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HDAC6 mediates the acetylation of TRIM50

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ABSTRACT

The E3 Ubiquitin ligase TRIM50 promotes the formation and clearance of aggresome-associated polyubiquitinated proteins through HDAC6 interaction, a tubulin specific deacetylase that regulates microtubule-dependent aggresome formation. In this report we showed that TRIM50 is a target of HDAC6 with Lys-372 as a critical residue for acetylation. We identified p300 and PCAF as two TRIM50 acetyltransferases and we further showed that a balance between ubiquitination and acetylation regulates TRIM50 degradation.

1. Introduction

The TRIM family comprises proteins that act as E3 ubiquitin ligases in regulating a plethora of biological processes [1]. TRIM50 has been recently associated with the dynamic movement of intracellular vesicles and of gastric parietal cells in mice knock out model [2]. We previously reported that *TRIM50* encodes an E3 ubiquitin ligase [3] that interacts with the adapter protein p62/SQSTM1 and with the Histone Deacetylase 6 (HDAC6) [4]. Furthermore we showed that TRIM50 promotes the recruitment of polyubiquitinated proteins into the aggresome, contributing to their clearance [4].

Protein lysine acetylation has emerged as a key posttranslational modification (PTM) in gene expression regulation, protein stability and signal transduction [5], through the modification of histones and nuclear and cytoplasmic components, ranging from transcription factors to receptor proteins.

Protein acetylation is a dynamic process controlled by the antagonistic actions of two large families of enzymes: the histone acetyltransferases (HATs) and the histone deacetylases (HDACs). The balance between the activities of these enzymes serves as a key regulatory mechanism for gene expression and governs numerous developmental processes and disease states. HATs commonly catalyze the transfer of an acetyl group from acetyl-CoA to the terminal amine on the side chain of lysine residues of proteins. At least two independent HATs, namely p300 and PCAF, regulate the activity of non-histone proteins pointing out HATs as multifunctional factors [6]. In humans, there are 18 potential histone deacetylases enzymes, HDAC1 to HDAC11 and SIRT1 to SIRT7, which are responsible for the removal of acetyl groups and maintenance of the equilibrium of lysine acetylation in histones. Like HATs, histone deacetylases also possess substrate specificity and accumulating evidences suggest that many, if not all, HDACs can deacetylate also non-histone proteins [5].

Additionally, since lysines also provide specific sites for other PTMs, their acetylation would chemically lock the residue, conferring to this modification a regulatory potential with the ability to interfere with cellular functions relying on other lysine modifications, i.e., methylation, sumoylation, neddylation, biotinylation, and ubiquitination [7].

Ubiquitination is one of the most abundant and versatile PTMs in cells where the ubiquitin is covalently added to lysine residues. In humans, the ubiquitination is catalyzed by >500 E3 ligases. There are several types of ubiquitin modifications with different effects on target proteins, for instance the polyubiquitination is the triggering signal that promotes their proteasomal degradation. A cross-regulation between lysine acetylation and ubiquitination was found as a critical regulatory mechanism. Besides a direct competition for the lysine being modified, a more complex process connect the two signalling pathways, for instance the acetylation improves proteins stability by inhibiting ubiquitin-dependent degradation [7].

In the present study, we report that TRIM50 is acetylated by PCAF and p300. We found that HDAC6 deacetylases TRIM50 in a microtubule-dependent manner and that acetylation and ubiquitination compete for the same lysine residue.

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2. Materials and methods

2.1. Fusion plasmids and mutagenesis

The FLAG-TRIM50 stable cell line, pcDNA3-EGFP wild-type and mutants TRIM50 were described in [4]. TRIM50 Dom.1-2-3 were cloned into a pcDNA3-EGFP vector using a PCR based method with appropriate oligonucleotides followed by in-frame insertion into the vector. EGFP-TRIM50_K372R, EGFP-TRIM50_Del372-374, EGFP-TRIM50ΔNES1-2-3 and EGFP-TRIM50 Dom.1ΔNES3 were generated by site-directed mutagenesis via QuickChange II kit (Stratagene) using EGFP-TRIM50 and EGFP-TRIM50 Dom.1 as templates, respectively. HDAC6 mutants were a gift of Prof. P. Matthias (Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland). FLAG-PCAF and HA-p300 were a gift of Prof. E. Seto (Molecular Oncology Program, H. Lee Moffitt Cancer Center and Research Institute, Tampa, USA).

2.2. Cell lines

HEK293, HeLa (all from ATCC, Manassas, USA) and MEF *Hdac6* cell lines were maintained in DMEM with Glutamax medium supplemented with 10% fetal bovine serum and 1% antibiotics (Invitrogen, Carlsbad, CA). Fugene HD (Promega) was used for transfection according to the manufacturers' instructions.

2.3. Immunofluorescence microscopy

For immunofluorescence analyses, the cells transfected with EGFP-TRIM50 and EGFP-TRIM50 mutants were fixed before their incubation with the primary and secondary antibodies of interest, mounted in mowiol and examined on a Zeiss LSM 510 META confocal microscope (Carl Zeiss, Jena, Germany). All confocal images were obtained using the necessary filter sets for GFP, Alexafluor 488 and 546, using a Zeiss Plan-Neofluor 63× oil immersion objective (NA 1.4), with the pinhole set to one Airey unit. HDAC6 antibody (Santa Cruz) was used at 1:200 dilution, where is indicated.

2.4. Immunoprecipitation and western blot

Co-immunoprecipitation experiments were performed using Dynabeads magnetic beads (Invitrogen) following manufacturer's instructions. Complexes were analyzed by western blotting using indicated antibodies: anti-EGFP, anti-ubiquitin (Santa Cruz), anti-Ack (Cell Signaling), anti-FLAG (Sigma) and anti-HA (Covance). Horseradish peroxidase conjugated anti-mouse (GE Healthcare) and anti-rabbit (Santa Cruz) antibodies and the ECL chemiluminescence system (GE Healthcare) were used for detection. Where indicated the MG132 proteasome inhibitor (Calbiochem, USA) was added to the cell medium.

2.5. Statistical analysis

All microscopy experiments were performed at least in triplicate. Approximately 100 cells were analyzed for each experimental condition. Variations of localizations were evaluated on the basis of Student's *t*-test.

3. Results

3.1. TRIM50 is acetylated at K372 site

As elsewhere demonstrated, TRIM50 interacts with HDAC6, an interaction strengthens in the presence of the proteasome inhibitor MG132 [4]. To map the domain(s) of HDAC6 that binds TRIM50 we performed co-immunoprecipitation assays with different HDAC6 truncating mutants in the presence of MG132. As reported in Fig. 1(B–D) only the two constructs containing the first deacetylase (CAT1) domain, HDAC6_DBUZ and HDAC6 (1-503), interact with TRIM50 indicating that CAT1 is necessary for TRIM50 interaction.

The association between TRIM50 and the catalytic HDAC6 domain suggests that TRIM50 may be a deacetylation target of HDAC6. To assess this, two TRIM50 constructs, tagged with FLAG and EGFP respectively, were immunoprecipitated and an anti-acetyl-lysine specific antibody was used for immunodetection of acetylated-TRIM50. Acetylated form(s) of TRIM50 were detected showing that TRIM50 is an acetylated protein (Figs. 1E and 2B). Moreover a TRIM50 mutant construct lacking the C-Terminal region (EGFP-TRIM50_Dom1) (schematized in Fig. 2A) was unable of being acetylated, hinting that RFP domain is involved in acetylation (Fig. 2B). Next we searched for putative lysine residues of TRIM50 involved in acetylation. Computational prediction revealed the presence of two evolutionarily conserved lysines, K372 and K374, as the more likely acetylation sites in the C-terminal TRIM50 region (Fig. 2C). Therefore, to assess the acetylated residues in TRIM50 protein, by direct mutagenesis we generated the two EGFP-TRIM50_Del372-374 and EGFP-TRIM50_K372R constructs and performed immunoprecipitation assays using an EGFP antibody. These studies revealed that both TRIM50_Del372-374 and TRIM50_K372R mutant proteins did not undergo to acetylation modifications when compared to TRIM50 wild-type construct, hinting that K372 residue is a target of acetylation (Fig. 2D).

3.2. p300 and PCAF are acetyltransferases of TRIM50

Acetylation Set Enrichment-Based software (ASEB; http://202.38.126.151/hmdd/huac/) predicted CBP/p300 and PCAF as acetylase proteins associated to K372 residue of TRIM50. By immunoblotting assays we showed that ectopic expression of p300 and PCAF increased the level of acetylated TRIM50, although with different strength (Fig. 2E).

3.3. HDAC6 deacetylates TRIM50 in a microtubule-dependent manner

Since the catalytic CAT1 domain of HDAC6 is involved in TRIM50 interaction and TRIM50 is acetylated, we asked whether HDAC6 is a deacetylase of TRIM50. As shown in Fig. 2F, the overexpression of HA-HDAC6 abrogates TRIM50 acetylation. To confirm this result, we transfected Mouse Embryo Fibroblasts (MEFs) derived from *Hdac6* $^{+/+}$ and $^{-/y}$ mice with EGFP-TRIM50 and we found that TRIM50 acetylation increased in *Hdac6* $^{-/y}$ cell lines, providing additional evidence for a role of HDAC6 in mediating TRIM50 deacetylation (Fig. 2G). Finally, we used Trichostatin A (TSA), a deacetylase inhibitor of class I/II HDAC family enzymes (Fig. 2E and H). By immunoprecipitation assay we found that TSA increased TRIM50 acetylation level. Collectively, these data indicate that HDAC6, through its first catalytic domain, interacts with and regulates TRIM50 acetylation in K372 residue.

Since HDAC6 and TRIM50 possess both a microtubule-binding capacity we assessed whether microtubules integrity is required for TRIM50 deacetylation. We treated the FLAG-TRIM50#3, a HEK293 cell line stably expressing a FLAG-TRIM50 construct, in ice for 1 h, a treatment that leads to complete depolymerization of microtubule network [8]. After microtubule depolymerization, by immunoprecipitating TRIM50 protein and blotting with the acetyl-lysine antibody we observed an increase of TRIM50 acetylation level. This increase was persisting even in the presence of HDAC6 (Fig. 3A). Overall, these results indicate that an intact microtubule network is required for HDAC6 to deacetylase TRIM50.

3.4. TRIM50 acetylation antagonizes TRIM50 ubiquitination

TRIM50 is regulated by ubiquitination [3]. Since acetylation can compete with the ubiquitination in targeting proteins [9] we asked whether acetylation competes with and may inhibit TRIM50 ubiquitination. To assess this, we analyzed the ubiquitination level of TRIM50 and of K372-TRIM50 mutant protein. A higher ubiquitination level of TRIM50

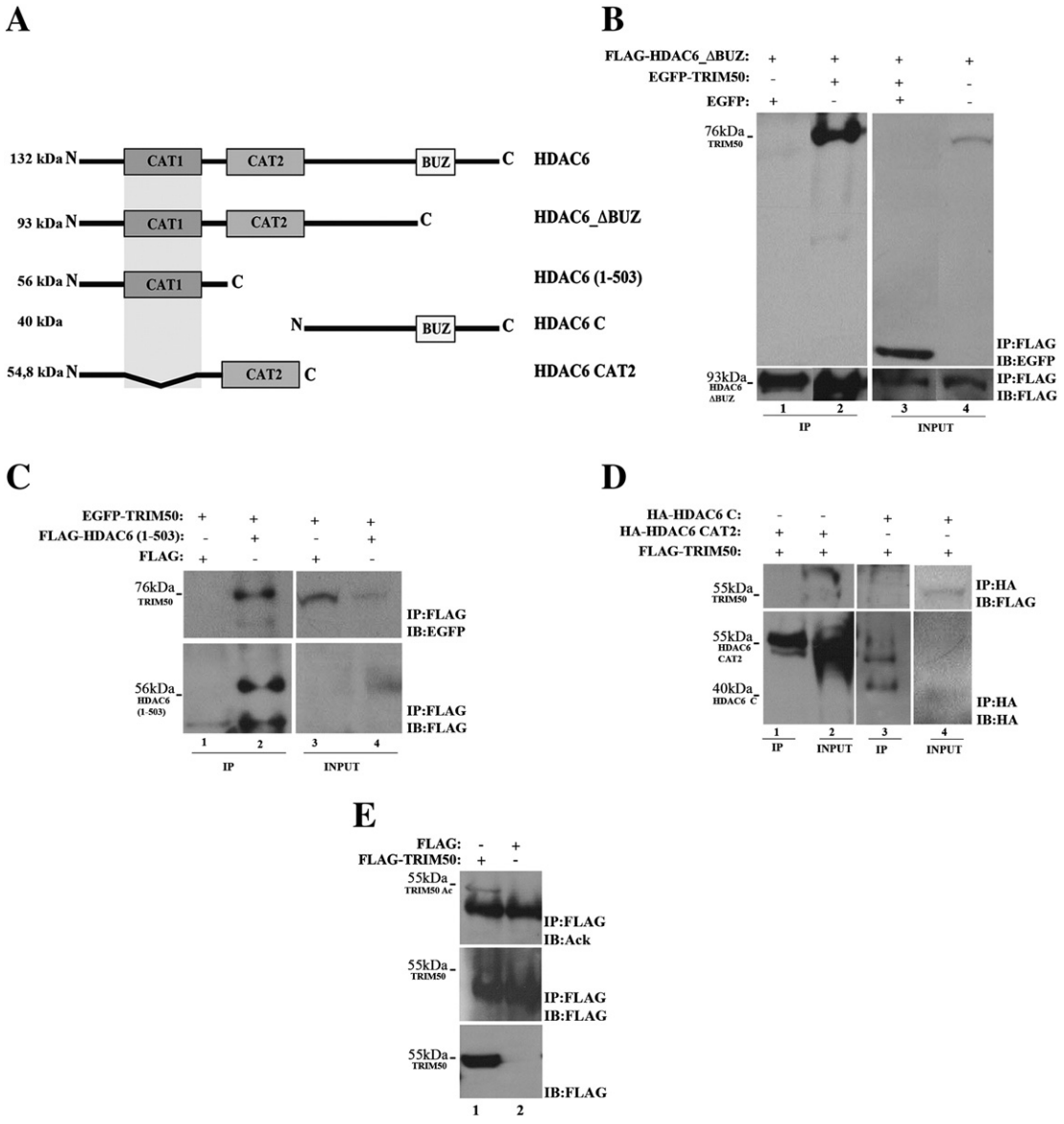

Fig. 1. TRIM50 is acetylated in vivo. (A) Schematic representation of HDAC6 deletion mutants with the minimal region of interaction between TRIM50 and HDAC6 depicted in gray. (B–C) HEK293 transiently transfected with EGFP-TRIM50 and FLAG-HDAC6 deletion mutants were treated with 25 μM of MG132 for 6 h, immunoprecipitated with anti-FLAG and immunoblotted with anti-GFP. (D) FLAG-TRIM50#3 transiently transfected with HA-HDAC6 deletion mutants were treated with 25 μM of MG132 for 6 h, immunoprecipitated with anti-HA and immunoblotted with anti-FLAG. (E) FLAG-TRIM50#3 and FLAG-#4 empty vector, were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Ack.

was observed in wild type construct when compared to K372R-TRIM50 proteins, suggesting that acetylation and ubiquitination compete for the same lysine residue (Fig. 2D). Corroborating these data, we also observed that after microtubule depolymerization, which causes an increase of TRIM50 acetylation level, the ubiquitination of TRIM50 protein decreased (Fig. 3A).

3.5. The RFP domain is involved in TRIM50 cytoplasmic localization

Since we have previously shown that the ablation of C-terminal end of TRIM50 determines a quite complete nuclear localization of TRIM50 [3,4], we speculate that the RING and Bb-Cc domains may contain specific Nuclear Localization Sequence(s) (NLS) that can mediate TRIM50 nuclear localization or that Nuclear Export Sequence(s) (NES) are located close to or within the RFP domain of TRIM50. Therefore, by bioinformatic tools we searched for NLS and NES (http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi; http://www.cbs.dtu.dk/services/NetNES/; http://www.sbc.su.se/~maccallr/nucpred/) in TRIM50 aminoacidic sequence, identifying three putative NES located in B-box (from amino acid 71 to 80), Coiled-coil (203–212) and just upstream to the RFP-like (277–289) domains, respectively. Using indirect immunofluorescence microscopy we measured the nucleus and cytoplasm localization of three TRIM50 mutant proteins carrying a deletion of each NES respectively (Fig. 2A). We found that only EGFP-TRIM50ΔNES3 construct showed a predominant nuclear localization (Fig. 3B) suggesting that this NES is the only functionally active. However, since an amount of EGFP-TRIM50ΔNES3 retains a cytoplasmic localization, we hypothesized that the C-terminal region and particularly the RFP domain contains some sequences that may cooperate with NES3 in determining TRIM50 cytoplasmic localization. To assess that, we generated a number of constructs carrying different deletions of the C-terminal end of TRIM50 and we measured the amount of nuclear and cytoplasmic localization of each construct (schematized in Fig. 2A). As reported in Fig. 3C, besides the TRIM50-ΔRFP mutant, the TRIM50_Dom1 construct retained only a

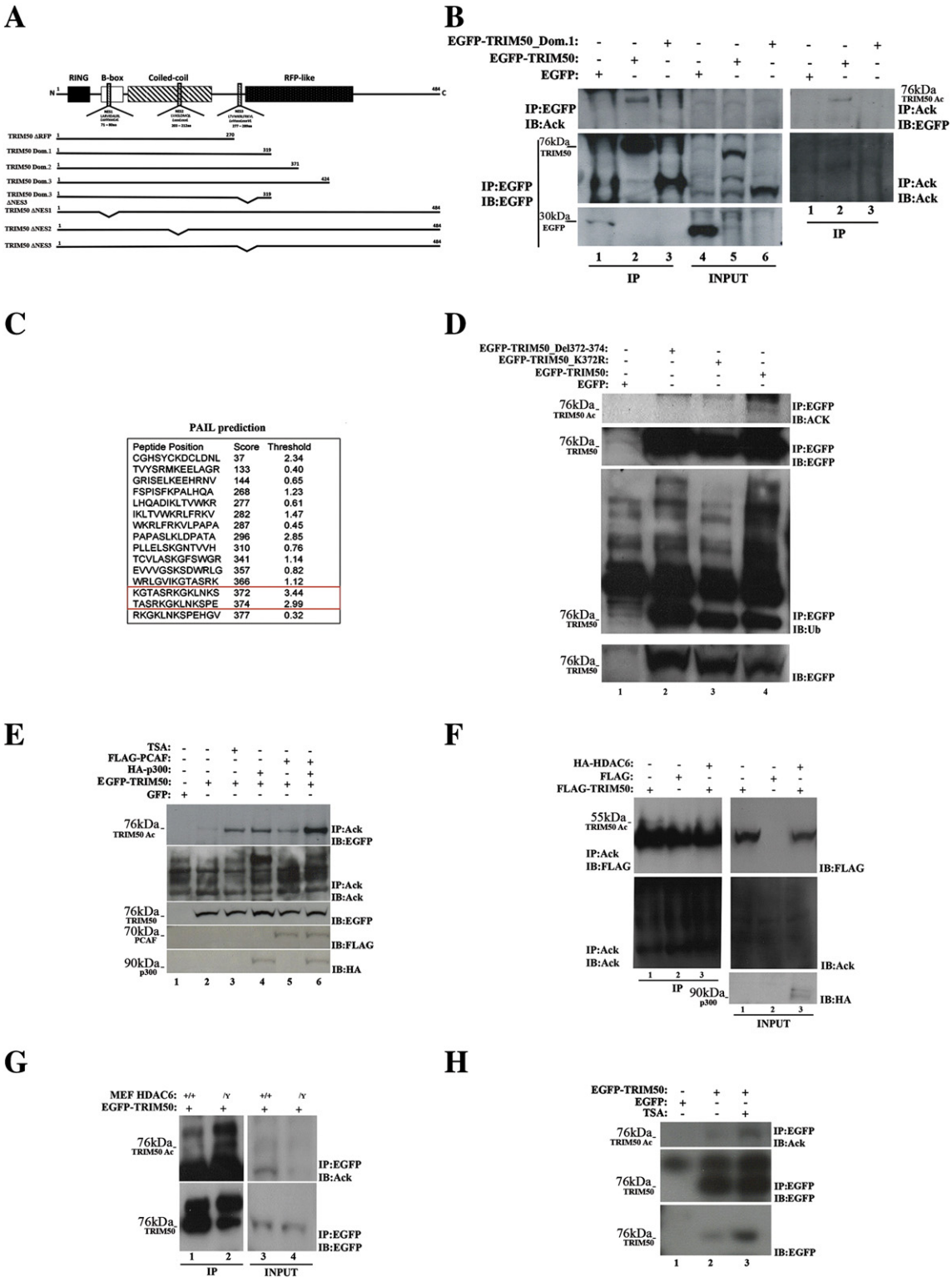

Fig. 2. HDAC6, PCAF and p300 regulate the acetylation and/or deacetylation of TRIM50. (A) Schematic representation of TRIM50 deletion mutants. (B) HEK293 transiently transfected with EGFP-TRIM50, EGFP-TRIM50_Dom1 and EGFP-empty vectors were immunoprecipitated with anti-EGFP and anti-Ack and immunoblotted with Ack and EGFP antibodies respectively. (C) PAIL bioinformatic analysis of TRIM50 sequence. (D) HEK293 transiently transfected with EGFP-TRIM50, EGFP-TRIM50 K372R and EGFP-TRIM50 Del372-374 vectors, were immunoprecipitated with anti-EGFP and immunoblotted with anti-Ack and anti-Ub antibodies. (E) HEK293 transiently transfected with EGFP-TRIM50 was treated with TSA 5 μM for 1 h and/or cotransfected with FLAG-PCAF or HA-p300, immunoprecipitated with anti-Ack and immunoblotted with anti-Ack and anti-EGFP antibodies. (F) FLAG-TRIM50#3 was transfected with HA-HDAC6, immunoprecipitated with anti-Ack antibody and immunoblotted with FLAG and anti-Ack antibodies. (G) *HDAC6*$^{+/+}$ and *HDAC6* $^{-/Y}$ MEFs were transfected with EGFP-TRIM50, immunoprecipatated with anti-EGFP and immunoblotted with Ack and EGFP antibodies, respectively. (H) HEK293 transiently transfected with EGFP-TRIM50 was treated with 5 μM of TSA for 1 h, immunoprecipitated with anti-EGFP antibody and immunoblotted with anti-Ack.

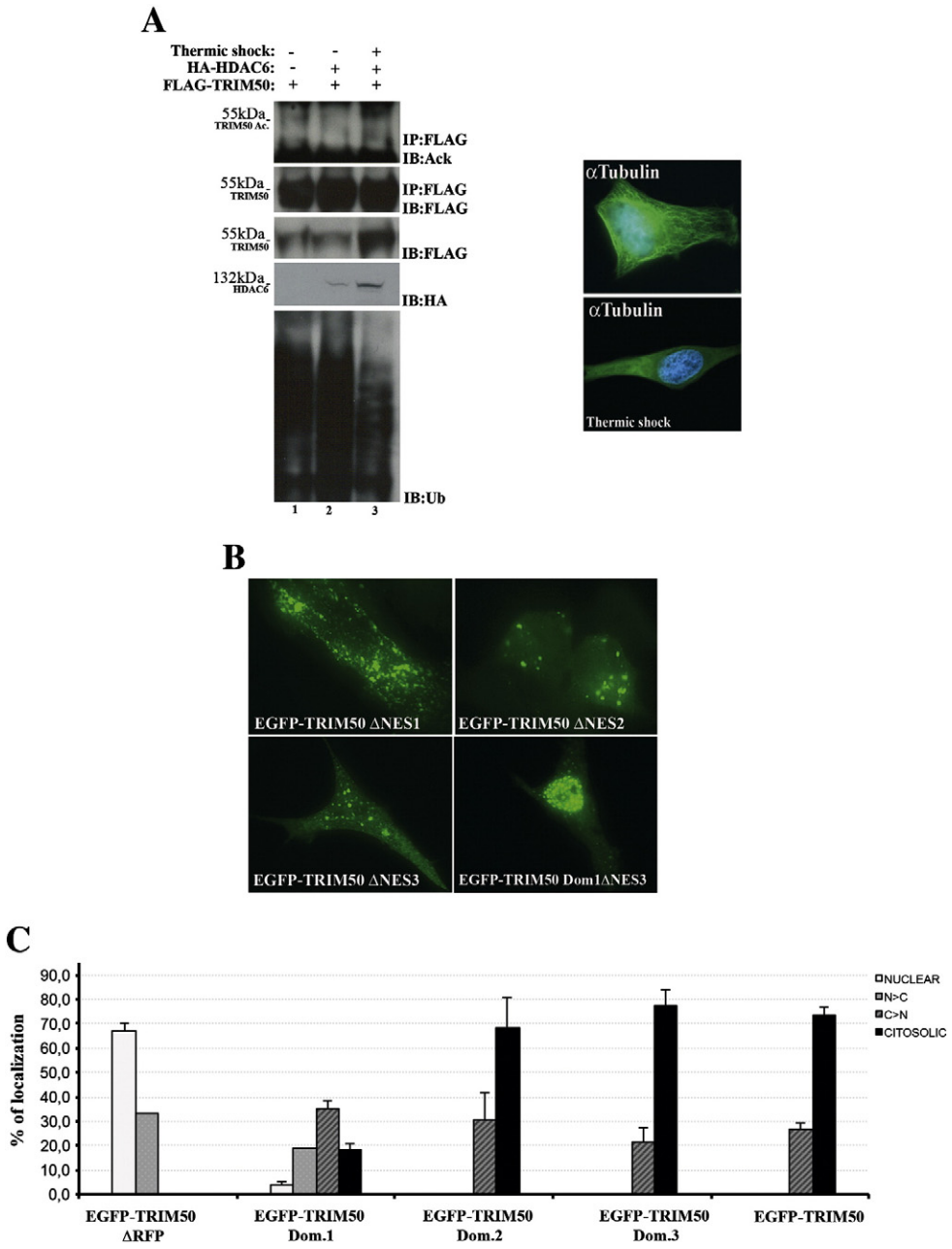

Fig. 3. TRIM50 acetylation reduces its ubiquitination in a microtubule-dependent manner. (A) FLAG-TRIM50#3 treated or not 1 h in ice, staining with αTubulin, was immunoprecipitated and immunoblotted with anti-Ack, FLAG, HA and Ub antibodies. Nuclear export of TRIM50 protein. (B) HeLa cells transfected with EGFP-TRIM50 mutants were analysed at confocal microscopy. (C) The diagram shows the percentage of localization of each mutant relative of three independent experiments.

marginal localization of TRIM50 into the nucleus (Fig. 3C) compared to the other domains that preserved a quite complete cytoplasmic localization of TRIM50.

In addition, ectopic expression of TRIM50_Dom1ΔNES3, a construct lacking the NES3, re-localized the protein exclusively in the nucleus (Fig. 3B). Overall, these results suggest that the region of TRIM50, which comprises a part of RFP domain and goes from residue 319 to 484, cooperates with NES3 to the TRIM50 cytoplasmic localization.

3.6. The RFP domain and acetylation are necessary for aggresome localization of TRIM50

Since TRIM50 re-localizes into aggresome upon proteasome inhibition [4], we determined the contribution of TRIM50 domains to aggresome localization. As elsewhere described TRIM50 aggresome-localization does not depend on the E3 Ubiquitin ligase activity, as a mutant lacking the RING domain retains the ability to localize to aggresome [4]. Likewise, MG132 treatment was able of inducing a main aggresome localization of TRIM50 in constructs carrying the B-box or the C-terminal domains (Fig. 4). Conversely, deletion of RFP domain induced a significant localization of TRIM50 protein outside the aggresome, in MG132 treated cells. Overall, these results substantiate the importance of RFP domain for TRIM50 aggresome localization. Next we asked whether the K372 residue has a role in TRIM50 aggresome localization. In untreated cells, ectopic K372R-TRIM50 construct localized in cytoplasmic structures as the wild-type protein; after proteasome inhibition, approximately 40% of K372R-TRIM50 resulted in a complete aggresome localization, while around 50% of the protein localizes just

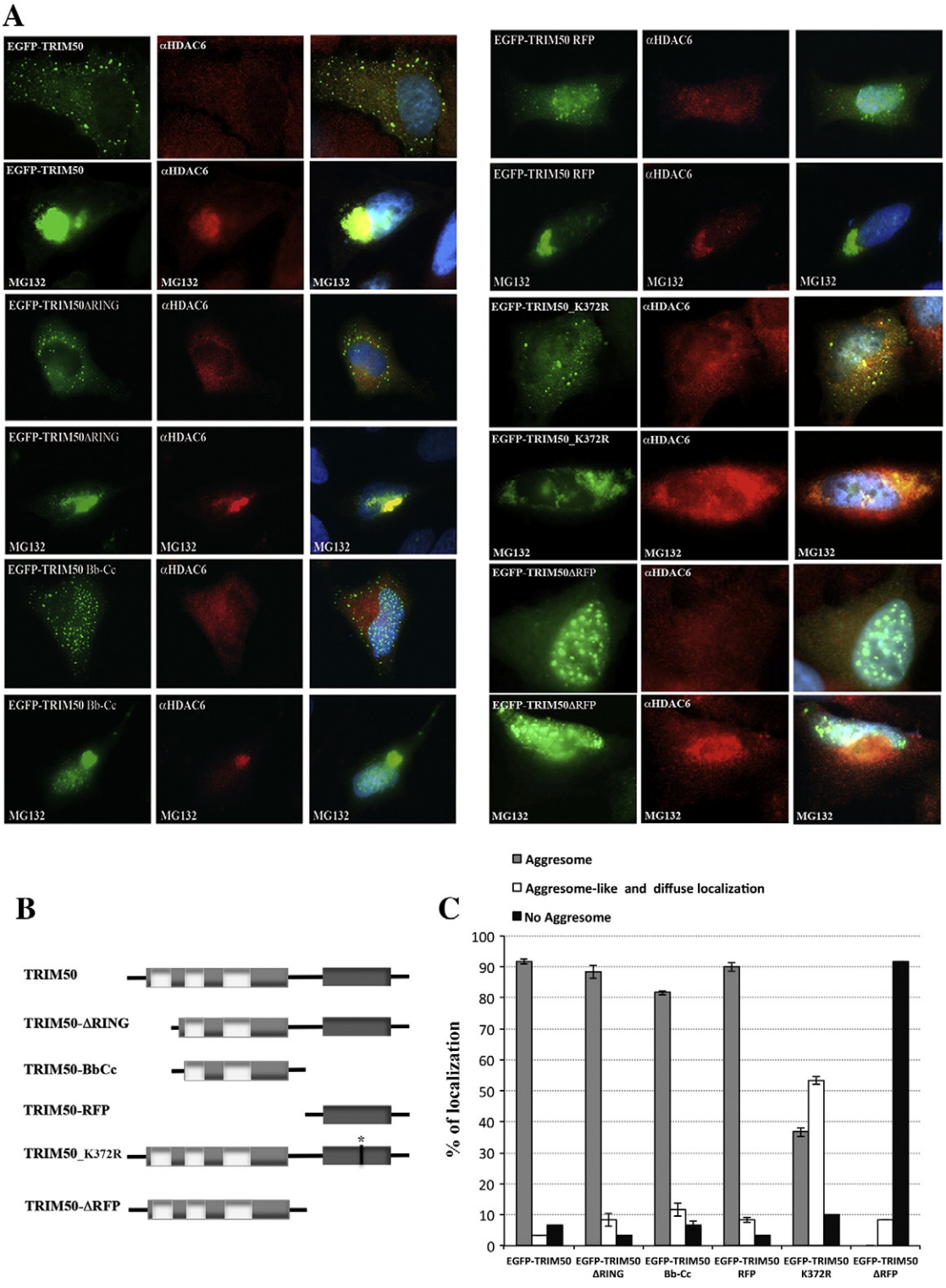

Fig. 4. Aggresome localization of TRIM50 domains. (A) HeLa cells transfected with the reported TRIM50 deletion mutants were stained using HDAC6 antibody and analysed at confocal microscopy. Where indicated the cells were treated with 25 μM MG132 for 6 h. (B) Schematic representation of TRIM50 deletion mutants used in immunofluorescence assays. (C) The diagram shows the percentage of TRIM50 localization of each mutant. The data comes from three independent experiments.

partially in the aggresome with the remaining fraction retaining a diffuse localization. Since elimination of K372 residue, involved in both ubiquitination and acetylation significantly affects the localization of TRIM50, we intimate that K372 although is neither sufficient nor necessary, contributes to drive TRIM50 aggresome localization.

4. Discussion

Lysine acetylation is best known for its key role in the histone code, wherein it dictates the dynamic output of gene transcription. Further studies have indicated that acetylation occurs not only on transcription

but also on a variety of other cellular processes such as ubiquitination, that do not directly involve histones [10]. In this report, we showed that the E3 Ubiquitin ligase TRIM50 is deacetylated by HDAC6 and that intact microtubules are necessary for TRIM50 deacetylation. Moreover this study also revealed that CBP/p300 and PCAF drive TRIM50 acetylation at Lysine 372, a residue also involved in TRIM50 auto-ubiquitination. Our results suggest that acetylation, by competing with the same lysine aminoacid residue, interferes with TRIM50 ubiquitination probably enhancing its own stability. Such acetylation and ubiquitination competition has been reported for other proteins. For instance investigations confirmed the role of lysine acetylation in the control of SMAD7 stability and showed that HDAC1 or SIRT1-dependent deacetylation, promotes SMAD7 ubiquitination and degradation [11]. Furthermore, MAML1-dependent acetylation of Notch1 ICD by p300 decreases the ubiquitination and proteosomal degradation of Notch1 ICD [12]; finally p14ARF, increases the level of p53 acetylated at lysine 382 in a nuclear chromatin-rich fraction increasing its stability and its transcription factor activity and inhibiting its degradation [13]. HDAC6 is a TRIM50-interactor that regulates the shuttle of TRIM50 to the aggresome when proteasome is inhibited. Findings suggest that HDAC6 is involved in the control of protein ubiquitination by deacetylating critical lysines [14]. Therefore, our hypothesis is that HDAC6 deacetylates TRIM50 at K372 favouring its ubiquitination and it is subsequently transported into aggresome associated to polyubiquitinated proteins.

TRIM proteins cluster into a constant and ordered N-Terminal region and a flexible C-Terminal tail that contain different types of functional domains. The TRIM50 C-terminal region, including the RFP domain, is essential for both TRIM50 cytoplasmic and aggresome localization. A combination of bioinformatics and fluorescence microscopy assays allowed to map a functional NES in the proximity of RFP domain which resulted necessary but not sufficient for cytoplasmic localization. Thus, we generated a number of constructs carrying deletions of TRIM50 within its C-terminal region and we defined the subcellular localization of such constructs. Immunofluorescence assays indicated that the amino acid stretch starting from residues 319 to the end of the protein contains additional sequences that might be involved in the cytoplasmic retention of the protein.

Therefore, we cannot exclude a functional interface between the C-Terminal TRIM50 region and NES3 sequence that work together for TRIM50 cytoplasmic localization. Alternatively TRIM50 can bind to an NES-containing protein, not yet identified.

Interestingly we demonstrated that TRIM50 interacts with p62 [4], a nucleus-cytoplasm shuttling protein that contains two NLSs and one NES. Nuclear p62 colocalizes with TRIM19/PML, by recruiting polyubiquitinated proteins to PML bodies and driving their proteasomal nuclear degradation [15]. Similarly, ectopically expressed TRIM50 and p62 show a nuclear colocalization in presence of Leptomycin B, an antibiotic that inhibits nuclear export of NES containing proteins (Fusco, unpublished data). Although in need of confirmation, we can speculate that TRIM50 by p62-mediated interaction may drive nucleus-cytosol shuttle and regulate the protein quality control degradation system.

5. Conclusions

By bioinformatic tools, biochemical and cellular assays we showed that the evolutionary conserved TRIM50 K372 residue competes for post-translational protein modifications such as acetylation and ubiquitination. TRIM50 is acetylated by PCAF and p300 and deacetylated by HDAC6 in a microtubule-dependent manner. Overall, the data presented here point to the existence of a potential crosstalk between acetylation and ubiquitination in the regulation of TRIM50. This study provides the basis for further elucidating the function of TRIM50 protein and highlights how TRIM50 acetylation is a fundamental posttranslational modification of this E3 Ubiquitin ligase protein.

Acknowledgments

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