BECA1 promotes radiation-induced G2/M arrest through regulation CDK1 activity: a potential role for autophagy in G2/M checkpoint

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Abstract

Autophagy and G2/M arrest are two important mechanistic responses of cells to ionizing radiation (IR), in particular the IR-induced fibrosis. However, what interplayer and how it links the autophagy and the G2/M arrest remains elusive. Here, we demonstrate that the autophagy-related protein BECA1 plays a critical role in ionizing radiation-induced G2/M arrest. The treatment of cells with autophagy inhibitor 3-methyladenine (3-MA) at 0–12 h but not 12 h postirradiation significantly sensitized them to IR, indicating a radio-protective role of autophagy in the early response of cells to radiation. 3-MA and BECA1 disruption inactivated the G2/M checkpoint following IR by abrogating the IR-induced phosphorylation of phosphatase CDC25C and its target CDK1, a key mediator of the G2/M transition in coordination with CCNB1. Irradiation increased the nuclear translocation of BECA1, and this process was inhibited by 3-MA. We confirmed that BECA1 interacts with CDC25C and CHK2, and which is mediated the amino acids 89–155 and 151–224 of BECA1, respectively. Importantly, BECA1 deficiency disrupted the interaction of CHK2 with CDC25C and the dissociation of CDC25C from CDK1 in response to irradiation, resulting in the dephosphorylation of CDK1 and overexpression of CDK1. In summary, IR induces the translocation of BECA1 to the nucleus, where it mediates the interaction between CDC25C and CHK2, resulting in the phosphorylation of CDC25C and its dissociation from CDK1. Consequently, the mitosis-promoting complex CDK1/CCNB1 is inactivated, resulting in the arrest of cells at the G2/M transition. Our findings demonstrated that BECA1 plays a role in promotion of radiation-induced G2/M arrest through regulation of CDK1 activity. Whether such functions of BECA1 in G2/M arrest is dependent or independent on its autophagy-related roles is necessary to further identify.

Introduction

Radiotherapy is a widely used strategy for the treatment of cancer patients. However, despite major advances in radiotherapy, the radioresistance of tumors remains the leading obstacle to their clinical treatment because it results in radiotherapy failure or tumor recurrence1. Approximately 10–45% of cancers are resistant to radiation, which greatly influences the outcomes of radiotherapy.

Autophagy is a process of cellular self-degradation that plays a critical role in maintaining the balance between cell survival and cell death3. Recent studies have indicated that the two roles of autophagy in cancer cells are associated with the initiation of a cascade of signaling pathways involving multiple molecules and transcription factors3. The suppression of BECA1 phosphorylation reduces autophagy in prostate cancer cells and thereby overcomes the radioresistance of these cells5. Conversely, the autophagy-mediated degradation of p62 and the c-Jun-mediated expression of BECA1 increase the...
radioresistance of lung cancer cells\textsuperscript{6}. We established BECN1-knockout human triple-negative breast cancer (TNBC) MDA-MB-231 cells using the CRISPR/Cas9 system, and functional analyses revealed that BECN1 deficiency suppressed MDA-MB-231 cell proliferation by inducing arrest of the cell cycle at the G\textsubscript{1} phase in vitro\textsuperscript{7}, which suggested that BECN1 regulates the cell cycle.

The activation of cell-cycle checkpoints is another fundamental process for controlling cellular homeostasis during exposure to radiation. During the cell cycle, various checkpoints play an important role in monitoring and regulating the progression as well as the genomic stability in response to DNA damage or other stresses. Li et al. reported that radiation induces G\textsubscript{2}/M phase arrest in primary human renal clear cell carcinoma cells, which might contribute to radioresistance\textsuperscript{8}. Carruthers et al. found that the suppression of ATM kinase, an upstream kinase of the G\textsubscript{2}/M checkpoint signal pathway involving CHK2/CDK25C/CDK1, by the inhibitor KU-55933 inactivates the G\textsubscript{2}/M checkpoint and radiosensitizes glioblastoma stem-like cells\textsuperscript{9}.

In the present study, we focused on BECN1 based on the following considerations. BECN1, the mammalian homolog of yeast Atg6 and a highly conserved eukaryotic protein\textsuperscript{10}, is a core autophagy-related protein that regulates vesicle nucleation during autophagosome formation. The blockage of autophagy using the autophagy inhibitor 3-methyladenine (3-MA) increases the number of cells in the G\textsubscript{2}/M state\textsuperscript{11}. BECN1 also associates with class III phosphoinositide 3-kinase (PI3K) to promote the generation of phosphatidylinositol 3-phosphate\textsuperscript{12}. BECN1 is also involved in the cellular response to radiation-induced damage or sensitivity\textsuperscript{13}. Moreover, our previous study showed that the knockdown of BECN1 suppresses TNBC cell proliferation and colony formation by inducing cell-cycle arrest at the G\textsubscript{1} phase\textsuperscript{7}. Our pilot study revealed that the inhibition of autophagy by 3-MA decreased the number of LC3 puncta and LC3B protein expression in ⧫-ray-irradiated cells (Fig. 1a). Thus, we hypothesized that autophagy-related BECN1 might play a markedly more important role in the activation of cell-cycle checkpoints in response to irradiation. Our findings demonstrated that BECN1 plays a role in promotion of radiation-induced G\textsubscript{2}/M arrest through regulation of CDK1 activity. Whether such functions of BECN1 in G\textsubscript{2}/M arrest is dependent or independent on its autophagy-related roles is necessary to further identify.

Results

BECN1 protein was remarkably upregulated in A549 cells, Hela cells, and mice lung fibrosis tissues

We performed immunofluorescence detection of the autophagy-related markers LC3 and WIPI2 (WD-repeat domain, phosphoinositide interacting 2), which is a member of the ATG18/WIPIs (WD-repeat protein interacting with phosphoinositide) family\textsuperscript{14}. As shown in Fig. 1a, b, c, upregulation of BECN1 was unexpectedly observed in these cell lines, revealing the potential association of autophagy with radiation-related development of lung fibrosis. After 4 Gy irradiation, the protein levels of LC3-II were significantly increased overtime after IR, whereas the SQSTM1/P62 level was decreased; this trend was largely reversed to a certain extent by 3-MA treatment (Fig. 1d). The numbers of LC3 puncta per cells increased postradiation, but this effect was inhibited by treatment with 3-MA and wortmannin (Fig. 1e). As shown in Fig. 1f, g, the numbers of LC3 and WIPI2 puncta/dots increased postradiation, but this effect was inhibited by treatment with 3-MA. The level of BECN1 in mice radiation-induced lung fibrosis tissues are remarkably upregulated compared with the control group without out radiation treatment (Fig. 2a). IHC staining was conducted and the BECN1 expression was verified to be upregulated in ice radiation-induced lung fibrosis tissues compared with the control group without radiation treatment (Fig. 2b). Collectively, these above data strongly demonstrate that autophagy and BECN1 protein expression are elevated in human cells in vitro, and mouse lung fibrosis tissues in vivo.

BECN1 and its mediated autophagy were essential for cell response to radiation and lung fibrosis

At the early stage (0–12 h) after irradiation, a series of survival-promoting DDRs, such as cell-cycle checkpoints and DNA repair, are activated and encourage cells to recover from radiation damage. Therefore, we designed experiments in which the cells were treated with the autophagy inhibitor 3-MA at the early (0–12 h post-IR) or later stages (12–24 h post-IR). Supplementary Fig. 1a suggest that autophagy might play a protective role during the early stage (0–12 h) but not at the later stage of the response to IR. After 2- or 4-Gy irradiation, the survival rates of 3-MA-treated HeLa cells were significantly decreased compared with those of the untreated cells (Supplementary Fig. 1b). As shown in Supplementary Fig. 1c, d, the apoptosis level was markedly increased in the group treated with 3-MA at 0 to 12 h post 4-Gy irradiation (4 Gy + 3-MA@0 h) compared with the group that was only irradiated with 4 Gy (4 Gy). These results further suggest that autophagy plays a dual-effect role in the early response of cells to irradiation, which at the early stage (0–12 h) of radiation exposure, autophagy is subjected to protective effect but at the later stage (12–48 h), it is subjected to inhibition effect.

The LC3-II level increased with increasing time post-IR, but a notably lower increase was observed in the cells cotreated with 3-MA (Fig. 2c). Autophagic flux was found to be active in irradiated BECN1-WT cells. BECN1
Deficiency resulted in a significantly decreased LC3B II/I ratio in BEC1-KO cells compared with BECN1-WT cells (Fig. 2d). We used cells transfected with the tandem GFP-LC3-mRFP-LC3ΔG plasmid to further confirm the effect of BECN1-KO on autophagic flux. This fusion protein can be cleaved by the endogenous ATG4 family protease to generate equal amounts of GFP-LC3 and mRFP-LC3. In the acidic environment of the lysosome, mRFP-LC3 (red) is markedly more stable than GFP-LC3 (green). MRFP red puncta represent the maturation of autophagolysosomes, and yellow puncta (indicating the expression of both GFP and mRFP) represent the formation of autophagosomes. A lower ratio of GFP-LC3 to RFP-LC3 puncta reflects autophagic flux. Both yellow and red puncta were observed in the cells, and a decreased ratio of GFP-LC3 to RFP-LC3 puncta was apparent following...
4-Gy irradiation (Fig. 2e). However, fewer LC3 puncta and a higher ratio of GFP-LC3 to RFP-LC3 puncta were observed in the irradiated BECN1-KO cells, particularly at 12 h, compared with the BECN1-WT cells (Fig. 2e, f). As shown in Fig. 2g, at indicated timepoints after 4 Gy IR treatment, the E-cadherin expression level was increased and E-cadherin and Vimentin expression levels were decreased at the BECN1 deficiency status. These data
reveal that BECN1-mediated autophagy is at least one of key events for BECN1 promotion of radiation-induced EMT.

G2/M checkpoint was regulated by BECN1 in response to IR

As shown in Fig. 3a, b, the percentages of G2- and M-phase cells increased in a time-dependent manner after 6-Gy irradiation, which indicated the induction of G2/M arrest and/or mitotic arrest. The cells pretreated with 3-MA exhibited relatively lower percentages of cells at the G2 and M phases compared with those found for the cells that were only irradiated alone (Fig. 3b). A similar result was also observed after pretreatment with chloroquine, another autophagy inhibitor (Supplementary Fig. 2). Because autophagy might participate in IR-induced cell-cycle arrest, we hypothesized that BECN1, a key regulator of autophagy, might play a critical role in the crosstalk between autophagy and cell-cycle progression. CRISPR/Cas9-mediated BECN1-knockout MDA-MB-231 cells (BECN1-KO) were used (Fig. 3d). As shown in Fig. 3c, e, BECN1 deficiency resulted in decreased G2/M arrest in response to IR compared with wild-type (WT) BECN1. Rescue experiments further showed that the restoration of BECN1 might increase G2/M arrest in response to IR. The silencing of BECN1 expression by specific siRNAs (Supplementary Fig. 3a) further demonstrated that autophagy-related BECN1 is involved in the regulation of irradiation-induced G2/M arrest (Supplementary Fig. 3b, c).

CRISPR/Cas9-mediated ATG7-knockout MDA-MB-231 cells were also used to further investigate the crosstalk between autophagy and cell-cycle progression, and the knockout of ATG7 expression was detected by western blotting (Fig. 3g). As shown in Fig. 3f, h, ATG7 deficiency resulted in decreased G2/M arrest in response to IR, which was similar to the effect observed with BECN1 deficiency.

To further clarify the role of autophagy-related BECN1 in radiation-induced G2/M arrest, the cells were analyzed by flow cytometry following the immunostaining of phospho-histone H3/Ser10 (pHH3), a marker of cells in mitosis. As shown in Fig. 3a, the ratio of pHH3-positive mitotic cells was sharply decreased in the population of control cells at 2–4 h postirradiation, which indicated that activation of the G2/M boundary checkpoint results in arrest at the G2/M transition. Eight hours post-IR, the mitotic cells in the population of cells treated with radiation and 3-MA were scarcely decreased (Fig. 4a, b). PHH3 immunofluorescence staining was also performed in BECN1-WT and BECN1-KO MDA-MB-231 cells (Fig. 4c, d). The proportion of pHH3-positive mitotic cells was sharply decreased in the population of BECN1-KO cells at 2–4 h postirradiation. In contrast, the proportion of mitotic cells in the population of BECN1-KO MDA-MB-231 cells was unaffected at the early stage (0–8 h) of the response to IR. At 12 h postirradiation, an increased proportion of mitotic BECN1-KO cells were observed, which suggested the occurrence of prolonged mitotic arrest in the population of BECN1-deficient cells. A representative image of MPM2-positive mitotic cells is shown in Fig. 4e. The MPM2 staining results further indicated that BECN1 deficiency liberated cells after IR-induced G2/M arrest, i.e., BECN1 deficiency resulted in inactivation of the G2/M checkpoint (Fig. 4e, f). Thus, the results clearly demonstrate that autophagy-associated BECN1 deficiency inactivates the G2/M checkpoint in response to IR.

BECN1 deficiency disrupts the responses of G2/M checkpoint proteins to IR

As shown in Fig. 5a, b, irradiated HeLa cells cotreated with 3-MA exhibited decreased levels of pATM, pCHK2, pCDK25C, and pCDK1 at the indicated timepoints post-irradiation compared with the control cells. CDK1 dephosphorylation and CCNB1 expression are required for activation of the CDK1-CCNB1 complex. This complex is maintained in an inactive state through phosphorylation of a conserved residue, tyrosine 15 (CDK1-pY15). As shown in Supplementary Fig. 4a, b,
Fig. 3 Involvement of autophagy in the regulation of IR-induced cell-cycle progression arrest. a Representative flow cytometry histogram of cell-cycle progression in the population of 4-Gy γ-ray-irradiated HeLa cells with or without cotreatment with 3-MA. b Effect of 3-MA on the G2 and M phase distribution in the population of irradiated cells. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 compared with the control group. c Representative flow cytometry histogram of cell-cycle progression in the population of 4-Gy γ-ray-irradiated BECN1-WT and BECN1-KO MDA-MB-231 cells. d Western blotting analysis of BECN1 in BECN1-WT MDA-MB-231 cells (control) and BECN1-KO cells generated by CRISPR/Cas9. GAPDH served as the internal loading control. e Quantitative measurement of the proportions of 4-Gy γ-ray-irradiated BECN1-KO and control cells in the G2 and M phases at the indicated times after IR. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 compared with the control group. f Representative flow cytometry histogram of cell-cycle progression in the population of 4-Gy γ-ray-irradiated ATG7-WT and ATG7-KO MDA-MB-231 cells. g Western blotting analysis of ATG7 in ATG7-WT MDA-MB-231 cells (control) and ATG7-KO cells generated by CRISPR/Cas9. GAPDH served as the internal loading control. h Quantitative measurement of the proportion of 4-Gy γ-ray-irradiated ATG7-KO and control cells in the G2 and M phases at the indicated times after IR. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 compared with the control group.
Fig. 4 Autophagy-related BECN1 deficiency inactivates the G2/M checkpoint in response to IR. 

**a** Representative flow cytometry histograms of phosphorylated histone H3 (pHH3)-positive mitotic cells in the population of γ-ray-irradiated HeLa cells with or without 3-MA cotreatment. The cells were immunostained with pHH3(Ser10) antibody to detect the proportion of pHH3-positive mitotic cells at the indicated times after IR.  

**b** Quantitative measurement of pHH3(Ser10)-positive cells. The data are presented as the means ± SDs from three independent experiments; *p < 0.01 compared with the control group.  

**c** Representative flow cytometry histogram of the population of γ-ray-irradiated BECN1-WT or BECN1-KO MDA-MB-231 cells. The cells were immunostained with pHH3(Ser10) antibody to detect the proportion of pHH3-positive cells at the indicated times after IR. A rescue experiment was conducted by transfecting BECN1-KO MDA-MB-231 cells with lentiviral vectors expressing BECN1.  

**d** Quantitative measurement of pHH3(Ser10)-positive cells. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 compared with the WT cells.  

**e** Representative image of mitotic protein monoclonal-2 (MPM2)-positive cells obtained through immunofluorescence staining (bars: ×20 magnification).  

**f** Quantification of MPM2 antibody-stained mitotic cells. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 compared with the WT cells.  

**g** pHH3 was detected by Western blotting analysis. β-actin served as the internal control.
Fig. 5 Autophagy inhibition and BECN1 deficiency disturb the response of G2/M checkpoint-related proteins to IR. 

(a) Western blotting analysis of G2/M checkpoint-regulating proteins (pATM, pCHK2, and CHK2) in the γ-ray-irradiated A549 cells that were cotreated or not cotreated with the autophagy inhibitor 3-MA. The proteins were detected at the indicated timepoints after IR, and GAPDH served as the internal control.

(b) Western blotting analysis of the phosphorylation of G2/M checkpoint-regulating proteins (pCDC25C, pCDK1, and CDK1) in the γ-ray-irradiated A549 cells with or without cotreatment with the autophagy inhibitor 3-MA. The phosphorylation analysis was performed at the indicated timepoints after IR, and GAPDH served as the internal control.

(c) Western blotting analysis of G2/M checkpoint-associated phosphorylated proteins (pCHK1, CHK1, pWEE1, and pMYT1) in the γ-ray-irradiated BECN1-WT or BECN1-KO MDA-MB-231 cells. GAPDH served as the internal control.

(d) Western blotting analysis of G2/M checkpoint-associated phosphorylated proteins (pCHK2, CHK2, pCDK1, and CDK1) in the γ-ray-irradiated BECN1-WT or BECN1-KO MDA-MB-231 cells. GAPDH served as the internal control.

(e) Quantitative measurement of the levels of pATM, pCHK2, pCDC25C, and pCDK1 in A549 cells with or without 3-MA cotreatment detected at the indicated time point after IR. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 between different groups.

(f) Quantitative measurement of phosphorylated protein levels of pCHK1, pWEE1, pMYT1, and pCDK1 in BECN1-WT and BECN1-KO MDA-MB-231 cells at the indicated timepoints after IR. The data are presented as the means ± SDs from three independent experiments; *p < 0.05 between different groups.
the proportion of pHH3-positive mitotic cells was sharply decreased in the population of WT cells at 2–4 h post-irradiation, whereas the numbers of mitotic cells in the population of ATG7-KO MDA-MB-231 cells was unaffected. ATG7 deficiency resulted in decreased G2/M arrest in response to IR. A rescue experiment showed that restoration of the expression of ATG7 could liberate cells from G2/M arrest in response to IR. Compared with WT cells, a notably lower increase in LC3-II and slower degradation of p62 protein were observed in ATG7 KO cells with increasing irradiation time (Supplementary Fig. 4c).

The levels of phosphorylated pCHK1, pCHK2, pWEE1, and pCDK1 were increased in BECN1-WT MDA-MB-231 cells after 6-Gy irradiation, whereas no obvious change or only a weak increase in the levels of nonphosphorylated CHK2, WEE1, and CDK1 were detected in BECN1-WT MDA-MB-231 cells. Overall, the expression of MYT1 in BECN1-KO cells was lower than that in WT cells at all examined times postirradiation (Supplementary Fig. 5a, b). The changes in the phosphorylation levels of the related proteins are shown in Fig. 5c, d. Lower levels of pCHK1, pCHK2, pWEE1, pMYT1, and pCDK1 were observed in BECN1-KO MDA-MB-231 cells compared with BECN1-WT cells at the indicated time-points postirradiation. These results suggest that (i) CDK1 is phosphorylated and inactivated in WT cells following irradiation but is maintained in a dephosphorylated state in BECN1-KO cells; (ii) BECN1 deficiency might promote the dephosphorylation of CDK1; and (iii) Inactivation of the G2/M checkpoint due to autophagy inhibition or BECN1 deficiency might be partially due to dysregulation of the ATM/CHK2/CDC25C/CDK1 signaling pathway.

**BECN1 deficiency disrupts the dissociation of CDC25C from CDK1 following irradiation**

As shown in Fig. 6a, the phosphorylated CDK1-pY15 levels postirradiation were increased in the BECN1-WT cells, and this effect is attributed to inactivation of the phosphatase CDC25C. However, the expression of CDK1-pY15 in BECN1-KO cells was barely changed after irradiation, which further indicated that CDK1 dephosphorylation in response to radiation is associated with BECN1. As shown in Fig. 5b, the interaction between CDK1 and CDC25C was sharply decreased in WT cells following irradiation, which suggested the dissociation of these two proteins. However, the cotreatment of the cells with the autophagy inhibitor 3-MA abrogated the radiation-induced dissociation of CDC25C from CDK1 (Fig. 6b). The interaction of CDK1 with CDC25C was detected in both BECN1-KO and BECN1-WT cells under normal growth conditions. CDC25C rapidly dissociated from CDK1 in BECN1-WT cells within 1 and 2 h after 4-Gy irradiation, whereas the interaction of CDC25C with CDK1 was not disrupted in the irradiated BECN1-KO cells (Fig. 6c), which is consistent with the effect of 3-MA treatment (Fig. 5b). Importantly, the restoration of BECN1 expression in BECN1-KO cells through adenovirus-mediated transfection of a BECN1-expressing vector resulted in the irradiation-induced dissociation of CDC25C from CDK1 (Fig. 6d). In addition, CDK1 CoIP results indicated that the interaction between CDK1 and WEE1 increased after 4-Gy irradiation in both BECN1-KO and BECN1-WT cells, which indicated that BECN1 does not influence the interaction between CKD1 and WEE1 (Fig. 6c, d).

As shown in Fig. 7a, increased levels of BECN1 in the nucleus and CDC25C in the cytoplasm were detected in BECN1-WT cells following irradiation. However, in BECN1-KO cells, the radiation-induced cytoplasmic translocation of CDC25C was largely blocked. Immunofluorescence staining also demonstrated that the cytoplasmic translocation of CDC25C was blocked in irradiated BECN1-KO cells and could be restored following transfection of an exogenous BECN1-expressing vector (Fig. 7b, c). These results are consistent with the effects of BECN1 on the interaction between CDC25C and CDK1 and the dephosphorylation of CDK1 (Fig. 6). The immunofluorescence staining assay results clearly showed that the radiation-induced nuclear translocation of BECN1 was largely attenuated by 3-MA (Fig. 7d). In addition, a western blot analysis indicated that irradiation increased the nuclear translocation of BECN1, and this effect was reduced by treatment with 3-MA (Fig. 7e). Moreover, the suppression of ATG5 and ATG7 expression by specific siRNAs inhibited radiation-induced autophagy, also prevented the radiation-induced nuclear translocation of BECN1 (Fig. 7f). These data demonstrate the following: (i) CDK1 interacts with WEE1 and CDC25C to form multi-protein complexes in BECN1-expressing MDA-MB-231 cells. (ii) BECN1 deficiency inhibition prevents the translocation of CDC25C from the nucleus to the cytoplasm. (iii) Consequently, CDK1 is found in a state of dephosphorylation in BECN1-deficient cells, leading to inactivation of the G2/M checkpoint and cell-cycle progression from the G2 to the M phase without arrest, even under the stress induced by radiation injury.

We then wondered whether the dissociation of CDC25C from CDK1 and its translocation from the nucleus to cytoplasm are directly mediated by BECN1. As shown in Fig. 8a, there exists interactions among BECN1, CDC25C, and CHK2, and these interactions increased following irradiation. We also observed an interaction between BECN1 and BCL2, and this interaction was weakened after irradiation. We did not detect any interactions between BECN1 and CHK1 or among CDK1, CCNB1, and WEE1 (Fig. 8a). As shown in Fig. 8b, the interaction of BECN1 with CDC25C and CHK2 only occurred in the nucleus, and this interaction increased...
following irradiation. To determine the domain(s) of BECN1 that mediate its interactions with CDC25C and CHK2, we generated a series of truncated BECN1 mutants (Fig. 8c). As shown in Fig. 8d, the BECN1-B mutant, in which amino acids 89–155 were deleted, was no longer able to interact with CHK2, and the BECN1-C mutant, in which amino acids 151–224 were deleted, was no longer able to interact with CDC25C.

As shown in Fig. 8e, the interaction between CDC25C and CHK2 was strengthened in BECN1-WT cells following irradiation. However, in BECN1-KO cells, the radiation-enhanced interaction between CDC25C and CHK2 was only observed after transfection of a vector directing the expression of full-length BECN1 or the BECN1-D or BECN1-E mutants. The BECN1-B and BECN1-C mutants did not interact with CHK2 or

Fig. 6 BECN1 deficiency and autophagy inhibition inhibits the dissociation of CDC25C from the CDK1 complex after irradiation. a The levels of phosphorylated CDK1-pY15 protein in BECN1-KO and BECN1-WT MDA-MB-231 cells were detected 1 and 2 h after 4-Gy irradiation by western blot analysis. β-actin served as the internal loading control. b Effects of 3-MA treatment on the protein–protein interactions of the CDK1 complex in MDA-MB-231 cells after irradiation. Cell lysates were collected from DMSO-treated MDA-MB-231 cells and 3-MA-treated cells at 2 h after 4-Gy irradiation, and immunoprecipitates were prepared with anti-CDK1 or anti-IgG antibodies. Western blot analysis was performed using anti-CDK1, anti-CDC25C, and anti-WEE1 antibodies. β-actin was used as the internal loading control. c Effects of BECN1 deficiency on the protein–protein interactions of the CDK1 complex after irradiation. Cell lysates were collected from BECN1-WT and BECN1-KO MDA-MB-231 cells at the indicated timepoints after 4-Gy irradiation, and immunoprecipitates were prepared with anti-CDK1 or anti-IgG antibodies. Western blot analysis was performed using anti-CDK1, anti-CDC25C, and anti-WEE1 antibodies. β-actin was used as the internal loading control. d Effects of BECN1 deficiency and overexpression on the protein–protein interactions of the CDK1 complex in MDA-MB-231 cells with or without 4-Gy irradiation. Cell lysates were collected from BECN1-WT cells, BECN1-KO cells, and BECN1-KO cells transfected with exogenous BECN1 mediated by lentiviral vectors with or without 4-Gy irradiation, and immunoprecipitates were prepared with anti-CDK1 or anti-IgG antibodies. Western blot analysis was performed using the indicated antibodies. β-actin was used as the internal loading control.
Fig. 7 (See legend on next page.)
CDC25C. These results indicate that the interaction/ phosphorylation of CDC25C with CHK2 in response to IR is mediated by BECN1.

Finally, we investigated the effects of BECN1 on the assembly/interaction of the G2/M checkpoint complex CHK2/CDC25C/CDK1/CCNB1 and its association with CHK2 kinase activity and the phosphorylation of CDC25C/S216 in response to IR (Fig. 8f). An HA-tagged CHK2 immunoprecipitation assay (IP:HA) showed that the interaction between exogenous HA-CHK2 and Flag-CDC25C in BECN1-WT cells increased following irradiation. The interaction between HA-CHK2 and Flag-CDC25C in BECN1-KO cells was lower than that in WT cells, regardless of irradiation. However, restoration of the expression of BECN1 allowed the radiation-induced interaction of CHK2 and CDC25C in BECN1-KO cells.

A Flag-tagged CDC25C immunoprecipitation assay (IP: Flag) showed that the interaction between exogenous HA-tagged CHK2 and Flag-tagged CDC25C in BECN1-WT cells was also enhanced following IR, whereas the interaction of Flag-tagged CDC25C with endogenous CDK1 in these cells decreased following irradiation (Fig. 8f). Similarly, in BECN1-KO cells, irradiation weakened the interaction of Flag-CDC25C with HA-CHK2 but did not decrease the interaction of Flag-CDC25C with CDK1. The phosphorylation-mimic mutant CDC25/S216E was no longer able to interact with CDK1. The restoration of BECN1 expression increased the interaction of CDC25C with CHK2 in BECN1-KO cells and decreased the interaction of CDC25C with CDK1 to a level similar to that observed in BECN1-WT cells in response to radiation. A high level of interaction was found between CDK1 and CCNB1 in BECN1-KO cells, and this level decreased following transfection of the phosphorylation-mimic CDC25C-S216E expression vector. Restoration of BECN1 expression decreased the interaction of CDK1 with CCNB1 in irradiated BECN1-KO cells.

The BECN1 and CDK1 expression levels are increased in breast cancer tissue samples

To determine whether the expression of BECN1 and CDK1 are altered in breast cancer tissues, gene expression data from the Gene Expression Omnibus (GEO) database (accession numbers GSE81838 and GSE65194) and the breast cancer patient dataset from the Cancer Genome Atlas (TCGA) were analyzed. As shown in Supplementary Fig. 6a, 93 genes overlapped among the three datasetsGSE65194, GSE81838, and TCGA datasets, of which BECN1 and CDK1 were both upregulated in breast cancer tissue compared with normal tissue. Supplementary Fig. 6b presents the relative expression levels of several essential autophagy-related genes, including BECN1 and G2/M-regulated genes, such as CDK1, CDC25C, and CHK1, in breast cancer and normal tissues in the TCGA dataset. We also found that both BECN1 and CDK1 are upregulated in breast cancer tissue compared with normal tissue (Supplementary Fig. 6c). Several essential autophagy-related and G2/M-regulating genes, including BECN1, CDK1, and CDC25C, are coexpressed; in particular, CDK1 is associated with both autophagy-related and G2/M-regulating genes (Supplementary Fig. 6d).

Therefore, BECN1 was translocated into the nucleus following IR, where it mediated the interaction of CDC25C with CHK2, prompting the phosphorylation of CDC25C and its dissociation from CDK1 and thus resulting in the inactivation of the CDK1/CCNB1 complex and arrest at the G2/M transition in the cell cycle, leading the CDK1 overexpression to promote the radiation-induced EMT (Supplementary Fig. 7).

Discussion

Autophagy and cell-cycle arrest are two critical cellular responses to IR, and autophagy is induced even as part of the radiation-induced bystander effect. Because initiation is potentiated by the impairment of autophagy through the disruption of core autophagy genes and autophagy-defective tumor cells also display a dysregulated cell cycle, we, in contrast to previous studies, used the autophagy inhibitor 3-MA and BECN1-KO cancer cells to directly determine the role of autophagy in G2/M arrest. The results of our study suggest that BECN1 deficiency enhances cellular sensitivity to IR, induces escape from the G2/M checkpoint after irradiation and promotes the G2/M transition without arrest. These two
events [(1) the suppression of autophagy post-IR promotes cell death and suppresses proliferation and (2) the suppression of autophagy induces escape from the G2/M checkpoint and promotes the G2/M transition] appear to be but are not actually contradictory. On the one hand, the inhibition of autophagy can promote the G2/M transition in unrepaired cells, and on the other hand, mitotic arrest can be induced in cells damaged by radiation. Moreover, the cells that escape G2/M arrest enter the M phase without undergoing adequate repair, which will likely result in mitotic catastrophic cell death26.
BECN1 is a key protein in the regulation of autophagy through the activation of VPS34. Xiao et al. demonstrated that macroautophagy is regulated by the cell-cycle protein Sdk1, which impairs the interaction of BECN1 with VPS34. CDK1 is an important player in macroautophagy suppression during the M phase. CDK1 can directly phosphorylate VPS34, which prevents formation of the BECN1-VPS34 complex and leads to decreased autophagy in M-phase cells. In contrast, CDK inhibitors stimulate autophagy by releasing BECN1, which results in the promotion of tumor growth. Our study revealed the involvement of autophagy in the regulation of the G2/M checkpoint. Autophagy dysregulation can disrupt arrest at the G2/M transition following irradiation, primarily by affecting the dissociation of CDC25C from CDK1 and decreasing the phosphorylation of ATM, CHK1, CHK2, and CDK1 in the absence of BECN1. To the best of our knowledge, this study provides the first demonstration that autophagy and the G2/M transition are associated with the dephosphorylation or phosphorylation of various G2/M-regulating proteins.

Progression from the G2 to the M phase is driven by activation of the CDK1/CCNB1 complex. Our study provides further evidence clarifying the mechanism through which autophagy regulates the G2/M transition. As indicated by previous studies, basal autophagy occurs at different phases of the cell cycle in response to environmental genotoxins such as IR. Li et al. demonstrated that increasing the expression of ATG5 in ATG5-deficient cells rescues the cells from G2/M arrest, whereas expression of the ATG5-K130R mutant does not produce this effect; the rescue from G2/M arrest coincides with increased levels of pCDK1 and CDKN1A/p21. Jia et al. demonstrated that autophagy regulates the negative cell-cycle regulator CDKN1B in naïve T cells and reduces its abundance in T cells after their activation to enter the cell cycle. Liu et al. reported that MJ-66, a tumor growth inhibitor, induces glioma cell-cycle arrest at the G2/M transition and increases the expression of PDK1 and CDK1. In the present study, we used CoIP assays to reveal that in BECN1-WT cells, CDC25C dissociates from the CDK1 complex after irradiation, which results in increased phosphorylation of CDK1 by WEEl and thus arrest at the G2/M transition in the cell cycle. However, in BECN1-deficient cells, the dissociation of CDC25C from CDK1 is attenuated, which results in CDK1 dephosphorylation and promotion of the G2/M transition even under the stress induced by radiation injury. Xu et al. reported that following B19V infection, activated ATR phosphorylates CDC25C, which in turn inactivates the CCNB1-CDK1 complex. Jaceosidin, isolated from Japanese mugwort, induces arrest at the G2/M transition in the cell cycle through inactivation of the CDC25C-CDK1 complex. Vera et al. reported that both CDK1 and WEE1 are mediators of G2/M arrest. Our study showed that BECN1 interacts with CDC25C and CHK2 using different domains. BECN1 deficiency decreased the interaction of CDC25C with CHK2 and stabilized the interaction of CDC25C with CDK1 even in the presence of IR. Our study elucidated that under IR stress, autophagy promotes G2/M arrest by targeting the formation of the CDC25C-CHK2, CDK1-CDC25C, and CDK1-WEE1 complexes.

In conclusion, our study demonstrates that BECN1 plays a role in promotion of radiation-induced G2/M arrest (Figs. 7g and 8). However, whether such functions of BECN1 in G2/M arrest is dependent or independent on its autophagy-related roles is necessary to further identify.

Materials and methods
Reagents and antibodies
The reagents used in this study were as follows: liposomes were purchased from Invitrogen (Carlsbad, CA, USA); glycine, lauryl sodium sulfate, tetramethylethylenediamine, TRizol, and tris(hydroxymethyl) aminomethane were purchased from Amresco (Solon, OH, USA); bovine serum albumin (BSA) was purchased from Roche (Basel, Switzerland); fluorescent protein solutions were purchased from Pierce (Rockford, IL, USA); ammonium peroxysulfate, dimethyl sulfoxide (DMSO), N, N’-methylenebisacrylamide, and puromycin were purchased from Sigma (St. Louis, MO, USA); trypsin, Dulbecco’s modified Eagle’s medium (DMEM) with high glucose, and fetal bovine serum were purchased from HyClone (Logan, UT, USA); PrimeSTAR DNA polymerase and T4 DNA ligase were purchased from TaKaRa (Tokyo, Japan); GAPDH, P62, CHK1, CHK2, MYT1, WEEI, CDC25, CDC25C, ATM, CDK1, LC3, p68-CHK1, p216-CDC25, p15-CDK1, p1981-ATM, SQSTM1/P62, pCHK2, pCHK1, CDC, pCDC25C, and MPM2 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX, USA), Cell Signaling Technology (Danvers, MA, USA), or Millipore/Upstate (NY, USA); and the pLKO.1 plasmid was purchased from Sigma (Darmstadt, Germany).

Cell lines, mouse model, and irradiation conditions
Human A549 cells and the human TNBC MDA-MB-231 cell line were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in DMEM (a high-glucose medium containing penicillin, streptomycin, and 10% FBS) and incubated at 37 °C under 5% CO2. The BECN1-KO MDA-MB-231 cell line was successfully established previously in our laboratory using the CRISPR/Cas9 system according to the free online design tool (http://crispr.mit.edu/) 7. The cells were irradiated with 60Co γ-rays at a...
dose rate of 127.15 cGy/min at room temperature at the Institute of Radiation Medicine, Academy of Military Medical Sciences (Beijing, China). Twenty C57BL/6 male mice (6–7 week old) were divided into two groups randomly, 20 Gy γ-rays were used to induce the lung fibrosis model. Mice were anesthetized with intraperitoneal sodium pentobarbital (80 mg/kg). Lung tissues were collected for further assay. The animal study has been approved by ethics committee of Xiangya School of Public Health, Central South University.

**Cell transfection**

The cells were passaged the day before transfection. After the cells were grown to 60% density, BECN1 siRNA knockdown was conducted through the transient transfection of validated BECN1 siRNA (sense, 5′-GCUGCC GUAUAUCUGUUCUTT-3′, antisense, 5′-AGAACAGUUAACGGCAGCTT-3′) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer’s instructions. Scrambled siRNA was used as the negative control. Forty-eight hours after transfection, the cells were collected for further experiments.

**Cell proliferation and colony formation assays**

Cell proliferation was assessed using the CCK-8 colorimetric assay (Dojindo Molecular Technologies, Kumamoto, Japan). The cells were divided into the following treatment groups: 0 Gy, 3-MA, 4 Gy + 3-MA, 3-MA at 0 h, 4 Gy, and 4 Gy + 3-MA. The cells were then cultured in a 96-well plate at a density of 4 × 10^3 cells/well at 37 °C in the presence of 5% CO_2 for 12, 24, 48, and 72 h, and the level of cell proliferation was then assessed. The optical density (OD) of each well at 450 nm was read using a Multiskan GO microplate reader (Thermo Fisher Scientific). Each experiment was performed in triplicate.

The colony formation ability was used to assess the cell survival percentage. After treatment with 3-MA, 2 Gy, 2 Gy + 3-MA, 4 Gy, or 4 Gy + 3-MA, the cells were seeded into 60-mm culture dishes at a density of 1000 cells/dish. After 2 weeks, the cells were stained with crystal violet. The number of microscopic colonies with more than 50 cells was counted. The cell survival ratio based on the number of colony-forming irradiated cells compared with that of the control cells was calculated.

**Western blotting analysis**

A Western blotting analysis was performed for the detection of proteins or phosphorylated proteins. Briefly, the samples were treated with lysis buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membranes. After the membranes were blocked with 5% nonfat milk in Tris-buffered saline containing Tween-20 (TBST) for 2 h, the membranes were incubated with primary and secondary antibodies overnight at 4 °C. An ImageQuant LAS5000 system (Molecular Dynamics, Sunnyvale, CA, USA) was used to visualize the bands. Details of the Western blot analysis can be found in our previous publications.14-16

**Apoptosis detection**

In this study, apoptosis was assessed using a Fluorescein Isothiocyanate (FITC)-Annexin V Apoptosis Detection Kit (BD Pharamingen, San Diego, CA, USA) following the manufacturer’s instructions. The cells were treated with or without 3-MA, subjected to 4-Gy irradiation and harvested at 24, 48, or 72 h after irradiation. The cells were washed twice with 3 ml of phosphate-buffered saline (PBS), and RNase A was then added. The cells were centrifuged, resuspended in PBS and transferred to clean Eppendorf tubes, and 100 μl of 1× Annexin V-binding solution was then added to the cells to form a suspension of 1 × 10^6 cells/ml. Subsequently, 5 μl of FITC-conjugated Annexin V and propidium iodide (PI) solution (40 μg/ml PI and 0.1% Triton X-100 in PBS buffer) was added. The cells were then incubated for 15 min at room temperature in the dark, and 400 μl of 1× binding buffer was then added. The cells were then analyzed by flow cytometry.14

**Cell-cycle analysis and G_2/M arrest assay**

The cells were seeded into 35-mm culture dishes at a density of 70–80% per dish. The cells were treated or not treated with 3-MA, subjected to irradiation 2 h after the pretreatment, and harvested at the indicated timepoints (0, 2, 4, 6, 8, or 12 h) after irradiation. The medium was removed, the cells were treated with RNase A (62 μg/ml) and incubated at 37 °C for 30 min. The cells were stained with PI solution, and the cell-cycle distribution was analyzed by flow cytometry. For the comparison of G_2/M arrest between radiation-induced, 3-MA-treated, and BECN1-knockout cells, the mitotic cells were counted using a FACSCalibur flow cytometer (BD Pharamingen). Cells that were treated or not treated with 3-MA or in which BECN1 was knocked out were subjected or not subjected to irradiation and harvested at the indicated timepoints (0, 2, 4, 6, 8, or 12 h) postirradiation. After irradiation, PBS was added to suspend the cells; the cells were then centrifuged at 2000 rpm, and 0.25% Triton X-100 was added to induce membrane rupture. After the addition of 40 μl of 1% BSA containing anti-Ser10-phosphorylated histone H3 antibody, the cells were incubated for 50 min at room temperature, and 80 μl of 1% BSA containing a FITC-tagged secondary antibody was then added. The cells were then incubated for 30 min and stained with PI solution (20 μg/ml) for 10–30 min at room temperature, and the mitosis stage was analyzed by flow cytometry. Two-dimensional dot plots were generated using ModFit LT software (Verity Software House, Inc., Topsham, ME, USA).
Coimmunoprecipitation (CoIP)

For the CoIP assay, normal MDA-MB-231 cells and BECN1-KO MDA-MB-231 cells that were subjected or not subjected to 4-Gy irradiation were washed, harvested, and lysed with PBS buffer containing 50 mM Tris-base, 1 mM EDTA, 1% NP-40, and 1× protease inhibitor cocktail. The lysates were centrifuged, and the supernatant was collected for the CoIP assay using the Pierce Classic IP Kit (Thermo Scientific). CDK1 antibody was used to form immunocomplexes with CDC25C and WEE1, and WEE1 antibody was used to form immunocomplexes with CDK1. These immunocomplexes were isolated by 8% SDS-PAGE, washed, and eluted, and the protein interactions were detected by western blotting.

Immunofluorescence staining and laser confocal microscopy

Autophagy and subcellular protein localization were analyzed by immunofluorescence staining and laser confocal microscopy observations. Autophagy was analyzed by quantifying the formation of puncta of the autophagy biomarker GFP-LC3 by immunofluorescence staining and laser confocal microscopy. HeLa cells were seeded in glass chamber slides. After 12 h, the cells were transfected with GFP-LC3 using Lipofectamine 2000. Four hours after transfection, the cells were irradiated with 4 Gy and treated with 0.25% Triton X-100, and the nuclei were stained with DAPI for visualization. The GFP-LC3 puncta per cell were counted. The samples were observed using an LSM 510 laser-scanning confocal microscope (Zeiss, Germany).

Coexpression network construction

To construct a coexpression network between autophagy-mediated genes and G2/M checkpoint genes, we analyzed the differential expression of autophagy-mediated mRNAs and G2/M checkpoint mRNAs (Supplementary Table 1) using gene expression data obtained from the GEO database (Accession Nos. GSE65194 and GSE81838) (https://www.ncbi.nlm.nih.gov/) and the breast cancer dataset from the TCGA (https://cancergenome.nih.gov/). The ranks of essential autophagy genes and G2/M checkpoint genes were determined by the absolute differences in their expression between the control and breast cancer groups. For each pair analyzed, we used the Pearson correlation test to detect significant correlations. Only Pearson correlation coefficients ≥0.9 (p < 0.01) were used to construct the network and generate visual representations.
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