

TRIM59 regulates autophagy through modulating both the transcription and the ubiquitination of BECN1

Tianyu Han^{a,b}, Meng Guo^a, Mingxi Gan^a, Bentong Yu^c, Xiaoli Tian^b, and Jian-Bin Wang^a

^aInstitute of Translational Medicine, Nanchang University, Nanchang, Jiangxi, China; ^bSchool of Life Sciences, Nanchang University, Nanchang, Jiangxi, China; ^cDepartment of Cardiovascular Surgery, The First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi, China

ABSTRACT

Macroautophagy/autophagy is a multistep cellular process that sequesters cytoplasmic components for lysosomal degradation. BECN1/Beclin1 is a central protein that assembles cofactors for the formation of a BECN1-PIK3C3-PIK3R4 complex to trigger the autophagy protein cascade. Discovering the regulators of BECN1 is important for understanding the mechanism of autophagy induction. Here, we demonstrate that TRIM59, a tripartite motif protein, plays an important role in autophagy regulation in non-small cell lung cancer (NSCLC). On the one hand, TRIM59 regulates the transcription of *BECN1* through negatively modulating the NFKB pathway. On the other hand, TRIM59 regulates TRAF6 induced K63-linked ubiquitination of BECN1, thus affecting the formation of the BECN1-PIK3C3 complex. We further demonstrate that TRIM59 can mediate K48-linked ubiquitination of TRAF6 and promote the proteasomal degradation of TRAF6. Taken together, our findings reveal novel dual roles for TRIM59 in autophagy regulation by affecting both the transcription and the ubiquitination of BECN1.

Abbreviations: ACTB: actin beta; BECN1: beclin 1; CHX: cycloheximide; CQ: chloroquine; GFP: green fluorescent protein; HA: haemagglutinin tag; His: polyhistidine tag; LC3B: microtubule associated protein 1 light chain 3 beta; NFKB: nuclear factor kappa B; NFKBIA: NFKB inhibitor alpha; NSCLC: non-small cell lung cancer; PIK3C3: phosphatidylinositol 3-kinase catalytic subunit type 3; RELA: RELA proto-oncogene, NF-κB subunit; SQSTM1: sequestosome 1; tGFP: Turbo green fluorescent protein; TRAF6: TNF receptor associated factor 6; TRIM59: tripartite motif containing 59; B: ubiquitin

ARTICLE HISTORY

Received 29 March 2017
Revised 7 June 2018
Accepted 14 June 2018

KEYWORDS

Autophagy; BECN1; NSCLC; TRAF6; TRIM59; ubiquitination

Introduction

Autophagy is an evolutionarily conserved cellular process that is involved in various aspects of cell functions [1,2]. In normal cells, autophagy is used to remove superfluous and damaged organelles and cytosolic proteins for degradation, serving as a surveillance mechanism and a survival strategy to maintain cell viability under stress conditions [3,4]. Disruption of autophagy is involved in diverse human diseases [5,6]. In cancer progression, autophagy plays dual roles, either as a tumor suppressor or promoting tumor progression [7].

Various proteins participate in autophagy. BECN1 is a coiled-coil protein that interacts with BCL2 and is also a central component of the BECN1-PIK3C3-PIK3R4 complex which is indispensable for autophagy induction [1]. BECN1 consists of 3 domains: BH3, coiled-coil domain (CCD) and BARA (the latter includes the ECD), and the roles of BECN1 in autophagy initiation depend on the proteins that bind to these domains [1,8]. For example, the class III phosphatidylinositol 3-kinase (PtdIns3K) interacts with the CCD and ECD to form a complex with BECN1 [9]. This complex increases the production of phosphatidylinositol-3-phosphate (PtdIns3P) that favors phagophore elongation and allows the recruitment of ATG proteins to the

phagophore [1]. Thus, exploring the regulation mechanisms of BECN1 is important for us to reveal the mysteries of autophagy and its functions in human diseases.

The RBCC/TRIM (tripartite motif) proteins is a large family that contains an N-terminal ring domain, 1 or 2 B-box domains and a CCD [10]. TRIM proteins participate in multiple cellular processes including cell proliferation, transcriptional regulation, immunity and cancer progression [11–14]. A series of studies indicated that many TRIM proteins are linked to autophagy [15–27]. Recently, several studies demonstrated that TRIM proteins can act as both autophagy cargo receptors and platforms assembling autophagosome-formation machinery. These TRIMs recruit ULK1 and BECN1 complexes and bind to mammalian Atg8-family paralogs via an LC3-interacting region, while recognizing cargo through the SPRY and potentially other domains [26,27]. In our previous studies, we explored the potential roles of TRIM proteins in non-small cell lung cancer (NSCLC) by the mRNA expression profiling of all TRIM proteins in NSCLC cells [28]. We identified TRIM59 as a potential tumor-promoting gene, but the precise molecular mechanism was obscure. TRIM59 has been reported to have oncogenic activity in mouse models and can be used as a multiple tumor marker for detecting early

tumorigenesis [29,30]. In gastric tumors, TRIM59 promotes gastric carcinogenesis through ubiquitinating and degrading TP53 [11]. However, the role of TRIM59 in regulating autophagy has not been studied yet. In this study, we found an unexpected role of TRIM59 in autophagy regulation. The expression of TRIM59 was reciprocally correlated with BECN1 expression in NSCLC, and TRIM59 knockdown significantly increased the basal levels of autophagy in NSCLC cells. We discovered that TRIM59 could affect the transcription of the *BECN1* gene by negatively regulating the NF κ B pathway. What is more intriguing was that TRIM59 could also regulate autophagy initiation through modulating TRAF6-mediated K63-linked ubiquitination of BECN1. TRIM59 overexpression significantly reduced the TRAF6-induced ubiquitination of BECN1 and disturbed the formation of the BECN1-PIK3C3-PIK3R4 complex, whereas TRIM59 knockdown had an opposite effect. Further studies demonstrated that TRIM59 could directly ubiquitinate TRAF6 in a K48-linkage for proteasomal degradation. Thus, our studies point out a new role of TRIM59 in NSCLC cells and elucidate a novel mechanism of autophagy regulation.

Results

TRIM59 knockdown induces autophagy

In our previous study, we demonstrated that TRIM59 promoted the growth of non-small cell lung cancer [28]. Here, we found an interesting phenomenon that the protein expression level of TRIM59 was inversely related to that of BECN1, an autophagy-related protein, in NSCLC tissues and cancer cells (Figure 1(a, b)). This prompted us to explore if TRIM59 could affect autophagy. To detect the formation of autophagosomes, we transfected a plasmid encoding MAP1LC3B/LC3B (microtubule associated protein 1 light chain 3 beta) fused with the green fluorescent protein (GFP) into H1299 cells. As can be seen from Figure 1c left panel, the GFP-LC3B puncta were significantly increased when TRIM59 was knocked down. The percentage of the cells with GFP-LC3B puncta increased significantly in TRIM59 knockdown cells compared with control H1299 cells (Figure 1(c), right panel).

We next assessed the expression levels of LC3-II and SQSTM1/p62 in TRIM59-silenced cells. As expected, the expression of the autophagosome-associated lipidated form, LC3-II, increased and the expression of SQSTM1 decreased when TRIM59 was knocked down (Figure 1(d)). As we know, autophagy is a highly dynamic

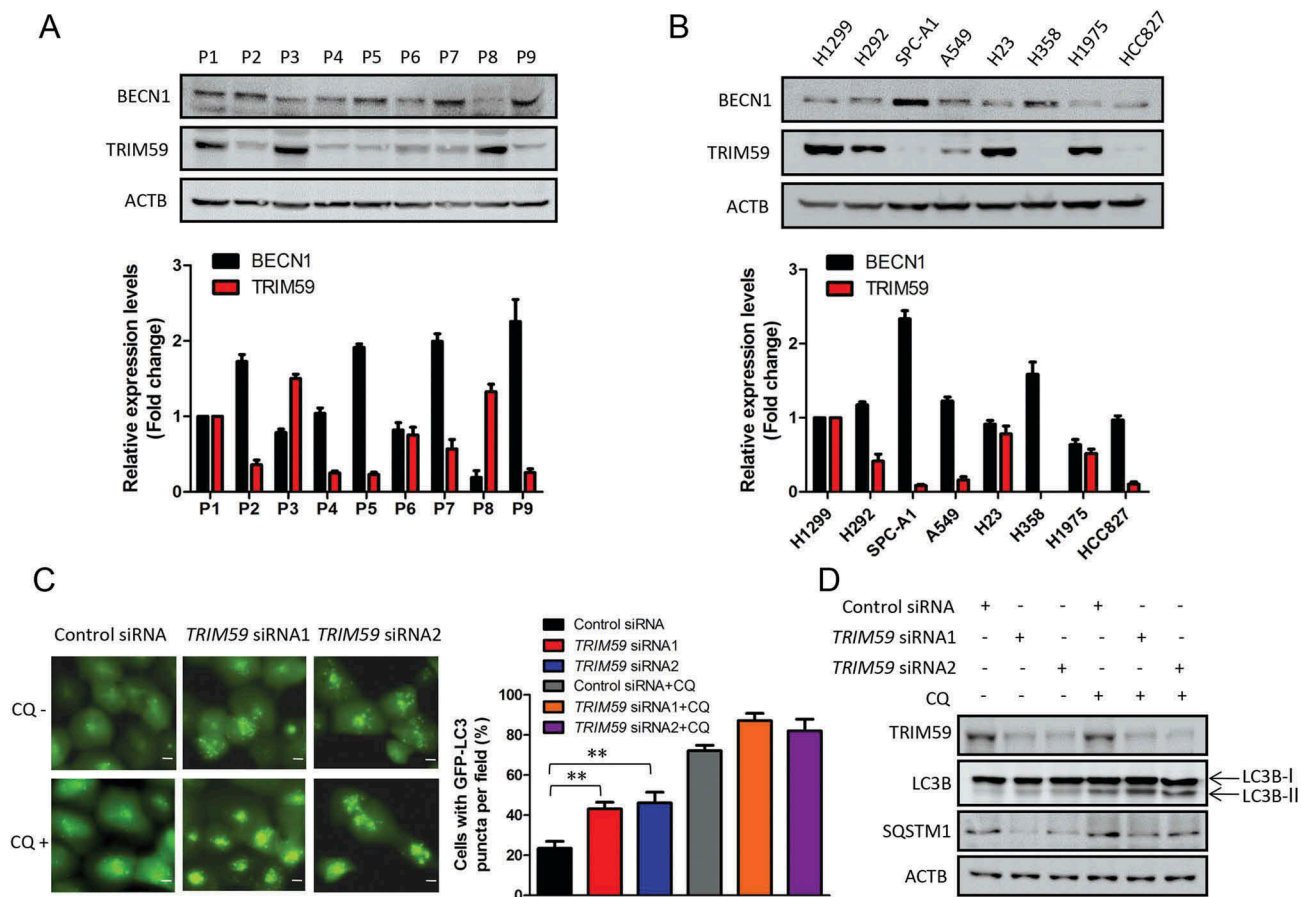


Figure 1. Knocking down TRIM59 induces autophagy. (a) Western blot analysis of the expression levels of BECN1 and TRIM59 in NSCLC tissues (top panel). BECN1 and TRIM59 expression relative to ACTB were quantified. Data represent the average of 3 independent experiments (mean \pm SD) (bottom panel). (b) Western blot analysis of the expression levels of BECN1 and TRIM59 in NSCLC cells (top panel). BECN1 and TRIM59 expression relative to ACTB were quantified. Data represents the average of 3 independent experiments (mean \pm SD) (bottom panel). (c) H1299 cells stably expressing pEGFP-C2-LC3B (GFP-LC3B) were transiently transfected with TRIM59 siRNAs. After 42 h, the cells were treated with or without 20 μ M chloroquine (CQ) for 6 h and then analyzed by fluorescence microscopy (Olympus IX83). Scale bar: 10 μ m (left panel). The cell numbers with GFP-LC3B puncta were counted under 200 \times magnification. $**P \leq 0.01$ (right panel). (d) TRIM59 was knocked down in H1299 cells stably expressing GFP-LC3B and treated with or without 20 μ M chloroquine (CQ) for 6 h. The autophagy-related proteins were analyzed using the indicated antibodies.

process, and the accumulation of autophagosomes and LC3-II levels can be caused by either the induction of autophagy or the inhibition of autophagic flux [31]. In order to discriminate between the 2 distinct mechanisms in our study, we used the autophagy inhibitor chloroquine (CQ) to treat cells. As can be seen in Figure 1(c), the percentage of the cells with LC3B puncta significantly increased when treated with CQ in both control and TRIM59-silenced cells. The expression of LC3-II also increased remarkably following CQ treatment compared with the CQ untreated group (Figure 1(d)). These data proved that TRIM59 knockdown promoted autophagy induction, not autophagy inhibition.

TRIM59 affects the transcription of BECN1 through regulating the NFKB pathway

As the expression of BECN1 was reciprocally correlated with TRIM59 (Figure 1(a, b)), we presumed that TRIM59 affected autophagy by regulating the expression of BECN1. With the increasing expression of exogenous TRIM59 in H1299 cells, the expression of BECN1 decreased in a concentration dependent manner (Figure 2(a)). We next examined the effects of TRIM59 on the mRNA level of BECN1. Overexpression of TRIM59 decreased the BECN1 mRNA level, and knocking down TRIM59 significantly increased the mRNA level of BECN1

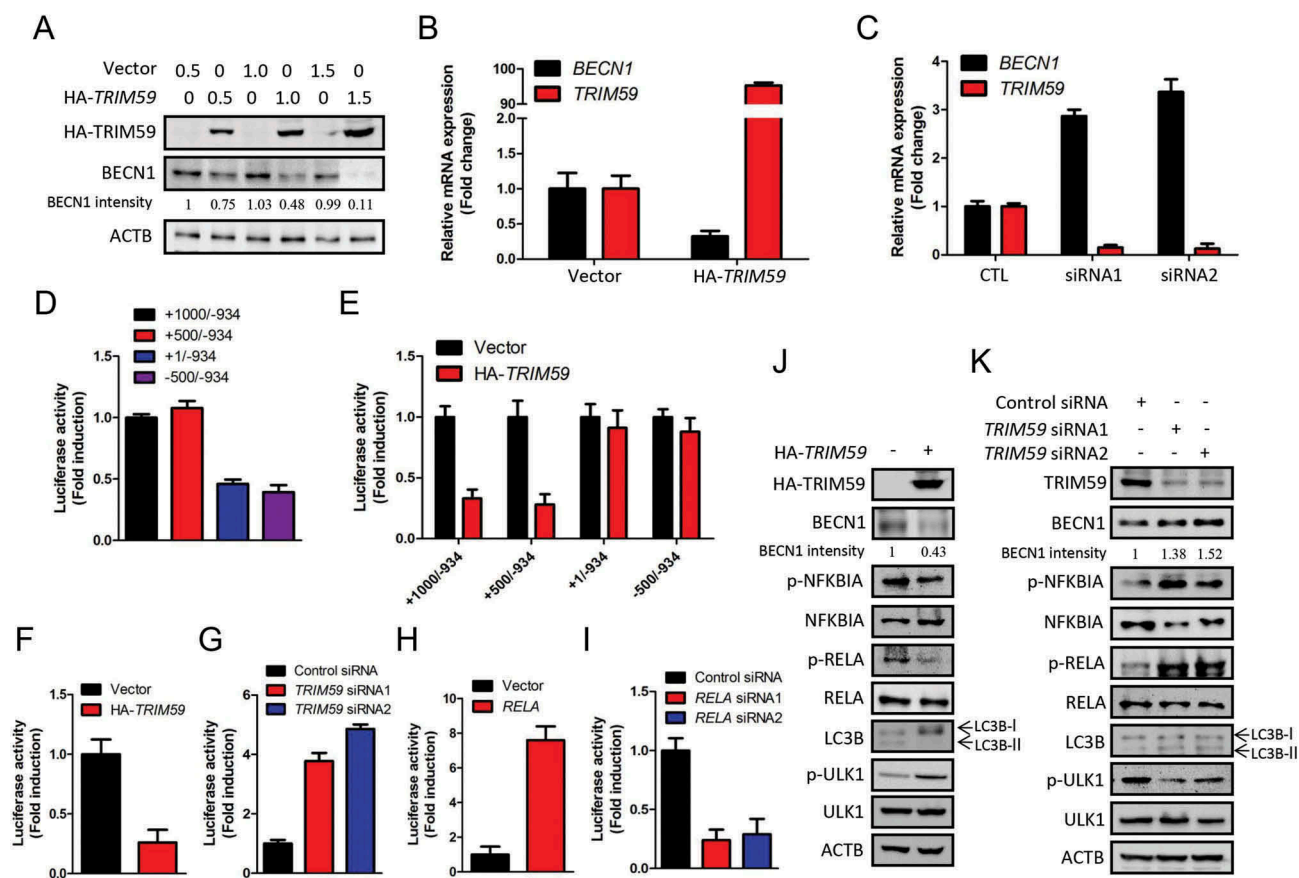


Figure 2. TRIM59 affects the transcription of BECN1. (a) H1299 cells were transfected with pCMV-HA-TRIM59 (HA-TRIM59) with a gradual increase in the amounts of the plasmids. The expressions of BECN1 and exogenous TRIM59 were detected by western blot using anti-BECN1 and anti-HA antibodies. The BECN1 intensity was calculated by normalizing against ACTB. (b) The mRNA expression of BECN1 and TRIM59 in control and TRIM59-overexpressing conditions were determined by Q-PCR. Data represent the average of 3 independent experiments (mean \pm SD). (c) The mRNA expression of BECN1 and TRIM59 in control (CTL) and TRIM59-silenced conditions were determined by Q-PCR. Data represent the average of 3 independent experiments (mean \pm SD). (d) pGL3-enhancer vectors containing different fragments of the BECN1 promoter and Renilla control plasmid were co-transfected into H1299 cells. The relative levels of luciferase activity were normalized to the levels of the + 1000 to -934 fragment and to the levels of luciferase activity of the Renilla control plasmid. Data represent the average of 3 independent experiments (mean \pm SD). (e) pGL3-enhancer vectors containing different fragments of the BECN1 promoter were transfected into H1299 cells, co-transfected with Renilla control plasmid and HA-TRIM59 or empty vector. The relative levels of luciferase activity were normalized to the levels of the vector control and to the levels of luciferase activity of the Renilla control plasmid. Data represent the average of 3 independent experiments (mean \pm SD). (f) pGL3-enhancer vector containing the + 500 to -130 region of the BECN1 promoter were transfected into H1299 cells, co-transfected with Renilla control plasmid and pCMV-HA-TRIM59 plasmid. The relative levels of luciferase activity were normalized to the levels of vector control and to the levels of luciferase activity of the Renilla control plasmid. Data represent the average of 3 independent experiments (mean \pm SD). (g) pGL3-enhancer vector containing the + 500 to -130 region of the BECN1 promoter were transfected into H1299 cells, co-transfected with Renilla control plasmid and control siRNA or TRIM59 siRNAs. The relative levels of luciferase activity were normalized to the levels of control siRNA and to the levels of luciferase activity of the Renilla control plasmid. Data represent the average of 3 independent experiments (mean \pm SD). (h) pGL3-enhancer vector containing the + 500 to -130 region of the BECN1 promoter was transfected into H1299 cells, co-transfected with Renilla control plasmid and pCDNA3.0-RELA plasmid. The relative levels of luciferase activity were normalized to the levels of vector control and to the levels of luciferase activity of the Renilla control plasmid. Data represent the average of 3 independent experiments (mean \pm SD). (i) pGL3-enhancer vector containing the + 500 to -130 region of the BECN1 promoter was transfected into H1299 cells, co-transfected with Renilla control plasmid and control siRNA or RELA siRNAs. The relative levels of luciferase activity were normalized to the levels of control siRNA and to the levels of luciferase activity of the Renilla control plasmid. Data represent the average of 3 independent experiments (mean \pm SD). (j) The pCMV-HA-TRIM59 plasmid was transfected into H1299 cells. After 48 h, the expression of the indicated proteins was determined by western blot. The BECN1 intensity was calculated by normalizing against ACTB. (k) The control siRNA or TRIM59 siRNAs were transfected into H1299 cells. After 48 h, the expression of the indicated proteins was determined by western blot. The BECN1 intensity was calculated by normalizing against ACTB.

(Figure 2(b, c)). These results indicated that TRIM59 might regulate the transcription of *BECN1*. We generated constructs with different fragments of the *BECN1* promoter region in the pGL3-enhancer vector and then performed a luciferase activity assay. From Figure 2d, we found that the promoter fragments + 1000 to -934 and + 500 to -934 showed higher activity than the others. We then detected the effects of TRIM59 on the activity of these promoter fragments. Overexpression of TRIM59 reduced the promoter activity of the + 1000 to -934 and + 500 to -934 fragments, whereas the + 1 to -934 and -500 to -934 fragments remained unchanged (Figure 2(e)). These results proved that the promoter region containing + 500 to + 1 might be important for TRIM59 to regulate the transcription of *BECN1*.

We next inserted the promoter region containing + 500 to -130 into the pGL3-enhancer vector and performed a luciferase activity assay in TRIM59-overexpressing and TRIM59-silenced conditions. Figure 2f and g show that the activity of this promoter region was sensitive to the expression level of TRIM59. The activity of this promoter fragment decreased when TRIM59 was overexpressed and increased when TRIM59 was knocked down. These results confirmed that TRIM59 regulated the transcription of *BECN1* through the + 500 to + 1 region of the *BECN1* promoter.

Because TRIM59 is not a transcription factor, we presumed that TRIM59 regulated the transcription of *BECN1* through modulating the activity of a certain transcription factor. By analysis of the promoter region in + 500 to + 1, we identified a RELA/p65 (a key subunit of NF κ B) binding site: GGGGATTTC, located in + 317 to + 308. We next detected the promoter activity of the + 500 to -130 fragment by modulating the expression of RELA. The promoter activity increased remarkably when RELA was overexpressed and decreased when RELA was knocked down (Figure 2(h, i)). Overexpressing RELA promoted the expression of *BECN1*, and knocking down RELA reduced the expression of *BECN1* (Fig. S1A-S1D). These results demonstrated that RELA could regulate the transcription of *BECN1*. A similar result was also reported by Copetti *et al.* in 2009 [32].

We next detected the effects of TRIM59 on the NF κ B pathway and autophagy. Overexpression of TRIM59 blocked the activation of the NF κ B pathway, as can be seen from the reduced expression of phosphorylated RELA and phosphorylated NF κ BIA. ULK1 (unc-51 like autophagy activating kinase 1) is an important regulator in the autophagy pathway, and the phosphorylation of ULK1 at Ser757 is closely related to the repression of autophagy induction [33]. TRIM59 overexpression increased the expression of p-ULK1 (Ser757) and decreased the expression of LC3-II, indicating the repression of autophagy (Figure 2(j)), whereas TRIM59 knockdown activated the NF κ B pathway and induced autophagy (Figure 2(k)). According to these results, we demonstrated that TRIM59 could affect the transcription of *BECN1* through regulating the NF κ B pathway, and thus affected autophagy.

TRIM59 affects the ubiquitination level of *BECN1*

As TRIM59 possessed E3 ligase function [11], we examined if TRIM59 could affect the stability of the *BECN1* protein. We first tested the stability of *BECN1* in physiological conditions

by adding cycloheximide (CHX) to block protein translation in H1299 cells. The protein level of *BECN1* decreased significantly when cells were treated with CHX for 6 h and this effect could be recovered by adding MG132, a proteasomal inhibitor, but not the lysosomal inhibitor chloroquine (CQ) (Figure 3(a, b)). These results indicated that *BECN1* was degraded via a proteasomal degradation pathway. To determine whether TRIM59 regulates the stability of *BECN1*, we examined the protein levels of *BECN1* in the presence of CHX under TRIM59-overexpressing or -silenced conditions. Figure 3c and d showed that TRIM59 did not affect the stability of *BECN1*. We next detected whether the ubiquitination of *BECN1* was affected by TRIM59. Unexpectedly, the ubiquitination level of *BECN1* was inversely related to TRIM59 expression levels. Knocking down TRIM59 remarkably increased the ubiquitination level of *BECN1* and overexpressing TRIM59 had an opposite effect (Figure 3(e, f) and Fig. S2A, S2B). From these results, we concluded that TRIM59 did not affect the stability of the *BECN1* protein through a proteasomal degradation pathway. However, TRIM59 strongly affected the ubiquitination level of *BECN1* and these changes in ubiquitination did not influence the stability of the *BECN1* protein.

TRIM59 affects traf6-mediated ubiquitination of *BECN1*

Previous studies have demonstrated that upregulating K63-linked ubiquitination of *BECN1* promotes autophagy [34]. According to our previous results in Figure 3e and f, we supposed that TRIM59 might also regulate autophagy through modifying K63-linked ubiquitination of *BECN1*. To test this hypothesis, we used a K63R mutant of ubiquitin to test its effects on the ubiquitination of *BECN1* when TRIM59 was knocked down. Figure 4a showed that the increased ubiquitination of *BECN1* induced by TRIM59 knockdown was not observed when the wild-type ubiquitin was replaced with the K63R mutant. We used an antibody specific for K63-linked ubiquitin for western blot. The results revealed that TRIM59 knockdown significantly increased the K63-linked ubiquitination of *BECN1* (Figure 4(b)). TRAF6 was reported to directly ubiquitinate *BECN1* through a K63 linkage [34]. In H1299 cells, we found that TRAF6 could bind to *BECN1* (Figure 4(c)). Thus, we examined if TRIM59 affected TRAF6-mediated ubiquitination. We found that overexpression of TRAF6 increased the ubiquitination of *BECN1*, and overexpression of TRIM59 remarkably reduced the TRAF6-induced ubiquitination (Figure 4(d)). These results indicated that, except for the influence on the transcription of *BECN1*, TRIM59 might also affect autophagy through interfering with TRAF6 mediated K63-linked ubiquitination of *BECN1*.

We wondered whether TRIM59-regulated TRAF6 induced autophagy. H1299 cells stably expressing GFP-LC3B were transfected with a plasmid encoding HA-TRAF6 alone or co-transfected with HA-TRAF6- and HA-TRIM59-encoding plasmids. The autophagy induced by TRAF6 overexpression was greatly prohibited by TRIM59 overexpression. The cell numbers with GFP-LC3B puncta were increased when transfecting with a TRAF6 plasmid alone, whereas a significant reduction was observed when the cells were co-transfected

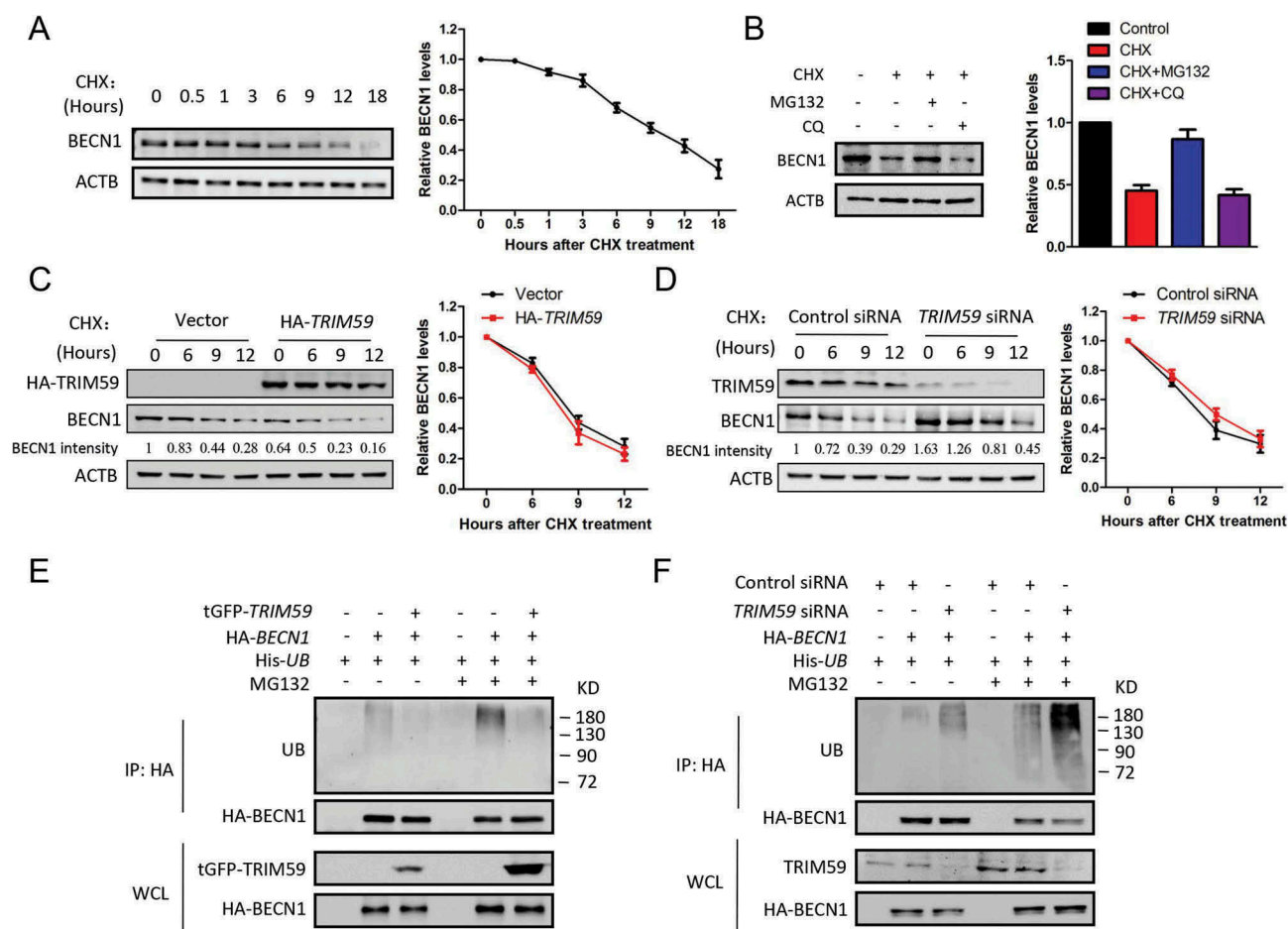


Figure 3. TRIM59 affects the ubiquitination of BECN1. (a) H1299 cells were treated with 25 μ g/ml cycloheximide (CHX) for different times and the expression of BECN1 was detected by western blot (left panel). BECN1 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (b) H1299 cells were treated with 25 μ g/ml CHX alone or 25 μ g/ml CHX plus 20 μ M MG132 or 25 μ g/ml CHX plus 20 μ M chloroquine (CQ) for 9 h. The BECN1 expression was detected by western blot (left panel). BECN1 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (c) H1299 cells were transfected with HA-TRIM59, treated with 25 μ g/ml CHX for different time points and then immunoblotted with antibodies against HA, BECN1 and ACTB. The BECN1 intensity was calculated by normalizing against ACTB (left panel). BECN1 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (d) H1299 cells were transfected with control or TRIM59 siRNA, treated with 25 μ g/ml CHX for different time points and then immunoblotted with antibodies against TRIM59, BECN1 and ACTB. The BECN1 intensity was calculated by normalizing against ACTB (left panel). BECN1 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (e) H1299 cells were co-transfected with plasmids encoding HA-BECN1, His-UB and with or without tGFP-TRIM59. After 42 h, the cells were treated with or without MG132 for 6 h. Proteins were immunoprecipitated with HA antibody. The ubiquitination was detected using anti-UB antibody. Exogenous BECN1 and TRIM59 were detected using anti-HA and anti-tGFP antibodies. (f) H1299 cells were co-transfected with plasmids encoding HA-BECN1, His-UB and with or without TRIM59 siRNA. After 42 h, the cells were treated with or without MG132 for 6 h. Then, proteins were immunoprecipitated with HA antibody, and the ubiquitination was detected using anti-UB antibody. The exogenous BECN1 and endogenous TRIM59 were detected using anti-HA and anti-TRIM59 antibodies.

with plasmids encoding TRAF6 and TRIM59 (Figure 4(e)). Overexpression of TRIM59 also decreased the expression of LC3B-II, whereas it increased SQSTM1 expression compared with overexpression of TRAF6 alone (Figure 4(f)).

BECN1 plays a central role in autophagy through participating in the formation of the class III phosphatidylinositol 3-kinase (PtdIns3K) complex [1]. PIK3C3 is the catalytic subunit of PtdIns3K and interacts with BECN1 to form a complex when autophagy is induced. As TRIM59 could affect autophagy by modifying the ubiquitination of BECN1, we tested the effects of TRIM59 on the formation of the BECN1-PIK3C3 complex. From Figure 4g, we could see that TRIM59 knockdown enhanced the interaction between BECN1 and PIK3C3. However, overexpression of TRIM59 significantly reduced the PIK3C3 protein levels precipitated by HA-BECN1 and this effect was recovered by TRAF6

overexpression (Figure 4h). These results demonstrated that TRIM59 could also affect autophagy through regulating the TRAF6-induced K63-linked ubiquitination of BECN1, thus modulating the formation of the PtdIns3K complex.

TRIM59 regulates the degradation of TRAF6 by ubiquitination

We already proved that TRIM59 could interfere with the E3 ubiquitin ligase function of TRAF6. An important question that needed to be answered was how TRIM59 regulated TRAF6. As shown in Figure 5a, knocking down TRIM59 apparently increased the expression of TRAF6. When the expression of exogenous TRIM59 increased, the expression of TRAF6 decreased in a concentration-dependent manner

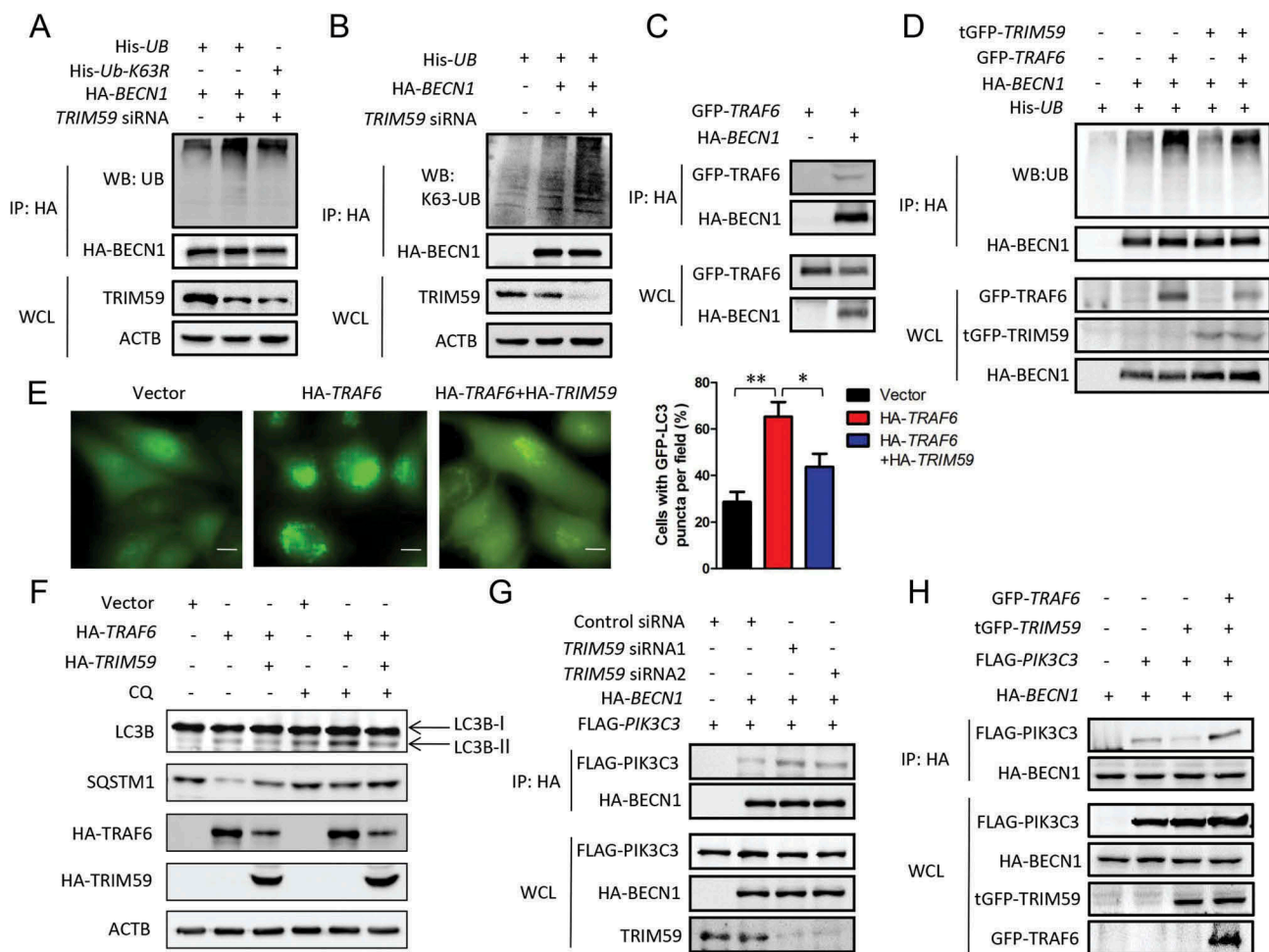


Figure 4. TRIM59 inhibits TRAF6-mediated ubiquitination of BECN1. (a) H1299 cells were co-transfected with plasmids encoding His-UB or His-UB-K63R, HA-BECN1 and either TRIM59 siRNA or control siRNA. Proteins were immunoprecipitated with HA antibody, and the ubiquitination was detected using an antibody specifically targeting ubiquitin. Exogenous BECN1 was detected using anti-HA antibody. Endogenous TRIM59 was detected using anti-TRIM59 antibody. (b) H1299 cells were co-transfected with His-UB, HA-BECN1 and either TRIM59 siRNA or control siRNA. Proteins were immunoprecipitated with HA antibody and the K63 linked ubiquitination was detected using an antibody specifically targeting ubiquitin that was K63 linked. (c) H1299 cells were co-transfected with plasmids encoding GFP-TRAF6 and either HA-BECN1 or empty vector. The cell lysates were immunoprecipitated with HA antibody and blotted with anti-HA or anti-GFP antibodies. (d) 293T cells were co-transfected with the indicated plasmids. Proteins were immunoprecipitated with HA antibody, and the ubiquitination was detected using an antibody specifically targeting ubiquitin. Exogenous BECN1, TRIM59 and TRAF6 were detected using anti-HA, anti-tGFP and anti-GFP antibodies. (e) H1299 cells stably expressing GFP-LC3B were co-transfected with pCMV-HA-TRAF6 (HA-TRAF6) and either pCMV-HA-TRIM59 (HA-TRIM59) or empty vector. After 48 h, the cells were analyzed by fluorescence microscopy (Olympus IX83). Scale bar: 10 μ m (left panel). The cell numbers with GFP-LC3B puncta were counted under 200 \times magnification. * $P \leq 0.05$, ** $P \leq 0.01$ (right panel). (f) H1299 cells stably expressing GFP-LC3B were co-transfected with a plasmid encoding HA-TRAF6 and either HA-TRIM59 or empty vector. After 46 h, the cells were treated with or without 20 μ M chloroquine (CQ) for 2 h. The autophagy-related proteins were analyzed by western blot. (g) H1299 cells were co-transfected with the indicated exogenous genes and siRNAs. After 48 h, the cells were lysed and immunoprecipitated with HA antibody. FLAG-PIK3C3 and HA-BECN1 were detected using anti-FLAG and anti-HA antibodies. TRIM59 was detected using anti-TRIM59 antibody. (h) 293T cells were co-transfected with HA-BECN1 and the indicated exogenous genes. Two days later, the cells were lysed and immunoprecipitated with HA antibody. FLAG-PIK3C3 and HA-BECN1 were detected using anti-FLAG and anti-HA antibodies. GFP-TRAF6 and tGFP-TRIM59 were detected with anti-GFP and anti-tGFP antibodies.

(Figure 5(b)). To investigate if TRIM59 affected the stability of TRAF6, we detected the stability of TRAF6 protein in physiological conditions by adding CHX for different times in H1299 cells. The expression of TRAF6 decreased significantly when treated with CHX for 3 h (Figure 5(c)) and the decreased expression of TRAF6 could be recovered by adding MG132, but not CQ (Fig. S3A). This observation indicated that TRAF6 protein was degraded in a proteasomal degradation pathway.

To determine whether TRIM59 regulates the stability of TRAF6, we examined the protein levels of TRAF6 in the presence of CHX under TRIM59-overexpressing or -silenced conditions. Figure 5d and e show that overexpression of TRIM59 accelerated the degradation rate of TRAF6, whereas

knocking down TRIM59 stabilized the protein level of TRAF6. In order to figure out if TRIM59 could interacted with TRAF6, we performed co-immunoprecipitation and GST affinity isolation experiments and found that TRIM59 could bind to TRAF6 (Figure 5(f-h) and Fig. S3B). As TRIM59 has an E3 ubiquitin ligase function, we detected the effect of TRIM59 on the ubiquitination of TRAF6. Figure 5i showed that TRIM59 knockdown reduced the ubiquitination level of endogenous TRAF6. Overexpression of TRIM59 increased the ubiquitination level of TRAF6, whereas it decreased the protein expression of TRAF6, indicating the ubiquitin-mediated degradation of TRAF6 (Figure 5j).

TRAF6 was reported to possess both K48-linked and K63-linked ubiquitination [35–39], we next used ubiquitin mutants

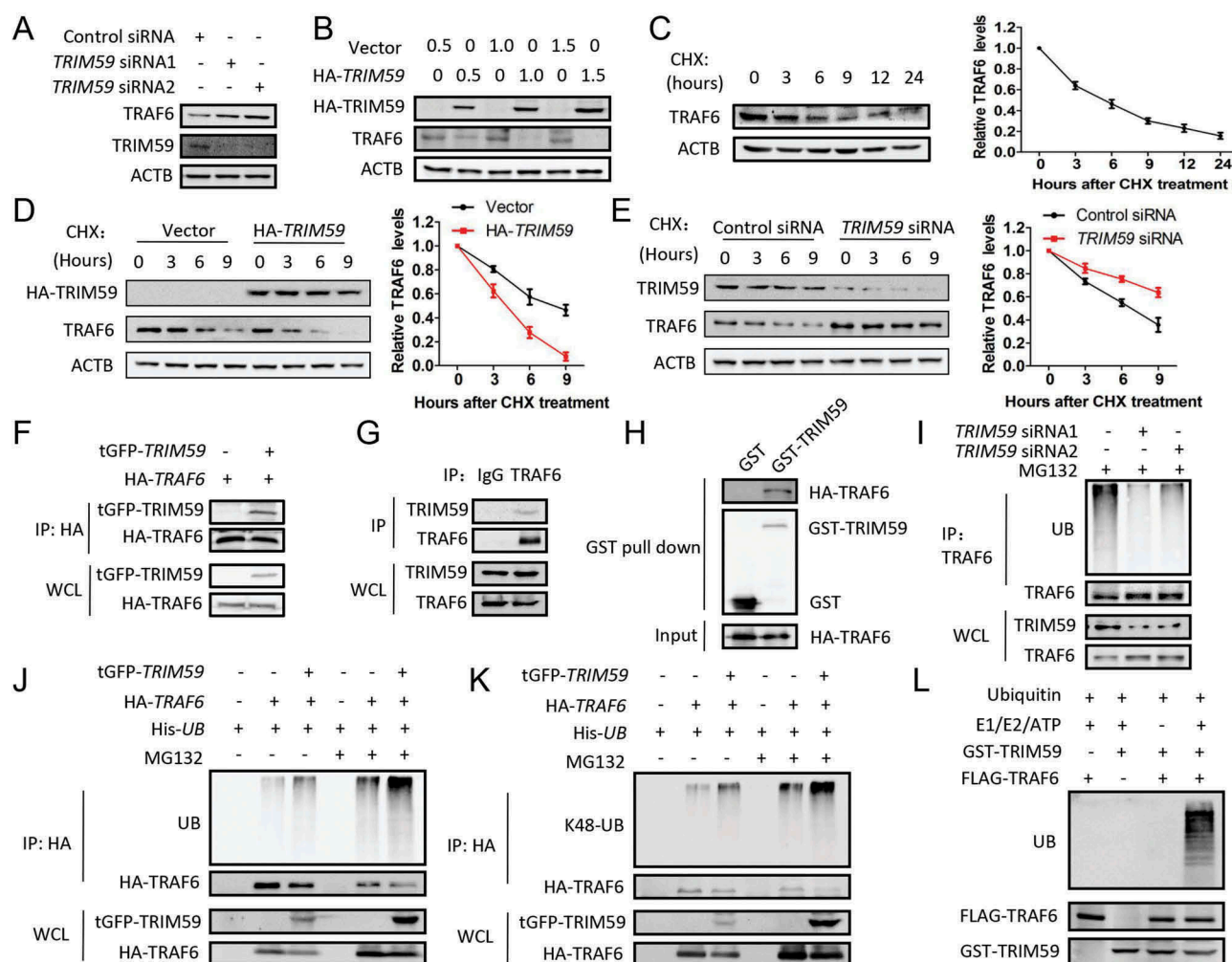


Figure 5. TRIM59 regulates the degradation of TRAF6 by ubiquitination. (a) TRIM59 was knocked down in H1299 cells and protein expression was detected using the indicated antibodies. (b) H1299 cells were transfected with a plasmid encoding HA-TRIM59 with gradually increasing the amount of the plasmid. The expression of TRAF6 and TRIM59 was detected by western blot using anti-TRAF6 and anti-HA antibodies. (c) H1299 cells were treated with 25 μ M cycloheximide (CHX) for different times and the TRAF6 expression was detected by western blot (left panel). TRAF6 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (d) H1299 cells were transfected with a plasmid encoding HA-TRIM59, treated with 25 μ M CHX for different time points and then immunoblotted with antibodies against HA, TRAF6 and ACTB (left panel). TRAF6 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (e) H1299 cells were transfected with control or TRIM59 siRNA, treated with 25 μ M CHX for different time points and then immunoblotted with antibodies against TRIM59, TRAF6 and ACTB (left panel). TRAF6 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (f) H1299 cells were co-transfected with a plasmid encoding HA-TRAF6 and either tGFP-TRIM59 or empty vector. The cell lysates were immunoprecipitated with HA antibody and blotted with HA and tGFP antibodies. (g) The lysate of H1299 cells was immunoprecipitated using TRAF6 antibody or normal rabbit IgG, and blotted with TRAF6 and TRIM59 antibodies. (h) GST-TRIM59 purified with glutathione Sepharose beads was incubated with extracts from HA-TRAF6-transfected 293T cells. Western blot was performed to detect the indicated proteins using anti-HA and anti-GST antibodies. (i) H1299 cells were transfected with TRIM59 siRNAs. After 42 h, the cells were treated with or without MG132 for 6 h. The lysates were immunoprecipitated using TRAF6 antibody and blotted with the indicated antibodies. (j) H1299 cells were co-transfected with a plasmid encoding His-UB, HA-TRAF6 and either tGFP-TRIM59 or empty vector. After 42 h, the cells were treated with or without MG132 for 6 h. Proteins were immunoprecipitated with HA antibody. The ubiquitination was detected using an antibody specific for ubiquitin, and TRAF6 and TRIM59 were detected using anti-HA and anti-tGFP antibodies. (k) H1299 cells were co-transfected with a plasmid encoding His-UB, HA-TRAF6 and either tGFP-TRIM59 or empty vector. Proteins were immunoprecipitated with HA antibody. The K48-linked ubiquitination was detected using an antibody specifically targeting ubiquitin that was K48 linked. TRAF6 and TRIM59 were detected using anti-HA and anti-tGFP antibodies. (l) The purified GST-TRIM59 was incubated with the indicated reaction component. The reaction mixture was subjected to western blot using antibodies against ubiquitin, Flag and GST.

specific for K48 (K48R) or K63 (K63R) to check the changes in the ubiquitination level of TRAF6 induced by TRIM59. The increased ubiquitination levels of TRAF6 induced by TRIM59 overexpression was not observed when transfecting with a plasmid encoding the K48R mutant; however, the increased ubiquitination levels of TRAF6 could still be observed when transfecting with a plasmid encoding the K63R mutant, indicating that TRIM59 induced a K48-linked ubiquitination of TRAF6 (Fig. S4A, S4B). We further examined these results using an antibody specific for K48-linked ubiquitination.

Figure 5k showed that the enhanced ubiquitination of TRAF6 induced by TRIM59 was indeed K48-linked. To provide evidence that TRIM59 directly ubiquitinated TRAF6, we performed an in vitro ubiquitination assay. As can be seen in Figure 5l, the polyubiquitin bands were observed when purified TRAF6 was added to the reaction system containing ubiquitin, UBA1/UBE1, UBE2D1, ATP and TRIM59. All these results pointed out that TRIM59 could directly regulate the degradation of TRAF6 in a ubiquitin-mediated proteasomal degradation process.

As TRAF6 was originally described as an important regulator for the NFKB pathway [40,41], we wanted to explore if the inhibition of BECN1 expression was also dependent on TRAF6 degradation induced by TRIM59. Figure S5A and S5B show that TRAF6 overexpression could not rescue the reduction of both the mRNA and protein level of *BECN1*/*BECN1* triggered by TRIM59. These results demonstrated that TRIM59 affected the expression of *BECN1* through a TRAF6-independent NFKB pathway.

The ring domain of TRIM59 is important for regulating traf6-induced autophagy

The ring domain of TRIM family proteins has been shown to be essential for their protein degradation function [10,11]. To get further insight into the mechanism for TRIM59 to ubiquitinate TRAF6, we constructed a TRIM59 mutant lacking the ring domain (ΔR). We found that this mutant could still interact with TRAF6 (Figure 6(a)). However, the mutant lost the ability to destabilize the TRAF6 protein (Figure 6(b)). Besides, the ability to ubiquitinate TRAF6 was also abolished (Figure 6(c)). These findings demonstrated that the ring domain of TRIM59 was important for the degradation of TRAF6.

We next detected the inhibitory effect of ΔR on the TRAF6-induced autophagy. In cells co-transfected with plasmids encoding TRAF6 and ΔR , the expression of LC3-II was not changed when compared with the cells transfected with the TRAF6 plasmid alone. Also, the expression levels of SQSTM1 were similar between these 2 groups (Figure 6(d)). In the group co-transfected with plasmids for HA-TRAF6 and

HA- ΔR , the percentage of the cells with GFP-LC3B puncta was not changed significantly compared with the group transfected with the HA-TRAF6 plasmid alone (Fig. S6A). These data demonstrated that overexpressing ΔR did not block the autophagy process induced by TRAF6. Then, we tested the ubiquitination function of this mutant on BECN1. Overexpressing ΔR did not change the ubiquitination of BECN1, and the inhibitory effect of TRIM59 on autophagy was not observed for this mutant (Fig. S6B, S6C). Taken together, the ring domain of TRIM59 is important for regulating the ubiquitination of TRAF6 and the TRAF6-induced autophagy.

Both the transcription and the ubiquitination of BECN1 are necessary for the autophagy induced by TRIM59 knockdown

As TRIM59 affects both the expression and the K63-linked ubiquitination of BECN1, we next wanted to examine whether both effects were necessary for autophagy induced by TRIM59 knockdown. *RELA* siRNAs were co-transfected with *TRIM59* siRNA into H1299 cells stably expressing GFP-LC3B. Figure 7a showed that the GFP-LC3B puncta were significantly increased when TRIM59 was knocked down. In addition, the percentage of the cells with GFP-LC3B puncta increased significantly; however, knocking down *RELA* decreased the total GFP-LC3B puncta and the cell numbers with GFP-LC3B puncta. The expression of LC3-II was increased when knocking down TRIM59, whereas knocking down *RELA* reversed these effects. In Figure 7b, *TRAF6* siRNAs were transfected into H1299 cells with TRIM59 knockdown. We discovered increased GFP-LC3B

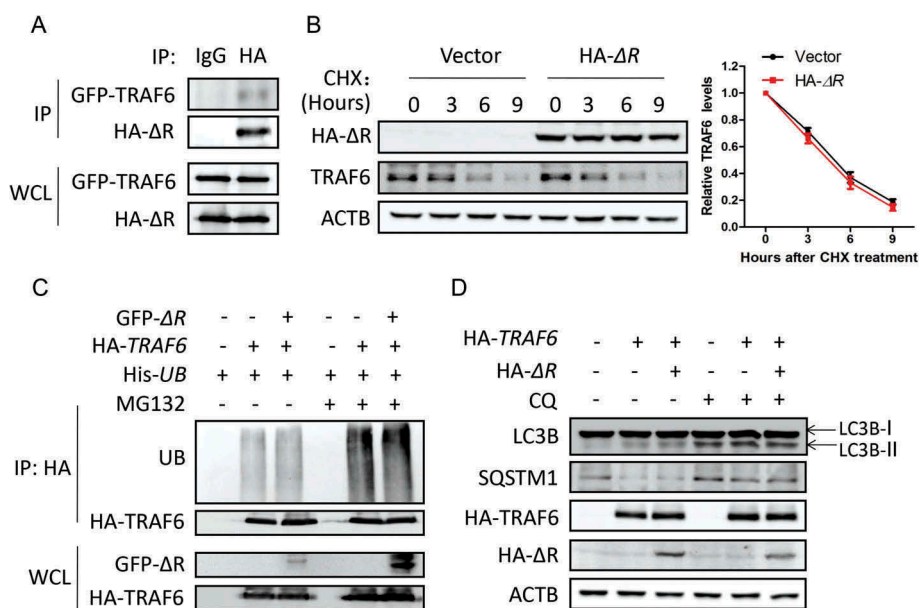


Figure 6. The ring domain of TRIM59 is important for regulating TRAF6-induced autophagy. (a) H1299 cells were co-transfected with a plasmid encoding GFP-TRAF6 and HA- ΔR . The cell lysates were immunoprecipitated with HA antibody or normal mouse IgG, and blotted with HA and GFP antibodies. (b) H1299 cells were transfected with HA- ΔR , treated with 25 μ M CHX for different time points and then immunoblotted with antibodies against HA, TRAF6 and ACTB (left panel). TRAF6 expression relative to ACTB was quantified. Data represent the average of 3 independent experiments (mean \pm SD) (right panel). (c) H1299 cells were co-transfected with a plasmid encoding His-UB, HA-TRAF6 and either GFP- ΔR or empty vector. After 42 h, the cells were treated with or without MG132 for 6 h. Proteins were immunoprecipitated with HA antibody. The ubiquitination was detected using an antibody specific for ubiquitin, and TRAF6 and ΔR were detected using anti-HA and anti-GFP antibodies. (d) H1299 cells stably expressing GFP-LC3B were co-transfected with a plasmid encoding HA-TRAF6 and either HA- ΔR or empty vector. After 42 h, the cells were treated with or without 20 μ M chloroquine (CQ) for 6 h. The autophagy-related proteins were analyzed by western blot.

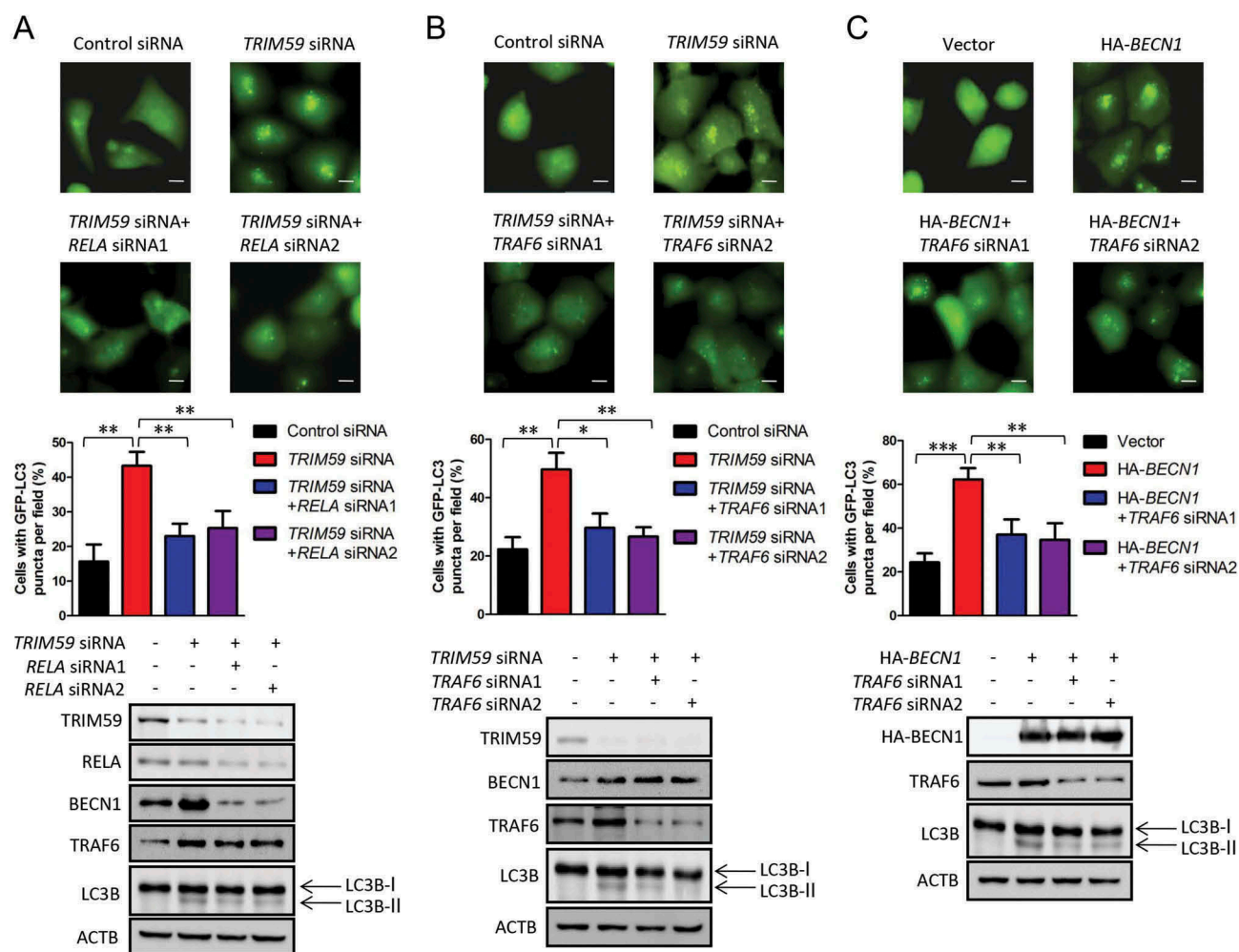


Figure 7. Both the transcription of *BECN1* and the ubiquitination of BECN1 are necessary for the autophagy induced by TRIM59 knockdown. (a) *TRIM59* siRNA was co-transfected with or without *RELA* siRNAs into H1299 cells stably expressing GFP-LC3B. After 48 h, the cells were analyzed by fluorescence microscopy (Olympus IX83). Scale bar: 10 μ m (upper panel). The cell numbers with GFP-LC3B puncta were counted under 200 \times magnification. $**P \leq 0.01$ (middle panel). The expression of related proteins was examined by western blot (bottom panel). (b) *TRIM59* siRNA was co-transfected with or without *TRAF6* siRNAs into H1299 cells stably expressing GFP-LC3B. After 48 h, the cells were analyzed by fluorescence microscopy (Olympus IX83). Scale bar: 10 μ m (upper panel). The cell numbers with GFP-LC3B puncta were counted under 200 \times magnification. $*P \leq 0.05$, $**P \leq 0.01$ (middle panel). The expression of related proteins was examined by western blot (bottom panel). (c) *HA-BECN1* was co-transfected with or without *TRAF6* siRNAs into H1299 cells stably expressing GFP-LC3B. After 48 h, the cells were analyzed by fluorescence microscopy (Olympus IX83). Scale bar: 10 μ m (upper panel). The cell numbers with GFP-LC3B puncta were counted under 200 \times magnification. $**P \leq 0.01$, $***P \leq 0.001$ (middle panel). The expression of related proteins was examined by western blot (bottom panel).

puncta and enhanced expression of LC3-II when TRIM59 was knocked down, whereas TRAF6 knockdown remarkably reduced the LC3B puncta and LC3-II expression induced by TRIM59 knockdown.

Noting that the expression level of BECN1 remained unchanged between the cells transfected with *TRIM59* siRNA and the cells co-transfected with *TRIM59* and *TRAF6* siRNAs, we proposed that both the expression and the ubiquitination of BECN1 were important for autophagy induction. To further demonstrate this, we overexpressed BECN1 in H1299 cells stably expressing GFP-LC3B with or without TRAF6 knockdown. We found that BECN1 overexpression strongly induced autophagy as determined by increased cell numbers with GFP-LC3B puncta and enhanced expression of LC3-II, whereas TRAF6 knockdown decreased the effects (Figure 7(c)). A previous study demonstrated that the Lys117 of BECN1 was the major ubiquitination site for K63-linkage and the Glu299 of BECN1 was important for the recruitment of TRAF6 to BECN1 [34]. We then mutated

these 2 residues of BECN1 and assessed the autophagy induction effects. As shown in Figure S7A-S7C, the autophagy induction effects for BECN1^{K117R} and BECN1^{E299A} were significantly attenuated compared with BECN1 wild-type. These results demonstrated that both the transcription and the ubiquitination of BECN1 were important for autophagy induced by TRIM59 knockdown.

Discussion

BECN1 plays a central role in autophagy that triggers a cascade of proteins involved in autophagosome formation [1]. The regulation of BECN1 can occur at the level of transcription, translation and post-translational modifications [8]. At the transcription level, the transcription factor E2F1 and NF κ B can bind to the promoter region of the *BECN1* gene to enhance transcription [32,42]. However, another transcription factor, SMAD2, negatively regulates the transcription of

BECN1 and thus represses autophagy in endothelial cells [43]. These studies demonstrate that modulating the expression of *BECN1* is an important event in autophagy induction. In the current study, we found that the expression of *TRIM59* and *BECN1* were inversely correlated in NSCLC. Further studies demonstrated that *TRIM59* repressed the transcription of *BECN1*. Although *TRIM59* is not a transcription factor, it acted as a negative regulator of the NF κ B pathway, and thus affected *BECN1* expression. This was one of the mechanisms for *TRIM59* to regulate autophagy determined in our study. *TRIM59* has now been accepted as an important oncoprotein, and the molecular mechanisms for its tumor-promoting functions are very preliminary. Previous studies proved that *TRIM59* could also affect the TP53 pathway, SMAD2/3 pathway and AKT pathway [11,44–46]. These findings together with ours indicated that *TRIM59* has many profound functions including the effect on autophagy, and these functions are important for cancer progression.

Post-translational modifications (PTMs) of *BECN1* including phosphorylation, acetylation and ubiquitination are essential for autophagy induction [47]. Ubiquitination of *BECN1* affects either its protein stability or functions. NEDD4 was reported to ubiquitinate *BECN1* with a K11-linkage for proteasomal degradation when *PIK3C3/VPS34* is depleted [48]. In another study, USP19 was reported to rescue the proteasomal degradation of *BECN1* through removing the K11-linked ubiquitination [49]. K63-linked ubiquitination of *BECN1* is critical for TLR4-induced autophagy, and TRAF6 functions as an E3 ligase in this process [34]. In our study, we found that except for the effect on the transcription of *BECN1*, *TRIM59* knockdown induced autophagy accompanied by the upregulation of *BECN1* ubiquitination. As *TRIM59* functions as an E3 ligase, this result was in contrast with our initial idea that *TRIM59* inhibited autophagy by ubiquitination and degradation of *BECN1*. What interesting results we found were that *TRIM59* regulated K63-linked ubiquitination of *BECN1* through direct ubiquitination and degradation of TRAF6, and the ring domain of *TRIM59* was essential in this process. We finally demonstrated that both the transcriptional effects on *BECN1* by *RELA* and the binding of TRAF6 to *BECN1* were essential for autophagy induced by *TRIM59* knockdown. In conclusion, we discovered the dual roles of *TRIM59* in autophagy regulation by regulating both the transcription and K63-linked ubiquitination of *BECN1*.

Until now, many studies have proved that TRIM proteins are associated with autophagy. The expression of *TRIM55* parallels that of autophagy proteins NBR1, SQSTM1 and LC3 during cardiac myofibril assembly and turnover [15]. *TRIM30* localizes in cytoplasmic bodies with ubiquitin chains and the autophagy marker LC3, and is degraded through both proteasome and autophagy pathways [16]. *TRIM11* can act as a receptor to recruit AIM2 to SQSTM1 for selective autophagy degradation upon DNA virus infection [17]. Another study demonstrated that *TRIM23* is essential for autophagy in response to viral infection and a *TRIM23*-TBK1-SQSTM1 axis is a key component in selective autophagy [18]. *TRIM13* regulates the initiation of autophagy during ER stress and *TRIM13*-induced autophagy is essential for ER stress-induced

caspase activation and cell death [19,20]. The interaction between *TRIM16* and *LGALS3* orchestrates the recruitment of core autophagic factors and activates selective autophagy in response to damaged endomembranes [21]. Besides, *TRIM16* can also act as a receptor for IL1 β and interact with *SEC22B* to recruit cargo to LC3-II-positive sequestration membrane in response to lysosomal damage, a process termed secretory autophagy [22]. In ATG5- or ATG7-deficient cells, *TRIM31* directly interacts with phosphatidylethanolamine (PE) in a palmitoylation-dependent manner, leading to the induction of autophagy. In this process, the B-box domain of *TRIM31* is required for the formation of *TRIM31*-positive puncta and colocalization with PE [23]. The E3 ubiquitin ligase complex of *TRIM28* with its regulators *MAGEA3* or *MAGEA6* (*MAGEA3/6*-*TRIM28*) can target AMPK for ubiquitination and proteasome-mediated degradation, resulting in significantly reduced autophagy. However, in cells not expressing *MAGEA3* or *MAGEA6*, *TRIM28* functions as a pro-autophagy factor through sumoylating *PIK3C3/VPS34* and promoting the formation of the *PIK3C3*-*BECN1* complex [24]. In another study, *TRIM17* can inhibit certain types of selective autophagy by stabilizing the *MCL1*-*BECN1* complex. However, *TRIM17* can also promote the removal of midbodies through autophagy because of the absence of *MCL1* from *TRIM17*-*BECN1* complexes at midbodies [25]. These 2 TRIM proteins (*TRIM17* and *TRIM28*) further expand the roles of TRIM proteins in regulating autophagy by showing that a single TRIM can either positively or negatively regulate autophagy depending upon different conditions.

A recent comprehensive analysis demonstrated that some TRIM proteins act as platforms assembling activated ULK1 and *BECN1* to induce autophagy. Besides, *TRIM5*/*TRIM5 α* can also act as an autophagy receptor by directly recognizing its cognate target without a need for ubiquitin tagging [26]. Another comprehensive study demonstrated that a subset of TRIM proteins (such as *MEFV*/*TRIM20* and *TRIM21*) function as regulators and receptors for autophagy-targeting components of the inflammasome and type I IFN response systems [27]. These studies demonstrate that the functions of TRIM proteins in autophagy induction are diverse and each TRIM protein may have a specific function in autophagosome formation, suggesting that a particular TRIM protein does not compensate for the function of other TRIM proteins in autophagy induction. Although the function of *TRIM59* in cancer progression has been intensively studied, the role of *TRIM59* in autophagy induction has not been clarified yet. Thus, our studies point out another new way for the TRIM proteins to regulate autophagy though regulating the transcription and modulating the post-translational modifications of autophagy-related proteins.

Materials and methods

Cell culture and NSCLC patient samples

The non-small cell lung cancer (NSCLC) cell lines H1299, A549, H292, H358, H1975, H23, HCC827 from ATCC were cultured in RPMI 1640 (Invitrogen, C11875500BT), containing 10% fetal bovine serum (Excel, FCS100). The SPC-A1 cells from National Infrastructure of Cell Line Resource were

cultured in RPMI 1640 (Invitrogen, C11875500BT), containing 10% fetal bovine serum (Excel, FCS100). The 293T cells were cultured in Dulbecco modified Eagle medium (DMEM, high glucose; Hyclone, SH30022.01B), containing 10% fetal bovine serum (Excel, FCS100). All the cells were incubated at 37°C with 5% CO₂. The NSCLC patient samples were kindly provided by Dr. Bentong Yu (Department of Cardiovascular Surgery, The First Affiliated Hospital of Nanchang University).

Reagents and plasmids

MG132 was purchased from Biovision (1791-5). Cycloheximide (CHX; C7698) and chloroquine (C7698) were bought from Sigma. Rapamycin was acquired from Gene Operation (IPA1021-0010MG). IPTG (isopropyl-β-D-thiogalactopyranoside) was purchased from Amresco (0487). *TRIM59* siRNAs, *RELA* siRNAs and *TRAF6* siRNAs were synthesized by Thermo Fisher Scientific. The plasmids pCMV-HA-*BECN1*, pEGFP-C2-*LC3B*, pEGFP-C2-*TRAF6*, pCMV-HA-*TRAF6*, pCMV-HA-*TRIM59*, pCMV-HA-Δ*R*, pEGFP-C2-Δ*R*, pcDNA3.0-*RELA*, pGEX-GST-*TRIM59* were constructed for this study as follows: the genes were amplified by PCR from cDNA prepared from H1299 cells (the primers used were: pCMV-HA-*BECN1* [EcoRI, KpnI], 5'-GCGAATTCGGATGGAAGGGTCTAAGACGT-3' [forward], 5'-GCGGTACCTCATTGTTATAAAATTGTG-3' [reverse]; pEGFP-C2-*LC3B* [XhoI, KpnI], 5'-GCCTCGAGCATGCCGTCGGAGAAGACCTT-3' [forward], 5'-GCGGTACCTTACACTGACAATTTTCATCC-3' [reverse]; pCMV-HA-*TRAF6* [EcoRI, KpnI], 5'-GCGAATTCGGATGAGTCTGCTAAA CTGTGA-3' [forward], 5'-GCGGTACCCTATAACCCCTGCA TCAGTAC-3' [reverse]; pEGFP-C2-*TRAF6* [EcoRI, KpnI], 5'-GCGAATTCATGAGTCTGCTAAACTGTGA-3' [forward], 5'-GCGGTACCCTATAACCCCTGCATCAGTAC-3' [reverse]; pCMV-HA-*TRIM59* [SalI, KpnI], 5'-GCGTCGACCATGCAC AATTTTGAGGAAGAG-3' [forward], 5'-GCGGTACCTC AATGGGAAACTATTTTCC-3' [reverse]; pGEX-GST-*TRIM59* [BamHI, SalI], 5'-GCGGATCCATGCACAATTTT GAGGAAGAG-3' [forward], 5'-GCGTCGACTCAATGGG AAATATTTTCC-3' [reverse]; pCMV-HA-Δ*R* [SalI, KpnI], 5'-GCGTCGACCAGAAGTATTACTGAAATTGC-3' [forward], 5'-GCGGTACCTCAATGGGAAACTATTTTCC-3' [reverse]; pEGFP-C2-Δ*R* [SalI, BamHI], 5'-GCGTC GACCAGAAGTATTACTGAAATTGC-3' [forward], 5'-GCGGATCCTCAATGGGAAACTATTTTCC-3' [reverse]; pcDNA3.0-*RELA* [KpnI, XhoI], 5'-GCGGTACCATGGACG AACTGTTCCCCCT-3' [forward], 5'-GCCTCGAGTTAG GAGCTGATCTGACTCA-3' [reverse]); the PCR products was purified using a Gel Extraction Kit (Omega Bio-tek, D2500-02), digested with the indicated restriction endonucleases, re-purified and ligated into the digested and purified vectors (pCMV-HA [Clontech, 631,604], pEGFP-C2 [Clontech, 6083-1], pcDNA3.0 [Invitrogen, A-150,228], pEGX-6P-1 [Amersham Pharmacia Biotech, 27-4597-01]) using T4 DNA ligase (New England Biolabs, M0202L). The pGL3-enhancer vectors containing different fragments of the *BECN1* promoter were constructed for this study as follows: the *BECN1* promoter fragments were amplified by PCR from

human genomic DNA extracted from H1299 cells and cloned into the KpnI-XhoI restriction sites of pGL3-enhancer vectors (Promega, E1771). The plasmid pcDNA3.1-FLAG-*PIK3C3* was kindly provided by Dr. Qimin Zhan (Chinese Academy of Medical Sciences and Peking Union Medical College). The plasmid pCMV6-AC-GFP-*TRIM59* was purchased from OriGene (PS100010). The mutant plasmids (*BECN1-K117R*, *BECN1-E299A*) were made by Generay Biotech.

Antibody against *BECN1* ordered from OriGene (TA502127) was used to detect the expression of *BECN1* in human tissues and cell lines. The *BECN1* antibody ordered from Proteintech (11,306-1-AP) was used to perform immunoprecipitation. The normal rabbit IgG and normal mouse IgG were purchased from Santa Cruz Biotechnology (sc-2027, sc-2025). Mouse anti-ACTB and anti-GFP were purchased from Proteintech (66,009-1-Ig, 66,002-1-Ig). Mouse anti-tGFP and anti-SQSTM1 monoclonal antibody were from OriGene (TA150041, TA502127). Mouse anti-HA monoclonal antibody was ordered from Thermo Fisher Scientific (26,183). Commercial rabbit polyclonal antibodies anti-LC3B, anti-TRAF6, anti-GST Tag and anti-UB were purchased from Proteintech (18,725-1-AP, 12,809-1-AP, 10,000-0-AP, 10,201-2-AP). The TRAF6 antibody purchased from Cell Signaling Technology (8028) was used for immunoprecipitation. Antibody against TRIM59 was purchased from Sigma (HPA017759). *RELA* antibody was ordered from Cell Signaling Technology (8242). The NFκB pathway sample kit was used to detect the activation of the NFκB pathway (Cell Signaling Technology, 9936). The K48-linkage-specific polyubiquitin antibody and K63-linkage-specific polyubiquitin antibody were purchased from Cell Signaling Technology (8081, 5621). The ULK1 and p-ULK1 (Ser757) antibodies were ordered from Cell Signaling Technology (6439, 14,202).

The recombinant human ubiquitin and His-tag ubiquitin activating enzyme (His-UBA1/UBE1) were purchased from UB-biotech (UB-100H-5M, UBE-024). The recombinant His-tag UBE2D1 was ordered from Sino Biological (11,432-H07E-50). The recombinant protein of TRAF6 was purchased from OriGene (TP319528). The Dual-Luciferase Reporter Assay System was purchased from Promega (E1910).

Western blot

After transfection with plasmids or siRNAs for 48 h, cells were lysed in RIPA buffer (Beyotime, P0013), containing protease inhibitor cocktail (Sigma, P2714) and phenylmethylsulfonyl fluoride (DINGGUO, WB0180), and the protein concentrations were measured using the BCA Protein Assay Kit (Pierce Biotechnology, 23,225). Total proteins were subjected to 10% or 12% SDS-PAGE and transferred to PVDF membranes (Millipore, IPVH00010). The membranes were blocked with 5% skim milk (BD, 232,100) for 1 h at room temperature and incubated with the indicated antibodies overnight at 4°C. The membranes were washed 3 times at room temperature with 1× TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20) for 10 min every time, followed by incubation for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse (Thermo Fisher Scientific, 31,430) or anti-rabbit

(Thermo Fisher Scientific, 31,460) secondary antibodies. Protein bands were visualized after incubation using the Pro-Light chemiluminescence detection kit (TIANGEN, PA112-01) and a digital gel image analysis system (TANON 5500), and the band intensities were quantified with Tanon GIS software.

Immunoprecipitation

After cells were lysed with RIPA buffer (Beyotime, P0013) containing PMSF (DINGGUO, WB0181) and cocktail, the cell lysates were precleared with protein G agarose (Roche, 11,243,233,001) at 4°C for 1 h, then the supernatant were incubated with the indicated antibodies and protein G agarose beads at 4°C overnight. On the second day, immunocomplexes combined with beads were washed with lysis buffer, followed by western blot.

GST affinity isolation

The GST-TRIM59 protein was induced in *E. coli* using 0.5 mM IPTG (isopropyl- β -D-thiogalactopyranoside [Amresco, 0487]) at 37°C for 5 h. The cells were harvested through centrifugation at 10000g for 10 min. After that, the cells were resuspended in phosphate-buffered saline (PBS [Solarbio, P1020] containing 1% Triton X-100 [Solarbio, T8200], 1 \times protease inhibitor), and lysozyme (Solarbio, L1080) was added to reach a concentration of 1mg/ml. The cells were then incubated on ice for 1 h and frozen completely at -80°C overnight. Then, samples were thawed on ice and the pellets were broken by sonication. After centrifugation at 12,000g for 20 min, the supernatants were transferred to fresh tubes and glutathione Sepharose beads (GE, 17-0756-01) were added and incubated at 4°C overnight. The GST fusion proteins conjugated to glutathione Sepharose beads were washed with PBS 3 times and then mixed with cell lysate at 4°C for 8 h. After that, the beads were washed with PBS 3 times and boiled with loading buffer. The binding proteins were detected using western blot.

Luciferase activity assay

The human genomic DNA was extracted from H1299 cells, the *BECN1* promoter fragments were amplified by PCR and cloned into the KpnI/XhoI restriction sites of pGL3-enhancer vectors. The primers used were:

Forward primer: 5'-GCGGTACCTTGCCAGGCTGGAGTGCAG-3' (+ 1000 to -934),
Reverse primer: 5'-GCCTCGAGCTCTGTGCTCTTGCTGTTCC-3' (+ 1000 to -934);
Forward primer: 5'-GCGGTACCGTGTCCAGTTTCAGGGGCTG-3' (+ 500 to -934),
Reverse primer: 5'-GCCTCGAGCTCTGTGCTCTTGCTGTTCC-3' (+ 500 to -934);
Forward primer: 5'-GCGGTACCCGCCGAGACCGGACGTGACG-3' (+ 1 to -934),
Reverse primer: 5'-GCCTCGAGCTCTGTGCTCTTGCTGTTCC-3' (+ 1 to -934);

Forward primer: 5'-GCGGTACCCAACAACAAAAGGCCGGGCA-3' (-500 to -934),
Reverse primer: 5'-GCCTCGAGCTCTGTGCTCTTGCTGTTCC-3' (-500 to -934);
Forward primer: 5'-GCGGTACCGTGTCCAGTTTCAGGGGCTG-3' (+ 500 to -130),
Reverse primer: 5'-GCCTCGAGGTGGTAACCTTGTTTCATCCG-3' (+ 500 to -130).

For transient transfection, pGL3-enhancer vector containing *BECN1* promoter fragments were transfected into H1299 cells using SuperFectin II in vitro DNA transfection reagent (Pufei, Shanghai, 2102-100). Forty-eight h after transfection, cells were lysed and luciferase activity was detected using the Dual-Luciferase reporter assay kit (Promega, E1910). The relative levels of luciferase activity were normalized to the levels of luciferase activity of the Renilla control plasmid.

RNA purification and Q-PCR analysis

Total RNA was extracted by TRIzol reagent (Invitrogen, 15,596-026). The cDNA was synthesized using PrimeScript RT reagent kit (Takara, RR047A). Q-PCR experiments were performed with a SYBR Green Premix Ex Taq II kit (Takara, RR820A), then we used the RT-PCR System-Applied Biosystem to detect mRNA expression of target genes using *GAPDH* as a control. The comparative Ct method was used to calculate the relative amount of mRNA expression of target genes. All Q-PCR data were obtained using an ABI ViiATM 7 Real-Time PCR System.

In vitro ubiquitination assay

The in vitro ubiquitination assay was performed as previously described with minor modifications [50]. The assay was performed in a 30- μ l reaction volume containing some or all of the following components: 50 ng of UBA1/UBE1, 200 ng of UBE2D1, 500 ng of purified TRAF6, 5 μ g of ubiquitin and 1.5 μ l 20 \times reaction buffer (1 M Tris, pH 7.5, 40 mM ATP [Sigma, A1852], 100 mM MgCl₂, 40m M DTT). The reaction was initiated by incubation with GST-tagged TRIM59 purified from bacteria with glutathione Sepharose beads. The reaction was performed at 30°C for 1.5 h with rotation and stopped by adding 2 \times loading buffer. The assay was analyzed by western blot.

Gene overexpression and knockdown

For gene knockdown experiments, the cells were seeded 18-24 h prior to transfection. A nonspecific oligonucleotide from Thermo Fisher Scientific was used as a negative control. The siRNAs were transiently transfected using SuperFectin siRNA Transfection Reagent (Pufei, 2103-100). After 48 h, the knockdown efficiency was determined by western blot using appropriate antibodies. For gene overexpression, the cells were transfected with the indicated plasmids using SuperFectin DNA Transfection Reagent kit (Pufei, 2102-100). After 48 h, the transfection efficiency was determined by western blot using relevant antibodies.

Statistical analysis

Data are presented as means \pm SD. The unpaired t-test was used to make the statistical comparisons, P -value \leq 0.05 was considered to be statistically significant.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work is supported by grants to J-B Wang from the National Natural Science Foundation of China [81372823, 31360282, 81672298], and the grant from Department of Education of Jiangxi Province [701, Science and Technology Luo Di program].

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