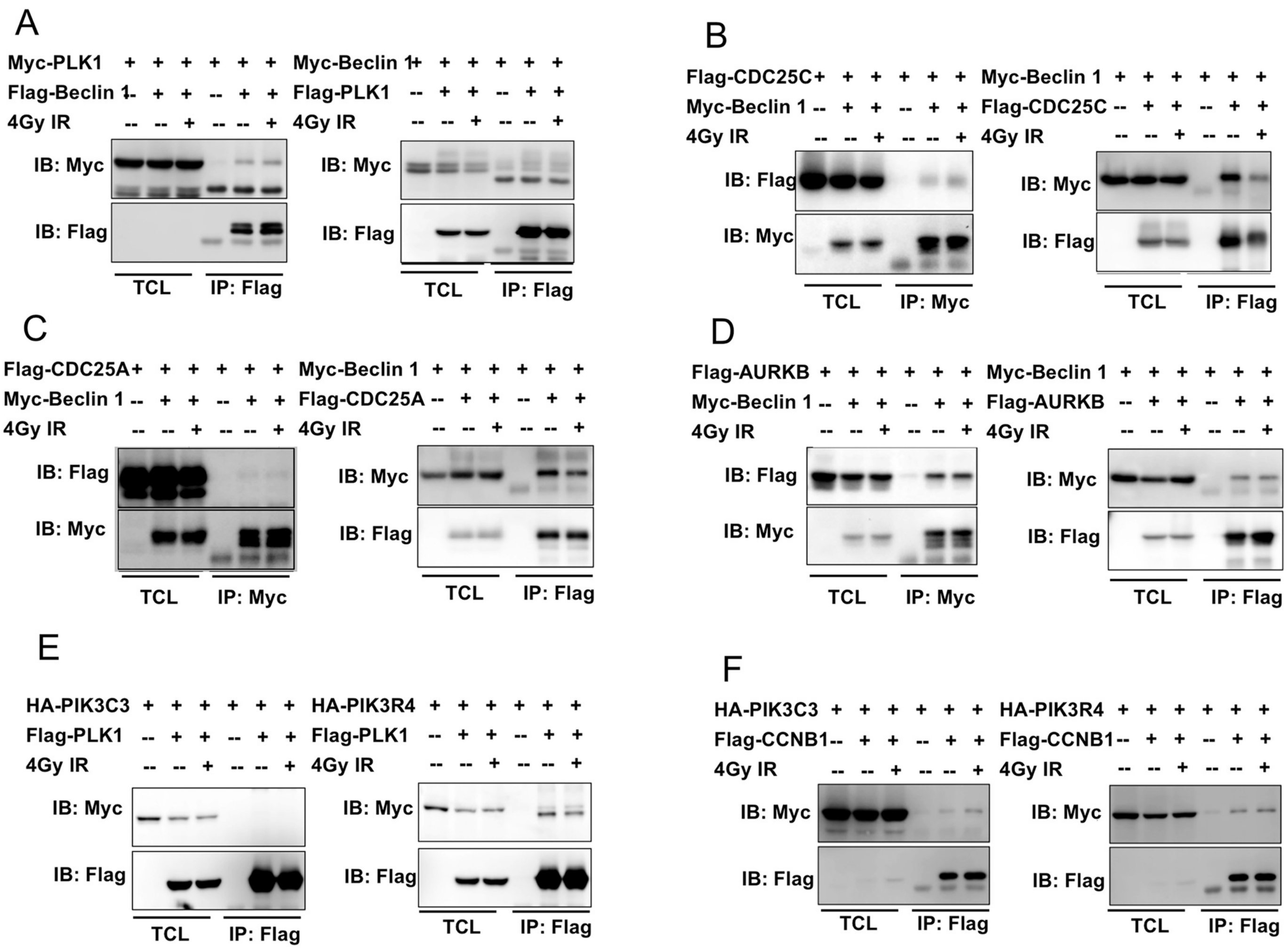

Fig. 5. Interactions between Beclin 1 and G2/M checkpoint-related protein.
A. HEK293T cells were cotransfected with plasmids encoding Flag-Beclin 1 and Myc-PLK1. Thirty-six hours after transfection, the cells were treated with 4 Gy IR. The cells were subsequently harvested at 1 h after IR. Partial of the total cell lysate were collected to detect basic protein expression level. The remained cell lysate was immunoprecipitated by Flag antibody and then immunoblotted with both Myc antibody and Flag antibody. The expression of Flag proteins was used as a loading control. IP, immunoprecipitation; TCL, total cell lysate. Similarly, the following interactions were also detected: Beclin 1 with CDC25C (**B**), Beclin 1 with CDC25A (**C**), Beclin 1 with AURKB (**D**), PIK3C3/PI3KR4 with PLK1 (**E**), and PIK3C3/PI3KR4 with CCNB1 (**F**). The abbreviations includes PLK1 (Polo-Like Kinase 1), CDC25C (Cell Division Cycle 25C), AURKB (Aurora Kinase B), and CCNB1 (Cyclin B1).

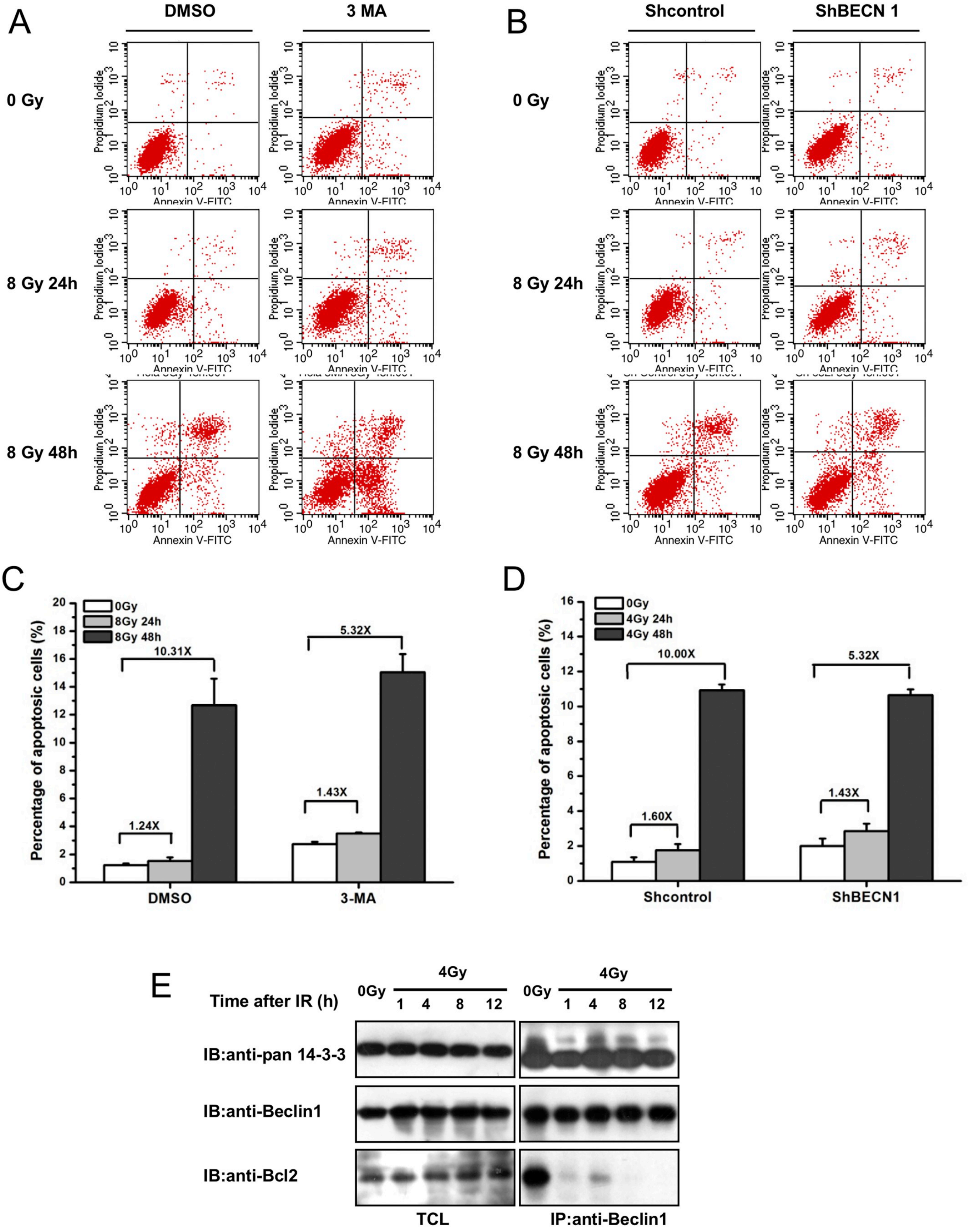

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Fig. 6. Role of Beclin 1 in the crosstalk between IR-induced autophagy and apoptosis.
A. *HeLa* cells were pretreated with 3-MA (2 mM) for 2 h and subsequently exposed to 8Gy γ-ray irradiation. The cells were harvested at 24 h and 48 h after IR. The cells were stained with PI and Annexin V-FITC, then analyzed apoptosis level via flow cytometry. Positive-stained cells were counted using FAC Scan. Representative histograms are shown. Cells treated with DMSO were used as a negative control. The mock IR group was not exposed to the γ-ray irradiation, labelled as 0 Gy. **B**. Both Shcontrol and ShBECN 1 stable cell lines were exposed to 8Gy γ-ray irradiation. The cells were harvested at 24 h and 48 h after IR. The cells were stained with PI and Annexin V-FITC, then analyzed apoptosis level via flow cytometry. Positive-stained cells were counted using FAC Scan. Representative histograms are shown. **C**. Quantitative analysis of (A). Data are presented as the means ± SD of three independent experiments. **D**. Quantitative analysis of (B). Data are presented as the means ± SD of three independent experiments. **E**. Cells were exposed to mock (0Gy) or IR (4 Gy) and collected at the indicated time points. The endogenic Beclin 1 was immunoprecipitated and immunoblotted with a pan14–3-3 or Bcl2 antibody. The expression of Beclin 1 was used as a loading control. IP, immunoprecipitation; TCL, total cell lysate.

the cell cycle. We also examined the relationship between Beclin 1 and CDC25A (Fig. 5C). Weak signals were detected in the Beclin 1 IP, while a slight decrease was observed in the CDC25A IP. No obvious difference was observed between cells without IR exposure and those with IR treatment in terms of the Beclin 1/AURKB interaction, either for the Beclin 1 IP or the AURKB IP (Fig. 5D). To further assess the possible function of the Beclin 1/PIK3CIII complex in G2/M arrest, we characterized the relationship between the G2/M checkpoint-related proteins and PIK3C3 or PIK3R4. In contrast to the observed Beclin 1/PLK1 interaction, no interaction was observed between PIK3C3 and PLK1 with or without IR pretreatment, while an observed PIK3R4/PLK1 interaction was slightly reduced by IR exposure (Fig. 5E). As a crucial controller of the G2/M transition, CCNB1 was selected to elucidate the associated mechanism. Both PIK3C3 and PIK3R4 interacted with CCNB1, and these interactions were further enhanced by IR (Fig. 5F). From the above co-IP results, we concluded that IR accelerates the interactions between Beclin 1 and a group of G2/M checkpoint-related proteins and that these interactions may be the mechanism of crosstalk between IR-induced autophagy and G2/M arrest.

3.6. Beclin 1 involvement in the crosstalk between IR-induced autophagy and apoptosis

The relationship between autophagy and apoptosis has always been controversial. In this study, we assessed whether Beclin 1 plays a role in the crosstalk between IR-induced autophagy and apoptosis. As is shown in Fig. 6A-D, 3MA pretreatment itself affected the percentage of late apoptotic cells (2.74% vs 1.23%), similar results was observed in Beclin1 silencing (2.00% vs 1.09%). That is to say, basal level of apoptosis was different in the various groups of cells. Aiming to compare variation in IR-induced apoptosis change, we took this into consideration. All changes were divided by the previous basal level, to better convey function of IR, which was represented as the fold change of after IR/before IR. As shown in Fig. 6A and C, in DMSO-pretreated control cells, IR increased apoptosis by 1.24- and 10.31-fold at 24 and 48 h, respectively. With regard to the 3MA-pretreated cells, apoptosis increased by 1.43- and 5.32-fold at 24 and 48 h, respectively. If we focused on the apoptosis level at 48 h, compared to the DMSO control cells, 3MA inhibited IR-induced apoptosis increase to some degree (5.32- vs. 10.31-fold, respectively). In addition, a similar change was observed in Beclin 1 knockdown cells. As presented in Fig. 6B and D, inhibition of Beclin 1 decreased IR-induced apoptosis increase (5.32- vs. 10.00-fold, respectively).

The anti-apoptotic protein BCL2 has typically been considered to be crucial protein for elucidating the crosstalk between autophagy and apoptosis, because BCL2 binds to Beclin 1 and inhibits autophagy by disrupting the interaction between Beclin 1 and class III PtdIns3K [18]. This theory was further evaluated in this study. An obvious interaction between Beclin 1 and BCL2 was discovered prior to IR, whereas this interaction remarkably disappeared at 1 h after IR and then reappeared and increased by 4 h (Fig. 6E). With the exception of this result, we were surprised to discover the interaction between Beclin 1 and pan14–3-3. Similarly, the interaction decreased immediately after IR exposure and then began to reappear beginning at 4 h after IR. Pan14-3-3 proteins are a group of highly conserved proteins that are involved in many vital cellular processes, such as metabolism, protein trafficking, signal transduction, apoptosis, and cell cycle regulation. These results suggest that pan14-3-3 may be another regulator that links autophagy, apoptosis and cell cycle progression, at least under IR conditions. However, further research is necessary to elucidate the details of the specific mechanism, such as the type of pan14-3-3 protein that participates in this process.

3.7. IR induces the redistribution of Beclin 1 and Tip60

Tip60, also known as KAT5, belongs to the MYST family of histone acetyltransferases (HATs). Tip60 plays a role in many cellular processes, including chromatin remodeling, DNA repair, gene transcription, apoptosis, and tumorigenesis [19]. Tip60 has been shown to directly acetylate and stimulate ULK1 to elicit autophagy in response to either growth factor deprivation [20] or ER stress [21], in which the GSK3β-Tip60-ULK1 pathway is activated to regulate autophagy [20] [21]. However, whether Tip60 participates in IR-induced autophagy has not been determined. To evaluate the specific function of Tip60 under IR, we observed the interaction between Tip60 and Beclin 1 after IR. Surprisingly, a specific signal for myc-Tip60 was detected after IR when HA-Beclin 1 was immunoprecipitated, whereas no signal was observed in cells without IR or in the control group (Fig. 7A). However, when myc-Tip60 was immunoprecipitated, the interaction between HA-Beclin 1 and myc-Tip60 was detected in cells regardless of whether they were exposed to IR (Fig. 7A). These interactions indicate that Tip60 may regulate IR-induced autophagy by interacting with Beclin 1.

To further confirm this interaction, we evaluated their colocalization under IR treatment by immunofluorescence. As shown in Fig. 7B, before IR exposure, Tip60 was diffused throughout the nucleoplasm, which was similar to the distribution of Beclin 1. Moreover, Tip60 and Beclin 1 were strongly colocalized. However, after IR exposure, Beclin 1 was exported from the nucleus to the perinuclear region or the cytoplasm. In contrast, IR induced Tip60 translocation into punctuate subnuclear structures named nuclear bodies (NBs), and this observation is consistent with that reported for UV irradiation [22]. Despite redistribution, colocalization was detected at 15 min after IR but disappeared 1 h after IR. This change in distribution and interaction strongly suggest that Tip60 may regulate IR-induced autophagy and G2/M arrest through Beclin 1.

3.8. ATM phosphorylates Beclin 1 at T57

Tip60 has been reported to form a complex with ATM by binding to its C-terminal FATC region, which is essential for the HAT activity of Tip60. Moreover, IR induces the colocalization of the Tip60-ATM complex [19,23]. In this study, we identified a novel phosphorylation site in Beclin 1, the T57 site. Based on the alignment of consensus substrate phosphorylation motifs, T57 in Beclin 1 was predicted to be a potential phosphorylation site of ATM (Fig. 8A). T57 in human Beclin 1 is highly conserved among the majority of mammals, including *Pongo abelii*, *Macaca mulatta*, *Mus musculus*, *Bos taurus*, *Sus scrofa*, but this site is not conserved in lower species, such as *Gallus*, *Xenopus tropicalis*, *Danio rerio, and Drosophila melanogaster*.

To determine whether Beclin 1 is a substrate of ATM, we further

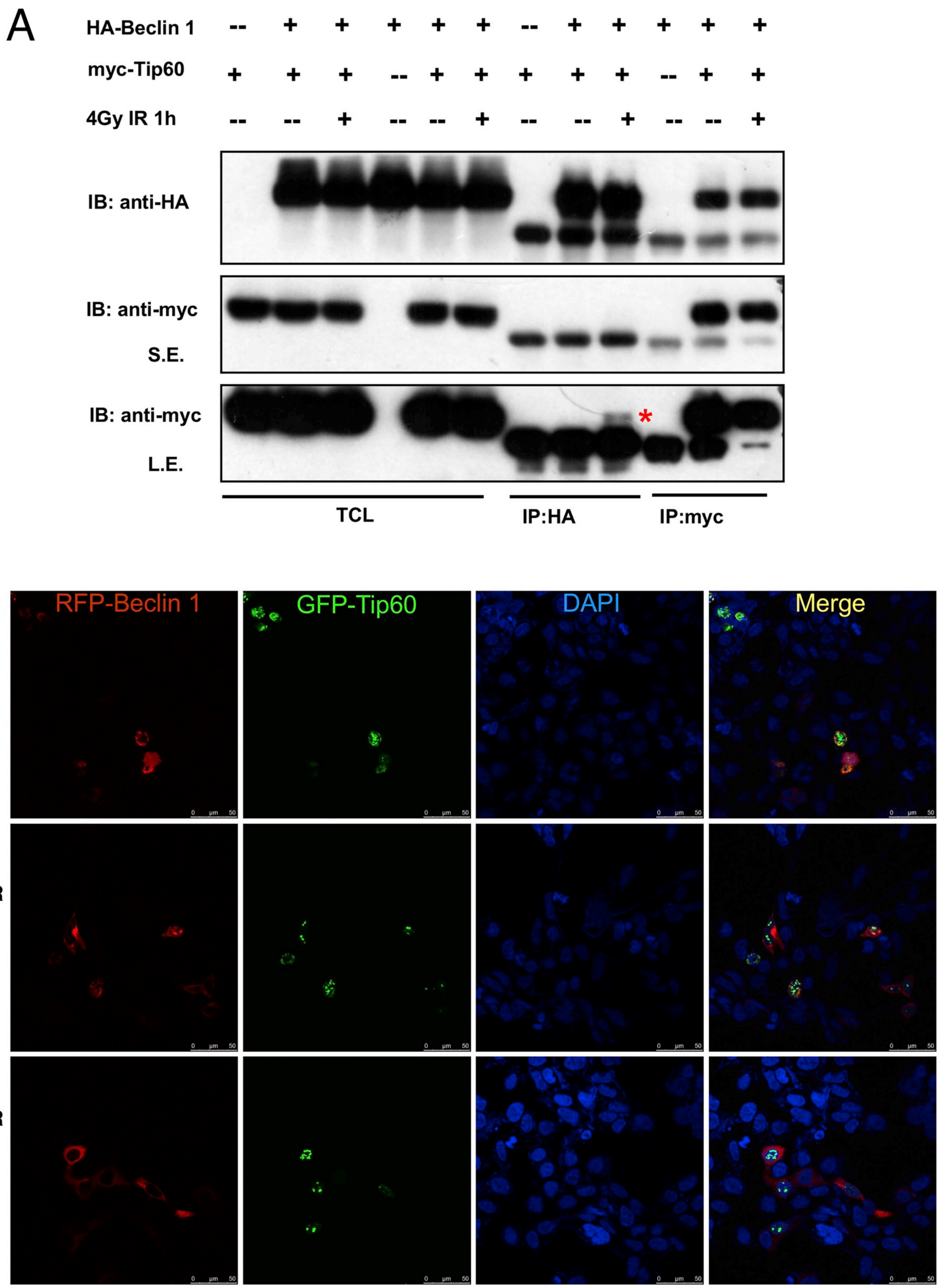

Fig. 7. Interaction between Tip60 and Beclin 1 after IR.

A. HEK293T cells were cotransfected with plasmids encoding HA-Beclin 1 and Myc-Tip60. Thirty-six hours after transfection, the cells were treated with 4 Gy IR. The cells were subsequently harvested at 1 h after IR. HA-Beclin 1 and Myc-Tip60 were separately immunoprecipitated and immunoblotted with an Myc or HA antibody. S.E: short exposure, L.E: longer exposure. **B**. HEK293T cells were cotransfected with plasmids encoding RFP-Beclin 1and GFP-Tip60. Thirty-six hours after transfection, the cells were treated with 4 Gy IR. Immunofluorescence staining image of colocalization between Beclin 1 and Tip 60 was observed using a confocal microscope.

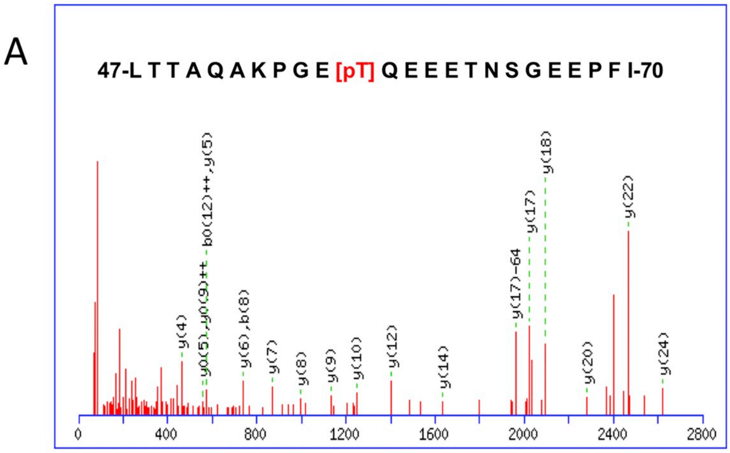

Table 1 The conservation of Thr 57 sites in Beclin 1

NO.	Entry	Organism	Sites	Motif	
1	Q14457	*Homo sapiens (Human)*	T57	* AQAKPGETQEEETNS	Mammals
2	Q5R878	*Pongo abelii (Sumatran orangutan)*	T57	* AQAKPGETQEEETNS	
3	F7HNU1	*Macaca mulatta (Rhesus macaque)*	T57	* AQAKPGETQEEETNS	
4	Q91XJ1	*Rattus norvegicus (Rat)*	S56	AQAKPGESQEEEANS *	
5	O88597	*Mus musculus (Mouse)*	T56	AQAKPGETQEEEANS *	
6	Q4A1L4	*Bos taurus (Bovine)*	T56	AQLKPGETQEEEANS *	
7	Q4A1L5	*Sus scrofa (Pig)*	T56	AQVKPGETQEEEANP *	
8	Q5ZKS6	*Gallus gallus (Chicken)*	NA	APARPGDAQEESALS *	Lower species
9	Q4A1L3	*Xenopus tropicalis (Western clawed frog)*	NA	VKPGDIQEVDSNIEE *	
10	F1RCP1	*Danio rerio (Zebrafish)*	NA	TANKQQDSGESSSFP *	
11	Q9VCE1	*Drosophila melanogaster (Fruit fly)*	NA	LDPQDASSFDHF VPP *	

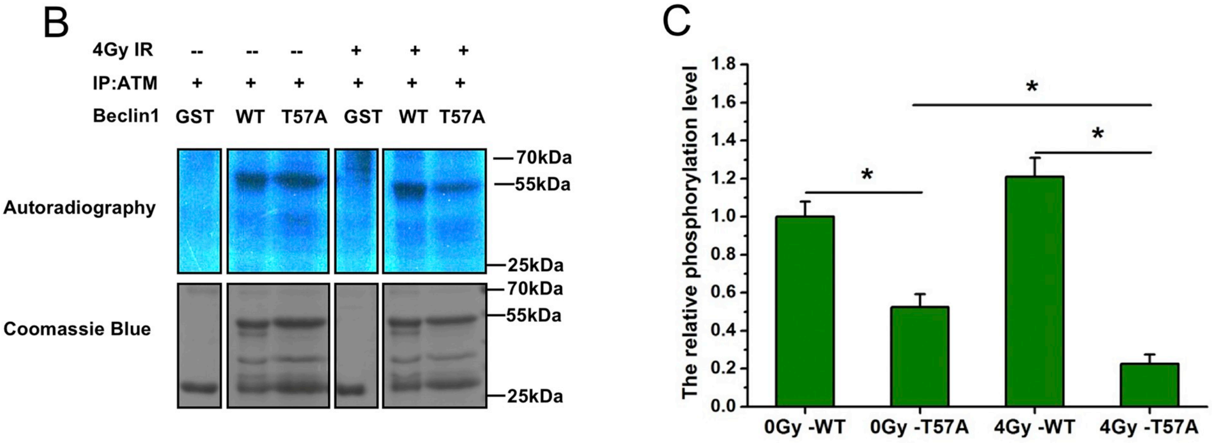

Fig. 8. ATM phosphorylates Beclin 1 at T57.

A. The mass spectrometric analysis was performed of a tryptic fragment, which matched to the peptide 47-LTTAQAKPGETQEEETNSGEEPFI-70. **Table 1 The conservation of Thr 57 sites in Beclin 1 from 11 species**. The conservation of Thr 57 sites in Beclin 1 was collected from 11 species, which included seven mammals and four lower species. **B.** The ATM kinase complex was immunoprecipitated from 293 T cells treated with or without IR. In the in vitro kinase assays, bacterially produced GST-P53, GST-Beclin 1 (WT), and GST-Beclin 1 (T57A) were incubated with ATM kinase complex in the presence of γ-^{32}P ATP. The proteins were resolved by SDS-PAGE and detected by autoradiography and Coomassie blue staining (CB). **C.** The quantification of relative levels of phosphorylated Beclin 1 in (**B**) is shown in the bar graph. * means $P < .05$ vesus two group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

assessed whether T57 is the exact phosphorylation site by performing in vitro kinase assays using ATM kinase immunoprecipitated from HEK293T cells that were exposed or unexposed to IR (Fig. 8B). To account for the different levels of Beclin 1-WT and Beclin 1-T57A expression, we evaluated the phosphorylation level by determining the ratio of the signal by autoradiography to that by Coomassie blue staining, and this ratio was then normalized to the phosphorylation level in the 0 Gy-WT group (Fig. 8C). Before IR exposure, the phosphorylation-defective mutation of Beclin 1 (T57A) abolished the incorporation of ^{32}P-ATP into Beclin 1 by ATM. However, after exposure to IR, the phosphorylation level was enhanced in the WT group but further abolished in the T57A group. Our data suggest that Beclin 1 T57 may be an ATM target phosphorylation site and that the function of the T57 site may be crucial upon IR exposure.

4. Discussion

In this study, we demonstrated the crucial roles of Beclin 1 in IR-induced autophagy, G2/M cell cycle arrest, and apoptosis and showed that Tip60 and ATM are important molecular regulators. Our findings reveal a more precise mechanism of crosstalk between IR-induced autophagy and G2/M cell cycle arrest.

The crucial roles that autophagy plays in maintaining cellular homeostasis have been well established, and autophagy dysfunction might result in human diseases, such as cancer. The function of autophagy in cancer is typically considered a double-edged sword in tumorigenesis and therapy [24]. On the one hand, autophagy enables cancer cells to survive under some stress conditions, such as starvation, hypoxia, exposure to chemotherapeutics, and radiotherapy [25]. On the other hand, autophagy acts as a tumor suppressor that induces autophagic cell death [26]. This divergent role is considered to be dependent on various factors, such as the genetic context, tumor type, and disease stage. Although much is unknown regarding the exact role of autophagy in cancer, it is clear that abnormal control of autophagy is one of the key hallmarks of cancer. Consequently, the modulation of autophagy can be a promising avenue for cancer therapy. Radiotherapy is a major strategy for cancer treatment. In this study, we demonstrated that IR regulates autophagy by enhancing Beclin 1/PIK3CIII complex activity and the association between Beclin 1 and ATG14L. The immunofluorescence results also indicated that IR induces autophagy and that Beclin 1 participates in the process of IR-induced autophagy, as detected using a 2FYVE domain fusion protein that binds PtdIns3P with high specificity.

Beclin 1 is considered to be a key component of the PtdIns3K complex to initiate autophagy in mammalian cells [7,27]. The MAPLC3-II/MAPLC3-I ratio is a protein marker that is widely used to label autophagosomes, since lipidated MAPLC3-II is converted from its unconjugated form (MAPLC3-I), and MAPLC3-II is reliably associated with phagophores and completed autophagosomes [28]. In the present study, we measured the dynamic change in MAPLC3 lipidation, as manifested by the ratio between LC3-II and LC3-I, after the data were normalized to the internal loading control. The results showed that no significant difference could be detected before 4 h after IR. That is, very early after IR, a lack of Beclin 1 did not substantially alter MAPLC3 lipidation. Additionally, SQSTM1/p62 degradation revealed that Beclin 1 inhibition damaged IR-induced autophagy. Similar observations have been reported previously [29]. The effect of Beclin 1 on MAPLC3 lipidation has been controversial [29–31] and depends on various situations, such as starvation, hypoxia, and IR. One study showed that the deletion of Beclin 1 in vitro did not block an autophagy response in human cells but did reduce the ability of cells to undergo DNA repair when exposed to IR [31]. This finding contrasts with our data, and an explanation for this discrepancy is as follows: the authors of the previous report observed only the earliest period after IR (3 h), while our study examined the dynamic change from 0 to 24 h, which comprehensively demonstrated the effect of Beclin 1 inhibition on IR-induced autophagy. Moreover, the function of Beclin 1 in starvation-induced autophagy was also evaluated in this study. Beclin 1 inhibition did not affect the process of starvation-induced autophagy in response to either serum starvation or amino acid deprivation. However, regarding autophagy recovery after starvation, Beclin 1 inhibition had an obvious effect after serum starvation but not after amino acid deprivation. With regard to a higher total level of MAPLC3, the background signal appeared higher in ShBeclin 1 cells than in Shcontrol cells. One plausible explanation for this result is that MAPLC3, especially its lipidated form (MAPLC3-II) that is located on the membranes of autophagosomes and is degraded following the maturation of autophagosomes, was readily accumulated in ShBeclin 1 cells because the malformed autophagosomes lose their ability to degrade macromolecules [31]. Therefore, our data strongly indicate the crucial role of Beclin 1 in IR-induced autophagy.

Cancer cells lose cell cycle control and exhibit infinite proliferation [32]. Blocking the cell cycle, especially at the G2/M checkpoint, may be an effective way to suppress the progression of tumors. In addition, G2/M arrest is a specific phenomenon after DNA damage, especially in response to IR. However, the relationship between G2/M arrest and IR-induced autophagy, as well as the associated regulatory mechanisms, remains to be elucidated. Genotoxic stress caused by DNA damage or stalled replication forks can lead to genomic instability. To guard against such instability, genotoxically stressed cells activate checkpoint factors that halt or slow cell cycle progression. Among the pathways affected are DNA replication via reduced firing of the replication origin and mitosis via prevention of cyclin-dependent kinase (Cdk) activation. Our data showed that the autophagy inhibitor 3MA markedly delayed IR-induced G2/M arrest, and this delay did not result from either the inhibition of ROS production or the blocking of ATM activity. Because the target of 3MA is the Beclin 1/PIK3CIII complex, there is growing evidence suggesting that Beclin 1 may be involved in mitosis or cell cycle progression [12,33]. This possibility motivated us to determine whether Beclin 1 is the possible link that connects autophagy to the cell cycle during IR exposure. As anticipated, similar to 3MA, Beclin 1 contributed to the IR-induced G2/M arrest, a result that is supported by those of previous studies [12,33]. To gain a better understanding of the specific mechanisms by which Beclin 1 is involved in IR-induced G2/M arrest, we examined the interactions between Beclin 1 and G2/M checkpoint-related proteins. IR elevated both the Beclin 1/PLK1 and Beclin 1/CDC25C interactions, which may show the role of Beclin 1 in IR-induced G2/M arrest. In contrast, no interaction was observed between PIK3C3 and PLK1 or between PIK3R4 and PLK1, indicating the possible existence of competition for the interaction with PLK1 between Beclin 1 and PIK3C3/PIK3R4. Thus, we concluded that Beclin 1 participates in IR-induced G2/M arrest by interacting with PLK1 and CDC25C to regulate cell cycle arrest.

Apoptosis is a canonical pathway that allows cells to undergo a highly regulated cell death process in response to pro-apoptotic stimuli and is triggered by most signaling pathways. The crosstalk between apoptosis and autophagy has been widely studied, and Beclin 1 has been reported to bridge autophagy and apoptosis [13]. The anti-apoptotic protein Bcl2 is widely known to inhibit autophagy by binding with Beclin 1. Our results further verified the interaction between Beclin 1 and Bcl2 and their dissociation induced by IR. In addition, we demonstrated the interaction between Beclin 1 and pan14-3-3 in response to IR. The major pan14-3-3 proteins comprise YWHAB, YWHAE, YWHAZ, YWHAQ, YWHAH, YWHAG, and SFN. Among them, YWHAG is highly expressed in the brain, in skeletal muscles, and in response to heat. YWHAQ is expressed in the cytoplasm of keratinocytes and in T cells. Most of the pan14-3-3 proteins are multifunctional regulators that are involved in cell cycle control (checkpoint) and signal transduction. In addition, they are also inhibitors of apoptosis that function by inhibiting the activation of p38 MAP kinase [34]. Pan14-3-3 proteins are adapter proteins that are implicated in the regulation of a large spectrum of both general and specialized signaling pathways, and they bind to a large number of partners, typically by recognition of a phosphoserine or phosphothreonine motif. Binding generally results in the modulation of the activity of the binding partner. For example, when SFN binds to KRT17, SFN regulates protein synthesis and epithelial cell growth by stimulating the AKT/mTOR pathway. Additionally, SFN regulates MDM2 autoubiquitination and degradation and thereby activates p53/TP53. However, which pan14-3-3 protein specifically interacts with Beclin 1 in response to IR requires further research. Therefore, the observed Beclin 1/pan14-3-3 interaction further demonstrates the importance of Beclin 1 as an regulator in the IR-induced crosstalk between autophagy, apoptosis, and G2/M arrest.

Recently, Tip60 was revealed to directly acetylate and stimulate ULK1 to elicit autophagy [20,21]. The recruitment of Tip60 depends on the MRN complex, and this requirement was demonstrated by the failure of Tip60 to be recruited to DSBs within 15 min of exposure to IR as a result of Rad50 depletion [23]. Tip60 forms a complex with ATM by binding to the C-terminal FATC region of ATM [23]. This complex is essential for the HAT activity of Tip60, and the colocalization of the Tip60-ATM complex occurs at IR-induced foci after cell exposure to a low dose of radiation. The absence of Tip60 reduces ATM acetylation, which in turn reduces ATM kinase activity after exposure to bleomycin or formaldehyde [23]. In our study, we observed the interaction between Beclin 1 and Tip60, which indicated that Tip60 may regulate IR-induced autophagy by interacting with Beclin 1. In addition, our immunofluorescence results demonstrated the colocalization of Tip60 with Beclin 1 as well as the translocation of Tip60 and Beclin 1. This result is consistent with the observation from a previous study [22]. UV irradiation has been reported to trigger Tip60 relocalization to NBs, which is a function of sumoylation, as suppression of Ubc9 abrogates sumoylation. Sumoylation is an active mechanism that is responsible for recruiting Tip60 to the NB. Based on this information, we hypothesized that the majority of Beclin 1 is exported to the cytoplasm to regulate autophagy in response to IR after 4 h. Thus, we conclude that Tip60 may regulate IR-induced autophagy and G2/M arrest through the interaction with Beclin 1 and through further translocation.

However, activation of the ATM kinase is dependent on the Tip60 protein [23]. Proteins accomplish a variety of functions via different posttranslational modifications, especially phosphorylation, which is involved throughout the process of autophagy. Taking Beclin 1 as an example, Wang et al. and Wei et al. observed that both EGFR (epidermal growth factor receptor) and AKT can individually regulate autophagy by phosphorylating Beclin 1 [35,36]. Beclin 1 has also been reported to be phosphorylated at S93/S96 by AMPK and at S15 by ULK1 in response to glucose starvation and amino acid deprivation, respectively [11,37]. In this study, we identified a novel ATM phosphorylation site, namely, T57, in Beclin 1 that regulates IR-induced autophagy. These data suggest that ATM may regulate autophagy via Beclin 1 through different molecular mechanisms.

In summary, in the present study, we demonstrated the crucial roles of Beclin 1 in IR-induced autophagy, G2/M cell cycle arrest, and apoptosis and showed that Tip60 and ATM are also important molecular regulators. Our findings reveal a more precise mechanism of crosstalk between IR-induced autophagy and G2/M cell cycle arrest.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

Acknowledgments

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