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TRIM50 regulates Beclin 1 proautophagic activity

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ABSTRACT

Autophagy is a catabolic process needed for maintaining cell viability and homeostasis in response to numerous stress conditions. Emerging evidence indicates that the ubiquitin system has a major role in this process. TRIMs, an E3 ligase protein family, contribute to selective autophagy acting as receptors and regulators of the autophagy proteins recognizing endogenous or exogenous targets through intermediary autophagic tags, such as ubiquitin. Here we report that TRIM50 fosters the initiation phase of starvation-induced autophagy and associates with Beclin1, a central component of autophagy initiation complex. We show that TRIM50, via the RING domain, ubiquitinates Beclin 1 in a K63-dependent manner enhancing its binding with ULK1 and autophagy activity. Finally, we found that the Lys-372 residue of TRIM50, critical for its own acetylation, is necessary for its E3 ligase activity that governs Beclin1 ubiquitination. Our study expands the roles of TRIMs in regulating selective autophagy, revealing an acetylation-ubiquitination dependent control for autophagy modulation.

1. Introduction

Autophagy has long been recognized as an important conserved protein degradation pathway required for cell survival and tissue homeostasis [1,2]. The autophagy pathway is mediated by a set of evolutionary conserved ATG proteins that include the ATG1/ULK1 serine/threonine kinase protein complex, the Beclin1/VPS34 class III phosphatidylinositol 3-kinase complex 1 (PI3KC3-C1), ATG5/ATG12/ATG16, and the Atg8/LC3 protein conjugation complexes [3,4]. Beclin 1, the mammalian ortholog of yeast Atg6/Vps30 [5], plays a pivotal role in the autophagic initiation process [6]. Beclin 1 governs the autophagic process by regulating PI3KC3-C1-dependent generation of phosphatidylinositol 3-phosphate (PtdIns(3)P) and the subsequent recruitment of additional Atg proteins that orchestrate autophagosome formation [7,8]. Accumulating evidence indicates that Beclin 1 is a substrate of versatile distinct ubiquitination modifications. For instance, the Lys48-linked polyubiquitination primes Beclin 1 for proteosomal degradation [9,10], while the Lys63-linked ubiquitin chains enables the Beclin 1-dependent autophagy induction [11,12]. The importance of acetylation in autophagy has only recently been recognized [4]. Nutrient starvation causing a rapid depletion of acetyl-coenzyme A results in a lower overall acetylation of cytoplasmic proteins with a subsequent induction of autophagy [13,14]. Particularly, Lys-437 of Beclin 1 has been identified as a p300-acetylated site inhibiting Beclin 1 pro-autophagy function [12,15].

TRIM E3 ubiquitin ligase proteins are a class of broad-repertoire, high-fidelity selective autophagic regulators/interactors [16–23]. We previously showed that TRIM50 is a p62/SQSTM1 interacting protein that promotes the formation and the autophagy clearance of aggresome-associated polyubiquitinated proteins through HDAC6 interaction [22,24,25].

Here, we show that the E3 ubiquitin ligase TRIM50 regulates positively the initiation phase of starvation-induced autophagy, binds Beclin 1 promoting its K63-linked ubiquitination and association to ULK1 that results in Beclin 1 pro-autophagic activation. We also demonstrate that Lys-372 residue of TRIM50, critical for its own ubiquitination and acetylation, is necessary for its E3 ligase activity that governs Beclin1 ubiquitination. Finally, we found that spermidine, by

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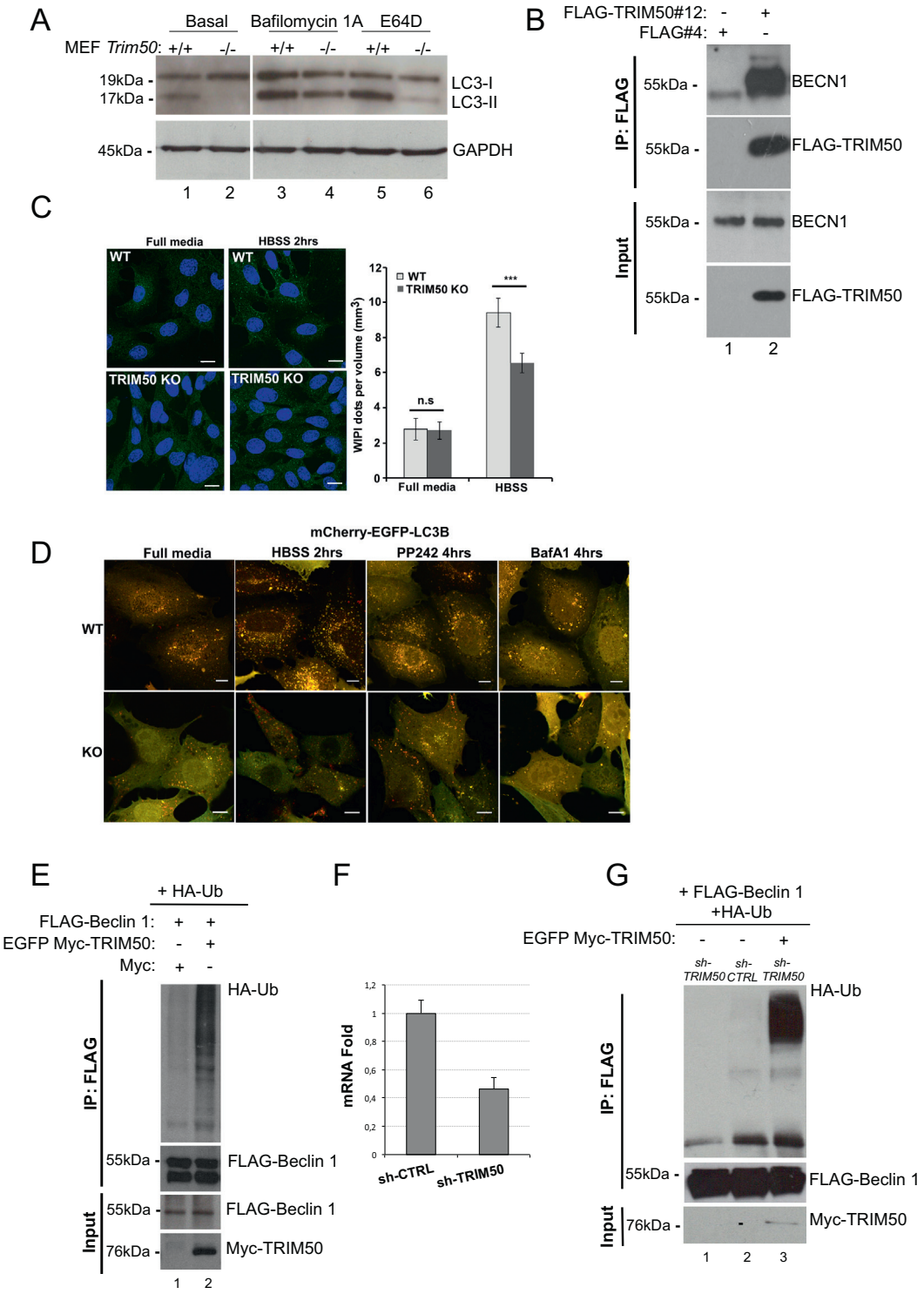


Fig. 1. TRIM50 as a modulator of the autophagic process. (A) Primary *Trim50* $^{+/+}$ and *Trim50* $^{-/-}$ MEF cells, were treated with 200 nM Bafilomycin 1A for 16 h or 10 μg/ml E64D for 14 h. LC3 and GAPDH were analysed by western blot. GAPDH was included as a loading control. (B) Total lysates of HeLa stable cell lines, FLAG#4 and FLAG-TRIM50#12 were immunoprecipitated using anti-FLAG antibody and immunoblotted with anti-BECN1 and anti-FLAG antibodies. (C) Representative images of cells stained for WIPI2 in full medium and upon starvation are shown (left) and the number of WIPI dots per cell volume quantified (right). For each condition, 10 images of 20–30 cells/frame (totally 200–300 cells per condition) were analysed using Volocity (Perkin Elmer). The graphs represent average values for each frame with standard deviations. The figure shows data from one independent experiment which was repeated with similar results. Statistical significance was evaluated using student *T*-Test. ***: $P < 0{,}005$. n.s. $P > 0{,}5$. (D) MEF cells were transfected with mCherry-EGFP-LC3B and imaged either in full medium or after induction of autophagy by starvation (HBSS) for 2 h, or treatment with 250 nM PP242 for 4 h. As a control, lysosomal degradation was blocked by treating with Bafilomycin for 4 h. The figure shows representative images from one independent experiment which was repeated with similar results. Scale bars = 10 μm. (E) HEK293 cell lines were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50 and HA-Ub, and total lysates were immunoprecipitated using anti-FLAG antibody and immunoblotted with anti-HA, anti-FLAG and anti-MYC antibodies. (F) Q-PCR to monitoring TRIM50 expression in SHSY5Y TRIM50 stable silenced lines. (G) SHSY5Y cells stable infected with lentiviral vectors encoding sh-TRIM50 and sh-Ctrl, were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50 (where indicated) and HA-Ub, immunoprecipitated using anti-FLAG antibody and immunoblotted with anti-HA, anti-FLAG, anti-MYC antibodies.

inhibiting the acetyltransferase activity of p300, enhances the acetylation of TRIM50, and thus the Beclin 1 activation.

2. Results

TRIM50 positively modulates the initiation phase of autophagy.

TRIM50 was previously reported to interact with p62/SQSTM1 [22], and it has been recently identified as readout of non-selective autophagy inductor [19]. Based on these evidences, we first assessed whether TRIM50 is able to modulate the autophagy process by tempering the level of the autophagy marker protein LC3 in *Trim50* $^{-/-}$ mouse embryonic fibroblast (MEF) cell lines [22]. By using Bafilomycin A1, a V-ATPase inhibitor responsible for neutralizing the lysosomal pH, and E64D, a membrane-permeable cysteine protease inibitor that blocks the activity of lysosomal hydrolases, we found that compared to MEF *Trim50* $^{+/+}$ littermates, MEF *Trim50* $^{-/-}$ showed lower LC3II/LC3I ratio levels in both basal conditions and upon autophagy inhibition [1] (Fig. 1A).

Furthermore, we tested whether TRIM50 is part of the autophagy machinery by checking the interaction of TRIM50 with well-known autophagy initiation proteins. Co-immunoprecipitation assay showed that TRIM50 interacts with the early positive autophagy regulator Beclin 1 [2] (Fig. 1B).

Staining of members of the human WD repeat protein interacting with phosphoinositides (WIPI) is used as a readout for autophagosome formation [26]. To test the effect of loss of TRIM50 on autophagy initiation MEF *Trim50* $^{+/+}$ and MEF *Trim50* $^{-/-}$ were either grown in full medium or starved in HBSS before immunostaining with a monoclonal antibody to WIPI2 (Fig. 1C). The results show a reduction of WIPI puncta upon induction of autophagy by starvation in the MEF *Trim50* $^{-/-}$ relative to the MEF *Trim50* $^{+/+}$ suggesting a less efficient initiation of autophagy upon loss of TRIM50. Consistently, MEF *Trim50* $^{+/+}$ contained more LC3B puncta than MEF *Trim50* $^{-/-}$ when transfected with a tandem tag LC3B construct [27] and starved for 2 h (Fig. 1D). The same was the case when autophagy was induced by treatment with the mTOR inhibhitor PP242. The autophagic flux was apparently not compromised in the MEF *Trim50* $^{-/-}$ cells since red-only dots were observed both in basal- and autophagy induced conditions (Fig. 1D). Overall these data indicate that TRIM50 fosters the initiation phase of starvation-induced autophagy and associates with Beclin1, a central component of autophagy initiation complex.

TRIM50 regulates K63-ubiquitination of Beclin 1 to promote autophagy induction.

Mounting evidence points to the importance of Beclin 1 as a component of a key complex required for autophagy induction [28]. In this complex Beclin 1 is the substrate of versatile distinct ubiquitination modifications [11,29–32] crucial for its role in the autophagy process.

Due to its intrinsic E3 Ubiquitin ligase activity [25], we asked whether TRIM50 ubiquitinates Beclin 1. To determine if TRIM50 acts as an E3 Ubiquitin ligase for Beclin 1, we performed an ubiquitination assay in HEK293 cell line co-transfected with FLAG-tagged-Beclin 1, EGFP-Myc-tagged TRIM50, and HA-tagged ubiquitin expressing vectors. Total cell lysates were immunoprecipitated with anti-FLAG and immunoblotted with anti-HA specific antibodies. We detected an increased level of Beclin 1-ubiquitinated forms in presence of exogenous TRIM50 (Fig. 1E). This evidence was confirmed by evaluating Beclin 1 ubiquitination level in a HeLa cell line where TRIM50 was stably silenced by siRNA. TRIM50-depleted cells showed a reduced Beclin 1 ubiquitination that was rescued upon TRIM50 over-expression (Fig. 1F–G). Moreover, the expression of TRIM50ΔRING, a mutant defective in E3 ligase activity [25], showed a decrease of ubiquitinated Beclin 1 forms (Fig. 2A), proving that TRIM50 is necessary for Beclin 1 ubiquitination. We then investigated which type of poly-ubiquitin chains are involved in Beclin 1 ubiquitination. Cells overexpressing HA-tagged-ubiquitin-K63R mutant, unable to form Lys-63-linked ubiquitin chains, showed a reduced Beclin 1 ubiquitination compared to cells expressing wild-type and K48R mutant ubiquitin, indicating that TRIM50 ubiquitinates Beclin 1 preferentially via K63 (Fig. 2A).

Since K63-ubiquitination of Beclin 1 results in autophagy induction [11,12], we tested whether TRIM50 modulates the autophagy process through K63-ubiquitination of Beclin 1. We observed a significant increase of basal autophagy level, measured by the LC3II/LC3I ratio, when TRIM50 ubiquitinates Beclin 1. Consistently, the expression of TRIM50ΔRING and Ub-K63R had no significant effect, and showed the same LC3II/LC3I ratio as the control (Fig. 2).

We supported these data by microscopy studies assessing the localization and the signal intensity of endogenous LC3 puncta. In line with western blot data, in cells transfected with EGFP-TRIM50, we observed an increase of LC3 puncta and intensity signal comparing with empty vector. Consistently, the intensity signal of LC3 puncta unchanged when TRIM50ΔRING and Ub-K63R were used (Fig. 3A–B). Even in MEF *Trim50* $^{-/-}$ and *Trim50* $^{+/+}$, through ectopic expression of ubiquitin proteins, a statistical reduction of LC3 relative intensity signal was observed in MEF cell lines depleted for *Trim50* when compared to wild-type cell lines. Interestingly, ectopic expression of UbK63R appreciated a reduction of LC3 intensity signal either in wild-type and in knock out MEF lines, suggesting the importance of K63 ubiquitination in the autophagy regulation. When we compared MEF *Trim50* $^{+/+}$ expressing ubiquin or UbK63R, we observed a more marked difference of LC3 signal than MEF *Trim50* $^{-/-}$, suggesting the importance of TRIM50-mediated K63-ubiquitination in order to promote the autophagy process (Fig. 3B–C). Overall these results point out the role of TRIM50 as an E3 Ubiquitin Ligase for Beclin 1.

Then we assessed the influence of TRIM50 on Beclin 1 ubiquitination during the induction of autophagy. Upon autophagy stimulation, achieved by serum starvation and inhibition of mTOR pathway by Rapamycin1A, the increased Beclin 1 ubiquitination resulted in a significant increase of autophagy flux levels in presence of TRIM50 (Fig. 4A–B). We confirmed these data by performing a proximity ligation assay (PLA) in HeLa cell lines quantifying the amount of K63-ubiquitinated Beclin 1 in presence or not of FLAG-TRIM50 in basal condition, EBSS and Rapamycin1A treatments. The number of dots representing K63-ubiquitinated Beclin 1 was higher in the presence of FLAG-TRIM50 compared to control cell line, with the highest fold increase upon autophagy induction and Rapamycin1A treatment (Fig. 4C).

Beclin 1 complexes with Vps34 to provide a PI(3)P-enriched domain for autophagosome formation. These core components bind different partners, including ULK1 [33]. Therefore, based on the ability of TRIM50 to associate with Beclin 1, we asked whether TRIM50 might promote the formation of the Vps34/Beclin 1/ULK1 complex. Immunoprecipitation analysis showed that, when TRIM50 is over-expressed, the association between Beclin 1 and VPS34 is not affected while we observed an increased strength of the association between Beclin 1 and ULK1; suggesting that TRIM50 facilitates the assembly of Beclin 1 with ULK1 (Fig. 5A).

We further investigated the role of TRIM50 E3 ligase activity in resolving Beclin 1-ULK1 assembly. MEF *Trim50* $^{-/-}$ and *Trim50* $^{+/+}$ were co-transfected with FLAG-tagged-Beclin 1, Myc-tagged ULK1, HA-tagged ubiquitin, and HA-tagged-ubiquitin-K63R mutant expressing vectors. Immunoprecipitation analysis showed that, when *Trim50* is depleted, the association between Beclin 1 and Ulk1 weakens as well as we observed the decrease of Beclin 1 ubiquitination (Fig. 5B). Overall, these results indicate that TRIM50 promotes Beclin 1 K63-linked ubiquitination, enhancing its interaction with ULK1.

The acetylation status of TRIM50 is central for Beclin 1 ubiquitination.

We have recently shown that TRIM50 is acetylated by p300 and deacetylated by HDAC6 at Lysine 372, a residue also involved in TRIM50 auto-ubiquitination [24]. Since it is known that acetylation and ubiquitination are two regulatory processes of enzyme activity and that acetylation has a role in autophagy regulation [34,35], we asked

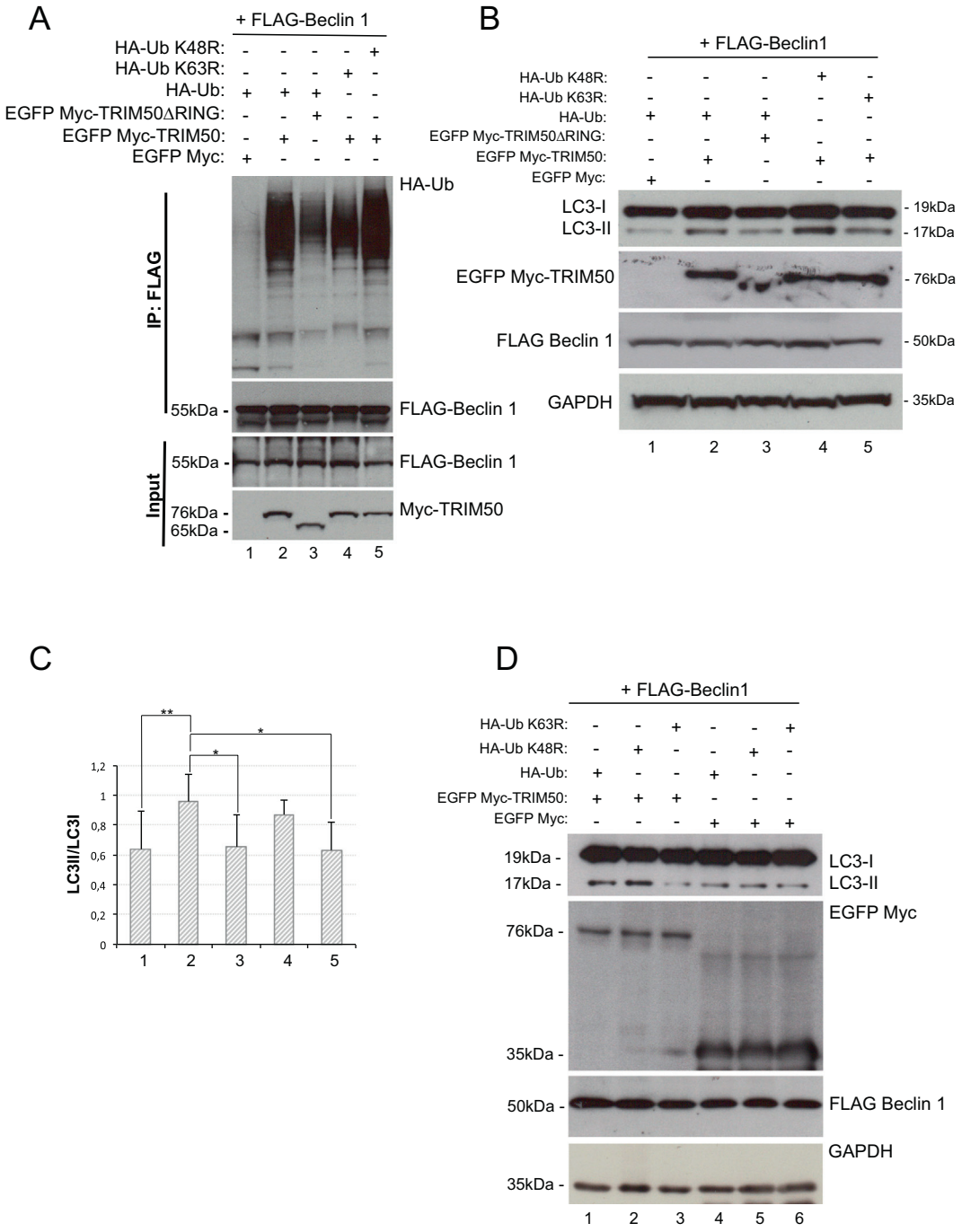


Fig. 2. Beclin 1 K63-ubiquitination is TRIM50-dependent. (A) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50 and EGFP Myc-TRIM50ΔRING in combination with wild type ubiquitin or its mutants (K48R; K63R) HA-tagged constructs. Protein extracts were immunoprecipitated using anti-FLAG antibody and immunoblotted using anti-HA, anti-FLAG, anti-MYC antibodies. (B) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50 and EGFP Myc-TRIM50ΔRING in combination with wild type ubiquitin or its mutant (K48R; K63R) HA-tagged constructs. Immunoblot analysis of Beclin 1, TRIM50, and LC3 were performed using anti-FLAG, anti-MYC and anti LC3 antibodies as indicated. GAPDH was used as a loading marker. The entire assay was made in triplicate, a representative one is shown. (C) Graph below, reports means ± s.d. of LC3 II/LC3I values from 3 independent experiments reported in the previous figure; The number 1 to the number 5 correspond to the same samples relative of previous experiment; * = $p^{<}0.05$, ** = $p^{<}0.03$. (D) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-empty and EGFP Myc-TRIM50 in combination with wild type ubiquitin or its mutant (K48R; K63R) HA-tagged constructs. Immunoblot analysis of Beclin 1, TRIM50, and LC3 protein has been determined as indicated. GAPDH was used as a loading marker. The entire assay were made in triplicate, a representative one is shown.

whether post-translational modifications of TRIM50 may impact the Beclin 1 activity.

We first analysed the ubiquitination levels of Beclin 1 in MEF *Hdac6* $^{-/y}$ cell lines and we did observed a very slight reduction of Beclin 1 ubiquitination compared to wild-type (Fig. 5C). Overexpression of TRIM50 or, with a stronger effect, along with HDAC6 caused an increased level of Beclin 1 ubiquitination in both *Hdac6* $^{+/y}$ and *Hdac6* $^{-/y}$ MEFs (Fig. 5C). We also observed that the K372R TRIM50 mutant [24] greatly reduced the ability to ubiquitinate Beclin 1 (Fig. 6A). Finally, we found that the overexpression of p300 acetylase, increasing

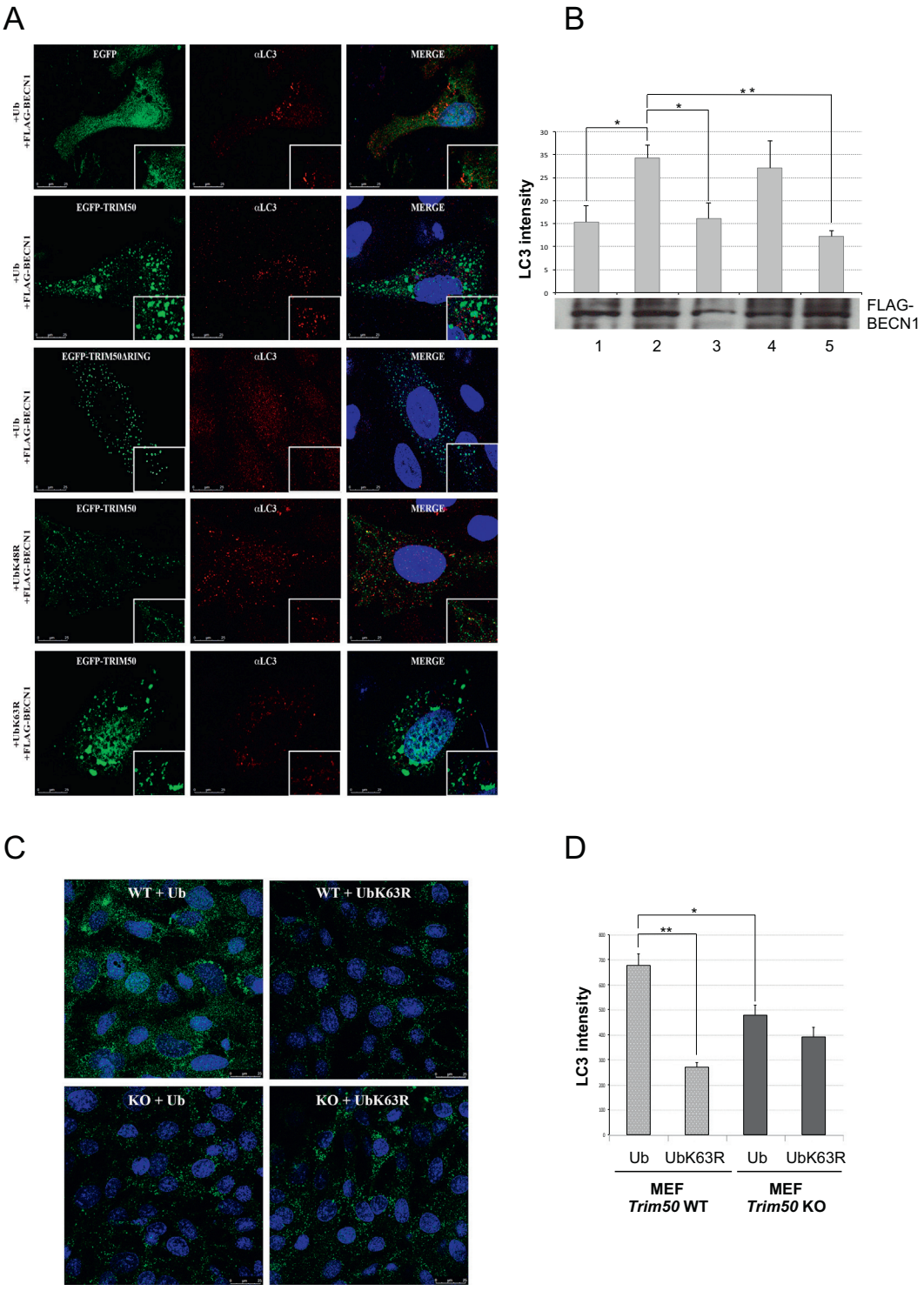


Fig. 3. Beclin 1 K63-ubiquitination is TRIM50-dependent. (A) HeLa cell lines cell lines were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc, EGFP Myc-TRIM50 and EGFP Myc-TRIM50ΔRING in combination with wild type ubiquitin or its mutants (K48R; K63R) HA-tagged constructs. The cells were fixed and processed for immunofluorescence study staining with anti-LC3 antibody and examined on a Leica TCS SP8 confocal microscopy (Leica, Wetzlar, Germany). (B) After acquisition, for all imagines we analysed the intensity of Alexafluor 568 signal, measuring the relative intensity of pixels representative for each ROI (regions of interest) corresponding of a single cell. The graph reports means ± s.d. of LC3 intensity values from 50 cells for each transfection reported in the previous figure; * = $p^{<}0.05$, ** = $p^{<}0.03$. Western blot reported the expression of Beclin 1 in this assay. (C) MEF *Trim50* $^{+/+}$ and MEF *TRIM50* $^{-/-}$ cells, transfected with vectors encoding wild type ubiquitin or its mutant K63R HA-tagged constructs, were fixed and processed for immunofluorescence study staining with anti-LC3 antibody and examined on a Leica TCS SP8 confocal microscopy (Leica, Wetzlar, Germany). (D) After acquisition, for all imagines we analysed the intensity of Alexafluor 568 signal, measuring the relative intensity of pixels representative for each ROI corresponding to a single cell. The graph reports means ± s.d. of LC3 intensity values from 50 cells for each transfection reported in the previous figure; ** = $p^{<}0.03$.

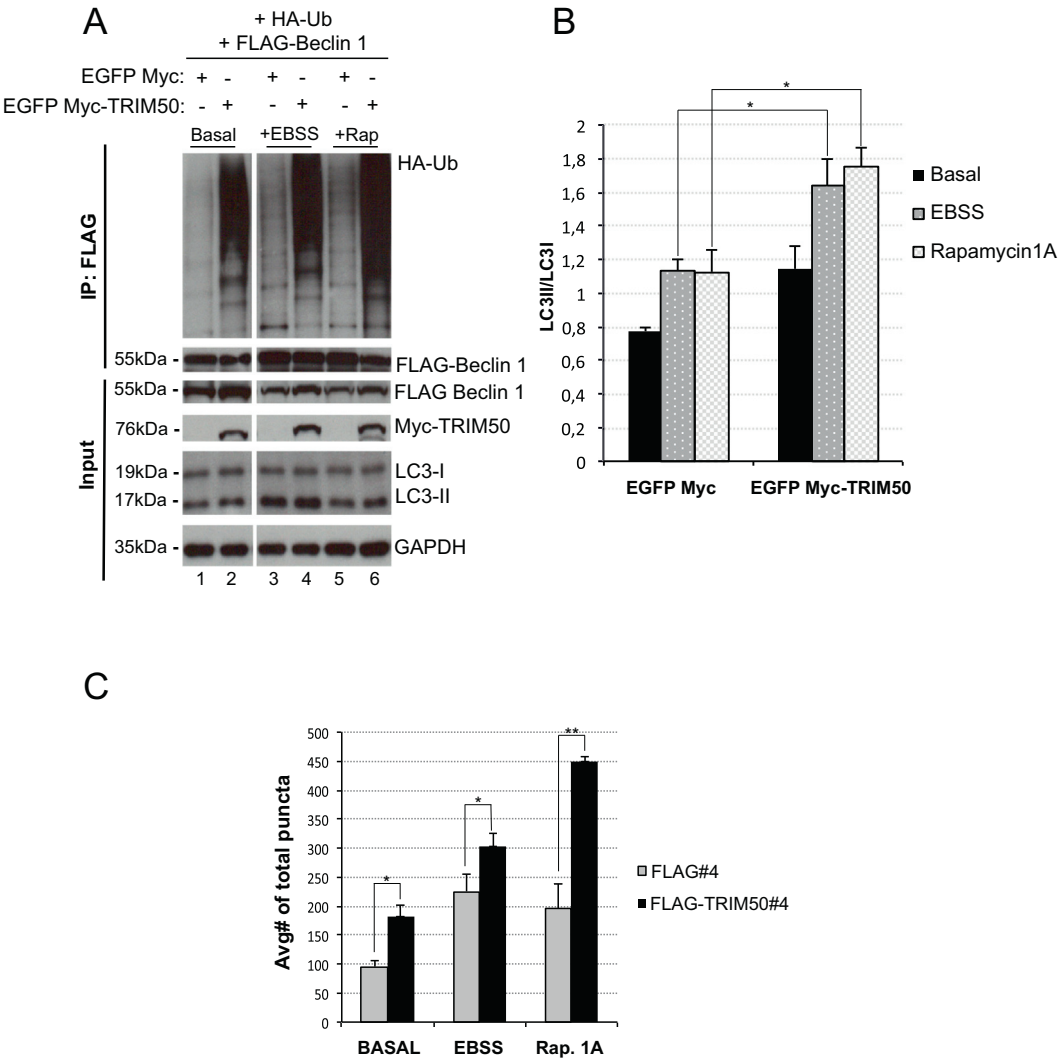


Fig. 4. TRIM50 E3 ligase activity enhancing autophagy process. (A) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, Myc-TRIM50 and HA-Ub and cultured in complete or starvation medium for 2 h or treated with 800 nM Rapamycin1A for 24 h. Immunoprecipitation assay was performed with anti-FLAG antibody and immunoblotted with anti-HA, anti-FLAG, anti-MYC, anti-LC3 and anti-GAPDH antibodies. (B) Graph reports means ± s.d. of LC3 II/LC3I values from 3 independent experiments reported in the previous figure; * = $p^{<}0.05$. (C) Proximity ligation assay (PLA) for direct in situ protein-protein ligation between the endogenous Beclin1 protein (using an anti-Beclin1 antibody) and endogenous K63-ubiquitinated proteins (using an anti-K63 antibody) in HeLa stable cell lines, FLAG#4 and FLAG-TRIM50#12. The graph represents a quantification of the average number of PLA puncta per cell elaborated by imageJ software. ** indicates *p*-value = 0.01; * indicates *p*-value = 0.05.

the acetylated level of TRIM50 at K372 (Fig. 7B) caused a decrease of ubiquitinated forms of Beclin 1 (Fig. 6B). Overall, these data confirmed that deacetylation of K372 of TRIM50 is necessary for Beclin 1 ubiquitination.

Recently, the role of spermidine as inhibitor of p300 acetyltrasferase activity has been reported [36]. We therefore investigated whether spermidine could modulate TRIM50 acetylation by inhibiting p300 activity. The incubation with spermidine caused a reduction of acetylated-TRIM50 forms with an increase of Beclin 1 ubiquitination that resulted in an increase of LC3II/LC3I ratio, confirming the induction of autophagic process (Fig. 6B).

Since it has been reported that Beclin 1 is acetylated by p300 and de-acetylated by SIRT1 [15], in order to test whether spermidine may per se regulate Beclin 1 modification, we evaluated the effect of spermidine on Beclin 1 ubiquitination in HEK293 cells that express a very low level of TRIM50. We observed an increase of Beclin 1 ubiquitination only in those samples treated with spermidine along with TRIM50, indicating that despite Beclin 1 acetylation/deacetylation level, the oscillation of Beclin 1 ubiquitination is related to TRIM50 expression (Figs. 6B and 7A).

We then used spermidine in MEF cell lines depleted for *Trim50*. In the presence of ectopic p300, spermidine, or both together, we found lower Beclin 1 ubiquitination levels in *Trim50* $^{-/-}$ MEFs than *Trim50* $^{+/+}$ MEFs (Fig. 7B). This result confirmed the central role of de/acetylation in regulating TRIM50 activity and that spermidine could be used to regulate TRIM50 activity through p300 inhibition.

3. Discussion

Beclin 1 is a key regulator of autophagy, playing a critical role in pro-autophagosomal protein complex formation at the phagophore assembly site [37,38]. The function of Beclin 1 is controlled by post-translation modifications including ubiquitination [10] [39–41]. Several E3 ubiquitin ligases contribute to Beclin 1 regulation: the HECT-type ligase Nedd4 can polyubiquitinate Beclin 1 for its proteasomal degradation with K11-linked chains [31]; TRAF6 catalyzes the formation of K63-linked polyubiquitin on Beclin 1 in macrophages, resulting in the induction of the autophagy [11]; Ambra1-containing Rbx1/Cul4-

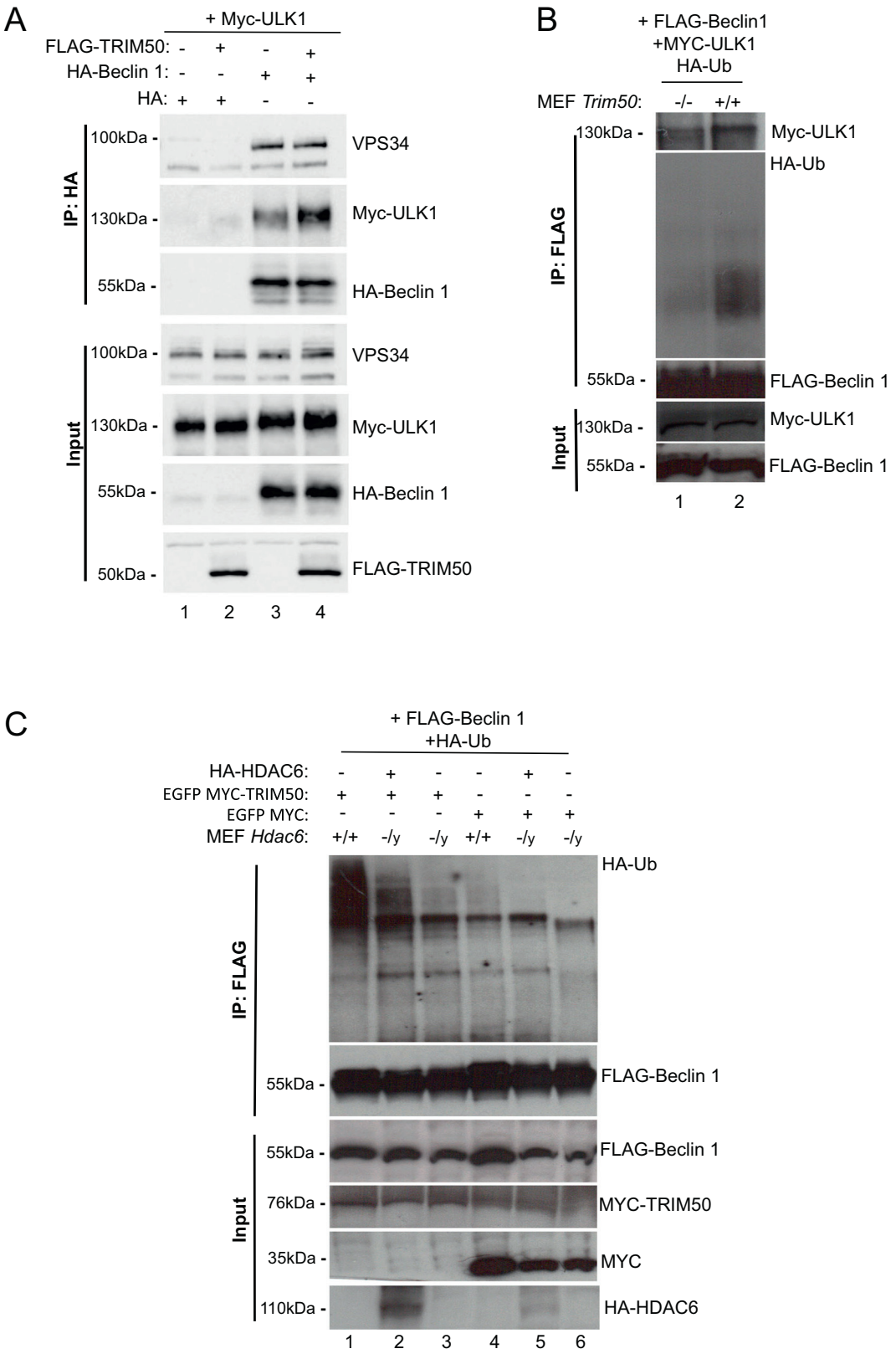
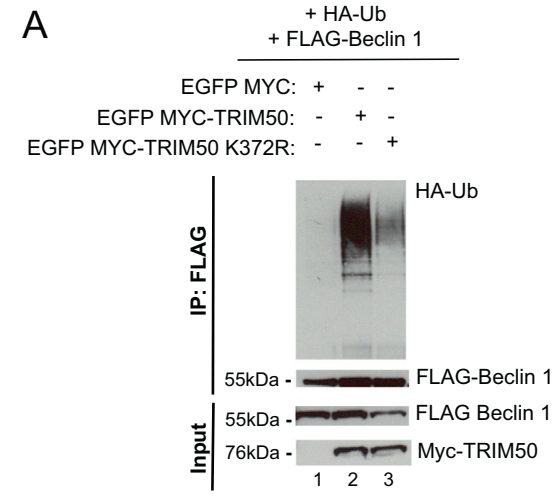


Fig. 5. TRIM50 mediates the assembly of Beclin 1 and ULK1 in a complex.
(A) HEK293 cells were transfected with vectors encoding HA-Beclin 1, FLAG-TRIM50 and Myc-ULK1. Protein extracts were immunoprecipitated using anti-HA antibody and immunoblotted using anti-HA, anti-FLAG, anti-MYC and anti-VPS34 antibodies. (B) MEF *Trim50* $^{+/+}$ and MEF *TRIM50* $^{-/-}$ cells were transfected with vectors encoding FLAG-Beclin 1 and Myc-ULK1 with HA-ubiquitin. Protein extracts were immunoprecipitated using anti-FLAG antibody and immunoblotted using anti-HA, anti-FLAG, anti-MYC antibodies. (C) MEF *Hdac6* $^{+/+}$ and MEF *Hdac6* $^{+/-}$ were transfected with vectors encoding FLAG-Beclin 1 and HA-Ub; and with EGFP Myc-TRIM50, EGFP-Myc and HA-HDAC6 where is indicated. Protein extracts were immunoprecipitated using an anti-FLAG, and immunoblotted using anti-HA, anti-FLAG, anti-MYC antibodies.



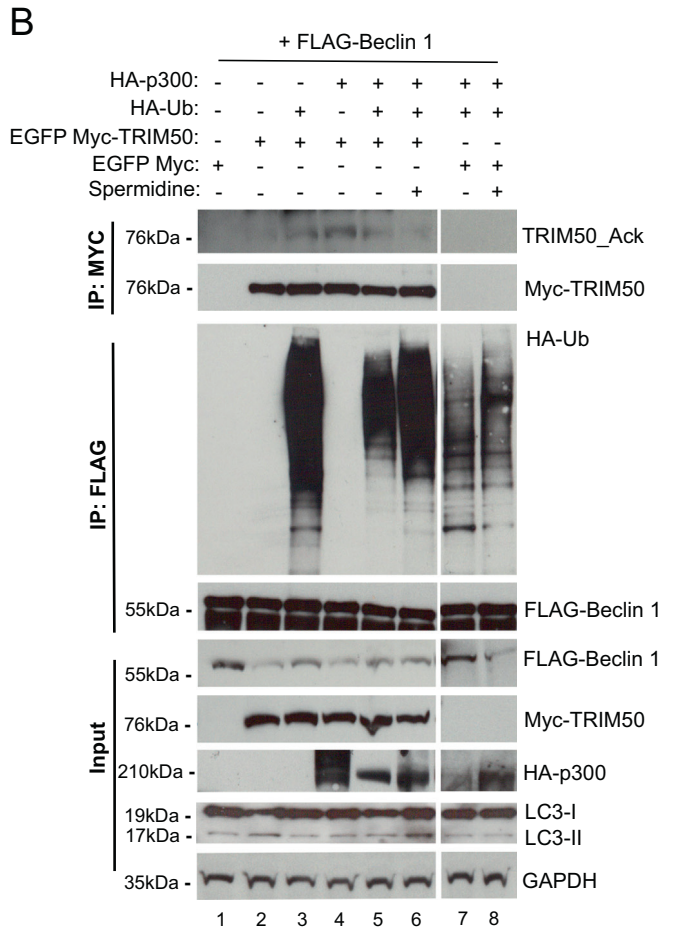
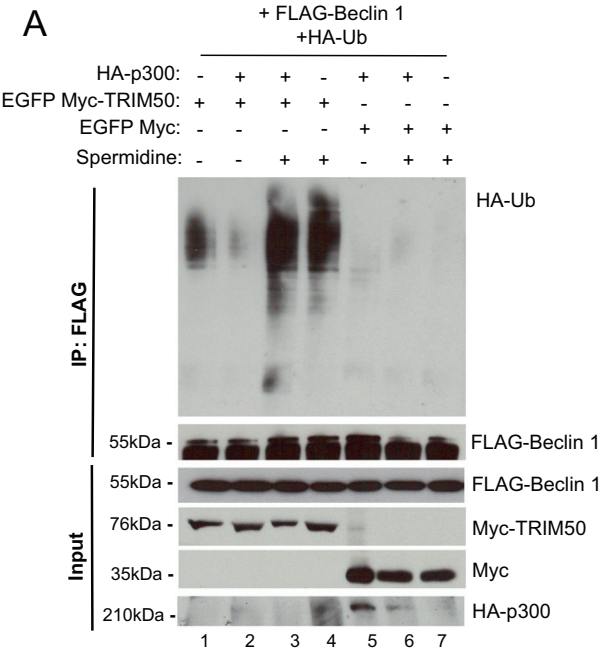


Fig. 6. Acetylation of TRIM50 regulates Beclin 1 ubiquitination. (A) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50, EGFP Myc-TRIM50 K372R and HA-Ub. Protein extracts were immunoprecipitated using an anti-FLAG, and immunoblotted using anti-HA, anti-FLAG, anti-MYC antibodies. (B) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50, HA-Ub and HA-p300, cultured in complete medium or incubated with 2 nM spermidine for 24 h, and immunoprecipitated using anti-Myc antibody to detect the acetylated form of TRIM50 and immunoblotted with anti-Ack antibody; or immunoprecipitated with an anti-FLAG antibody and immunoblotted with anti-HA, anti-FLAG, anti-MYC, anti-LC3 and anti-GAPDH antibodies.



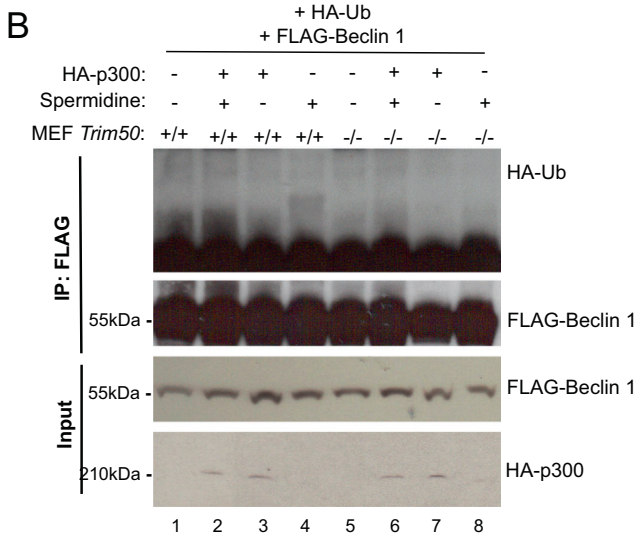


Fig. 7. Spermidine induces TRIM50 pro-autophagic activity. (A) HEK293 cells were transfected with vectors encoding FLAG-Beclin 1, EGFP Myc-TRIM50, EGFP Myc –empty, HA-Ub and HA-p300, cultured in complete medium or incubated with 2 nM spermidine for 24 h, and immunoprecipitated using anti-FLAG antibody and immunoblotted with anti-HA, anti-FLAG, anti-MYC antibodies. (B) MEF *Trim50* $^{+/+}$ and MEF *Trim50* $^{+/-}$ were transfected with vectors encoding FLAG-Beclin 1, HA-Ub and HA-p300 as indicated. Protein extracts were immunoprecipitated using an anti-FLAG, and immunoblotted using anti-HA, anti-FLAG antibodies.

ligase complex has been reported to ubiquitinate K437 of Beclin 1 [12]; and finally, the E3 ligase RNF216 mediates the K48-linked ubiquitination of Beclin 1, as a negative pathway for the autophagy regulation [42].

Here we show that the E3 ubiquitin ligase TRIM50 regulates positively the initiation phase of starvation-induced autophagy, binds Beclin 1, promoting its K63-linked ubiquitination and association to ULK1 that results in Beclin 1 pro-autophagic activation.

TRIM50 belongs to the TRIM protein family, a large family of proteins involved in different processes [43], including the regulation of autophagy processes as shown in a number of recent studies [17–21,44–52].

The change of the level of phosphatidylethanolamine (PE)-modified-LC3 (LC3II), through immunoblotting and microscopy studies, allows to estimate the overall autophagic flux. Here we showed that TRIM50 increases the LC3II level confirming the link between TRIM50 E3 ligase activity and autophagy flux.

The existence of a mutual relationship between ubiquitination and acetylation in the regulation of the TRIM50 E3 ligase activity is another important finding in our study. In line with our previous work [24], we show that p300-mediated acetylation of TRIM50 reduces its E3 ligase activity on Beclin 1. Conversely in *Hdac6* depleted cell lines, the capability of TRIM50 to ubiquitinate Beclin 1 is reduced, demonstrating that the TRIM50 E3 ligase activity may depend, at least in part, on the presence of its de-acetylase HDAC6. In this context, p300-dependent control of TRIM50 acetylation and activity might provide a further refinement of the proposed role for acetylation in the control of autophagy. As for TRIM50, p300 has been shown to regulate the acetylation of known components of the autophagy machinery such as Atg5, Atg7, Atg12, and Beclin 1, inhibiting autophagy activation and competing with other deacetylases, like Sirt1 [12,53].

Furthermore starvation contributes to control protein acetylation, as reported for the histone acetyltransferase Esa1, which acetylates Atg3, a key component of the autophagy machinery [54]. However whether and how acetylation and starvation are regulating each other remains largely unknown and deserves further studies.

In a previous study, Deretic and co-workers proposed the concept of the TRIMosome as a platform characterized by different TRIMs involved in the selective autophagy regulation [19,50,51,55,56]. The role of TRIMs in autophagy is not limited to the selective recognition of autophagy cargos by cooperating with ATGs family members and/or p62 scaffold protein, but, as reported in this work for TRIM50 and Beclin 1, also in the post translation modification of autophagy-related proteins. Therefore, TRIM family proteins are highly promising candidates for satisfying the requirements of multiple target recognition and signal transduction activation, two needed aspects to regulate autophagy in response to specific types of damage and stimuli. Our work highlights the importance of TRIMs as autophagy regulators, reporting how the two systems, TRIMs and autophagy machinery, interact. The definition of the role of TRIM50 as autophagic regulator widens our understanding of the repertoire, versatility and capacity for selective autophagy in mammalian cells.

The present study suggests a functional model where in basal conditions TRIM50 interacts with Beclin 1 mediating its K63-ubiquitination. Such modification favours the Beclin 1 assembling to ULK1 resulting in autophagy activation. p300 and HDAC6, by acetylating or deacetylating TRIM50, modulate the Ubiquitin E3 ligase enzymatic activity of TRIM50 on Beclin 1. Notably, in such context spermidine reduces the capacity of p300 to acetylate TRIM50 with the ultimate results of increasing autophagy (Fig. 8).

Spermidine is a nontoxic autophagy-inducing natural compound, with an increasing interest for its applications as a nutraceutical tool for therapies [57]. The use of spermidine as a natural autophagy-inducer in those conditions where autophagy is impaired should be considered as an innovative opportunity for future researches aimed at finding possible therapies.

4. Methods

4.1. Antibodies and inhibitors

All antibodies were used at a dilution of 1:1000 for immunoblotting and 1:200 for immunofluoresence. Mouse monoclonal antibodies were from: Sigma-Aldrich (FLAG-F3165), Roche (c-myc-11,667,149,001), Cell Signalling (anti-Ack #9681), Millipore (anti-Ubiquitin Lys63-Specific #051308), Life Technologies (anti-VPS34 #382100). Rabbit polyclonal antibodies were from: Sigma (TRIM50 HPA019862), Santa Cruz (HA-Y11 sc-805), Novus (LC3 NB100-2331), Millipore (GAPDH #2272157); anti-WIPI2 antibody was kind gifted from Sharon Tooze and diluted 1:500. Goat polyclonal antibody: Santa Cruz (BECN1-D18 sc-10,086). Inhibitors were purchased from Sigma, respectively: Rapamycin1A, Bafilomycin A1, EBSS, E64D and Spermidine, HBSS.

4.1.1. Fusion Plasmids

The pcDNA3-HA-Ub, pcDNA3-EGFP Myc, pcDNA3-EGFP Myc-TRIM50 and pcDNA3-EGFP Myc-TRIM50ΔRING were described in [25]. HA-p300 and HA-HDAC6 vectors were described in [22]. Human Beclin1 ORF and ULK1 ORF were cloned into pLPCX vector (Clontech) with FLAG MYC and HA as tag using a PCR based method with appropriate oligonucleotides followed by inframe insertion into the vectors.

4.1.2. Immunoprecipitation and western blotting

Cells were plated in 100 mm culture dishes at a density of 5×10^5 cells/ml. In transfection assays we included the appropriate empty vector in cases where a given plasmid was not included in the transfection. After treatments, cells were lysed in RIPA buffer plus protease inhibitors cocktail (Roche), sonicated and then centrifuged at 15000*g* for 5 min. Protein quantification was performed using the Bradford protein assay (BioRad) following manufacturer's instructions. 20 to 40 μg of protein extract per sample were run on a precast Nupage 10 or 12% Bistris Gel (Thermo Fischer scientific), transferred on a nitrocellulose membrane by iBLOT system (Thermo Fischer scientific) and blocked in Net-gelatin or TBS and 5% milk.

Co-immunoprecipitation experiments were performed using Dynabeads magnetic beads (Thermo Fischer scientific) following manufacturer's instructions. Complexes were analysed by western blotting using indicated antibodies. Detection was achieved using horseradish peroxidase conjugated anti-mouse (Santa Cruz) and anti-rabbit (Santa Cruz) antibodies. Signals were acquired using Amersham Hyperfilm ECL (GE Healthcare) chemiluminescence system. Protein bands densities were determined using densitometer Kodak digital software (Kodak).

4.1.3. Cell culture and reagents

HEK293, MEF and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM, LONZA) supplemented with 10% fetal calf serum (Life Technologies) and 1% penicillin/streptomycin solution (Lonza) at 37 °C under 5% CO_2.

For stable cell line, HeLa were transfected with CMV3X-FLAG-TRIM50 or empty vector and selected for 2 weeks with 1 mg/ml G418 (Invitrogen, Carlsbad, CA) selective agent. The expressing colonies were expanded and then used for protein extract preparations following standard procedures. HeLa cell line was used since the low level expression of endogenous TRIM50 protein. Hereafter the stable cell lines will be referred to as FLAG-TRIM50# 12 and FLAG#4, respectively. For stable SHSY5Y silenced TRIM50 cell lines, one lentiviral TRIM50 mRNA targeting pLKO.1 plasmid was used (TRCN0000007787, Sigma-Aldrich) adding 2μg/ml Puromycin (Sigma-Aldrich) as selective agent. The source of MEF *Hdac6* cells were described in [22].

For autophagy induction, cells were washed with PBS and cultured for 2 h in HBSS Medium (Sigma-Aldrich) or 2 h in Earle's balanced salt solution (EBSS, Sigma-Aldrich) for starvation, or treated with Rapamycin1A (800 nM, 24 h), pp242 (250 nM; 4 h), E64D (10μg/ml, 14 h) and Spermidine (2 nM 24 h). Plasmids transfections were performed using calcium phosphate method, Lipofectamine LTX (Thermo Fischer scientific) or TransIT-LT1 (Mirus Bio).

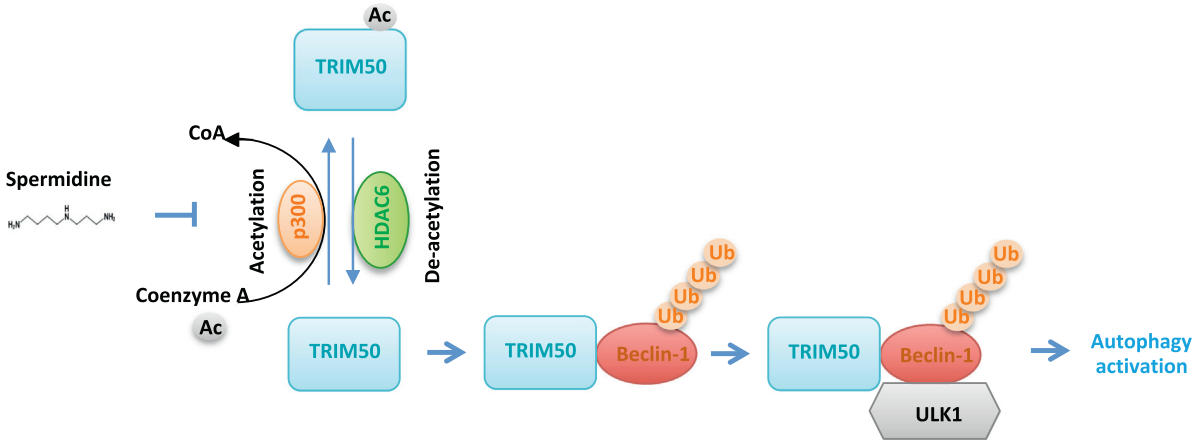


Fig. 8. Proposed model of TRIM50 regulation in Beclin 1 ubiquitination.
TRIM50 interacts with Beclin 1 mediating its K63-ubiquitination and in Beclin 1-ULK1 complex formation. p300-mediated acetylation of TRIM50; conversely the deacetylase HDAC6, promoting TRIM50 deacetylation, supports the ubiquitination activity of TRIM50 on Beclin 1. Spermidine treatment inhibits p300 activity, which decreases the level of TRIM50 acetylated forms, increasing TRIM50 activity on Beclin1 ubiquitination and its assembly with ULK1 to induce autophagy activation.

4.2. Confocal microscopy analysis

For immunofluorescence analyses, HeLa cells transfected with FLAG-Beclin 1, EGFP Myc, EGFP Myc-TRIM50, EGFP Myc-TRIM50ΔRING, HA-Ub or HA-UbK63R; MEF *Trim50* $^{-/-}$ and *Trim50* $^{+/+}$ were plated in 3 cm culture dishes and incubated with pp242 and/or HBSS medium. At 24 h after transfection or treatments incubations, were fixed with 100% methanol (chilled at −20 °C) for 20 min, incubated in blocking solution containing PBS and 1% bovine serum albumin (BSA). The cells were then incubated with anti α-LC3 (Nouvus, 1:200, #NB100–2331) for 2 h at room temperature followed by extensive washing with PBS. After an incubation with Alexa Fluor 568 goat anti-rabbit IgG (Termo Fisher Scientific, 1:500, #A11011) followed by DAPI (Molecular Probes, #D1306) staining, the cells were covered with a drop of mounting medium and examined on a Leica TCS SP8 confocal microscopy (Leica, Wetzlar, Germany). All confocal images in HeLa cell lines were obtained using the necessary filter sets for GFP and Alexafluor 568, using a × 63 (1.2 numerical aperture) water immersion objective. The acquisition of data has to be performed with the same intensity settings. Instead, MEF Trim50 cell lines were examined using a Zeiss Axiovert microscope equipped with LSM-780-META module. For quantification of WIPI dots, z-stack images (20–30 cells per image, 10 images per condition) were quantified using Volocity 6.3 (PerkinElmer).

4.2.1. Quantitative real-time PCR

RNA was extracted by using Trizol reagent (Life Technologies). cDNA synthesis was generated using a reverse transcription kit (Qiagen) according to the manufacturer's recommendations.

Specific primers in TRIM50 sequence for qPCR assays were designed using the Primer express program [58] with default parameters and based on the UCSC GRCh37/hg19 assembly. The real-time PCR reactions were performed on an Applied Biosystems TM 7900HT, as described by the manufacturer. *EEF1A1* and *GAPDH* were used as reference genes. qPCR reactions and calculations were made as reported in [59].

4.2.2. Proximity ligation assay

Proximity ligation assay (PLA) was performed using the Duolink kit (Sigma-Aldrich) following the manufacturer's protocol and using all the proprietary solutions from the kit. In brief, cells were grown in 12-well plates adding the chamber slides (Eppendorf) and fixed for endogenous protein detection as described for the standard immunofluorescence method above. Duolink blocking solution was added and incubated for 30 min at 37 °C in humidified chamber. The solution was tapped off and the two primary antibodies targeting the putative binding partners (anti-BECN1 and anti-Ubiquitin Lys63-Specific) were added in the antibody diluent solution from the kit, and incubated for 1 h at 37 °C in humidified chamber. Next wells were washed twice and the oligonucletide PLA probes were added and incubated for 1 h at 37 °C. Following this, probes were ligated using the provided ligase and ligation buffer, which was incubated on the slides at 37 °C for 100 min. Finally, incorporation of the red fluorophore and signal amplification was carried out at 37 °C for 100 min using the kit detection reagent, which incorporates DNA polymerase for rolling circle amplification.

PLA results were reported as average number of red puncta per total picture using Leica SP8 Confocal Microscope *Z*-stack software.

4.3. Statistical analysis

Statistical analysis of immunoblotting and PLA data were performed using unpaired, two-tailed Student's *t*-test (Excel software). Values are shown as mean ± standard deviation of at least 3 independent experiments. *P*-values ≤ 0.05 were marked by *. Densitometry analysis of immunoblots was performed using the ImageJ and Adobe Photoshop software. LC3 data are relative ratios of each sample without calibration.

Transparency Document

The Transparency document associated with this article can be found, in online version.

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Author contributions

C.F. G.M. conceived and designed the research. C.F., B.M., and E.S. performed most experiments with the help of M.D.R.; L.M.; N.N.; D.C.; B.A.; G.S.; M.T.P.; A.J.; T.J.

C.F. and G.M. wrote the manuscript with the help and suggestions of G.M.F. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare no conflicts of interest with the exception of GM who was a paid consultant for Takeda Pharmaceutical Company.

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