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TRIM8 regulated autophagy modulates the level of cleaved Caspase-3 subunit to inhibit genotoxic stress induced cell death

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ABSTRACT

In cancer patients, treatment modalities like chemotherapy and radiation exert their anticancer effects by inducing DNA damage. The cancer cells can survive under genotoxic stress by inducing DNA damage response (DDR) or can undergo cell death. The process of autophagy is emerging as crucial regulator of cell survival during different stress conditions. Post translational modification through ubiquitin plays an essential role in DDR during genotoxic stress conditions. Ubiquitin ligases regulate autophagy and cell death pathways however their role during genotoxic stress conditions is not understood. In the current study we identified TRIM8, RING E3 Ligase, as a novel regulator of autophagy during DDR. TRIM8 regulates lysosomal biogenesis and autophagy flux. The turnover of TRIM8 is high and is stabilized during genotoxic stress conditions. TRIM8 regulated autophagy is essential for its cytoprotective role during genotoxic stress induced cell death. TRIM8 stabilizes the turnover of XIAP during genotoxic stress and forms complex with XIAP and caspase-3 to inhibit its activation in presence of etoposide. TRIM8 mediated autophagy promotes degradation of cleaved caspase-3 subunits. This study described TRIM8, as a novel regulator of DDR-autophagy crosstalk, which may play role in survival of cancer cells in presence of genotoxic agents.

1. Introduction

The cell has evolved DNA damage response (DDR) to ensure genomic integrity and cell survival. DDR includes sensing of the DNA damage, recruitment of DNA repair proteins and repair. During DNA damage response (DDR), cell cycle check points are activated leading to either repair or cell death in irreversible DNA damage conditions. DNA double stranded breaks (DSBs) detection by ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) promotes activation of apoptosis by inducing p53 [1] however many other regulators still needs to be identified. The DNA repair process and cell survival/death pathway are intricately linked to maintain cellular homeostasis [2,3]. Any defect in this process can lead to genomic aberrations including chromosomal translocations, mutations deletions/additions leading to malignant transformation of the cells. Cancer cells often have compromised DDR which help them in avoiding cellular checkpoints and proliferate. During radiation and chemotherapy of cancer patients, the genomic DNA of malignant cells is the target and DDR induced apoptosis is initiated to eliminate cancer cells [4]. The mechanism of cross talk of different cell survival and death pathways and their regulators during genotoxic stress in cancer cells needs to be identified and investigated.

Genotoxic agent induced autophagy have been implicated both in initiation of cell death as well as cell survival, hence its role further needs more investigation for targeted therapeutic intervention [5,6]. The crosstalk between autophagy and apoptosis is crucial as apoptosis acts as the last resort for cells in severe or persistent genotoxic stress [7]. Autophagy maintains cellular fitness by elimination of defective organelles, dysfunctional proteins and aggregates [8–10]. Interestingly, autophagy has also been shown essential for removal of DNA damage induced extranuclear DNA that can lead to activation of inflammatory pathways. It is observed that damaged DNA is exported outside the nucleus and its autophagy mediated degradation in lysosomes is essential for cell survival. Failure in clearance of extranuclear DNA leads to activation of inflammatory pathways by intracellular DNA sensors like cGAS/STING [11]. It had been observed that knockout of p62/

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SQSTM1 (an autophagy adaptor protein) cells are autophagy deficient and show sequestration of RNF168, an E3 ligase essential for histone H2A ubiquitination for initiation of DDR. During DNA damage ubiquitination is a major event that regulates autophagy and DDR. Ubiquitination of histones at the DSB site by RNF8 is one of the earliest events promoting DDR. It also recruits other ubiquitin E3 ligases like RNF168, for sustained ubiquitination at DSB site. Ubiquitination at DSB sites promotes destabilization of nucleosome and provide docking signal for damage response and repair proteins [12,13]. Ubiquitin E3 ligases like $CRL4^{DDB2}$ ligase complex and TRIM2 also play critical role in regulation of DDR by regulating levels of p53 and BIM (Bcl-2-interacting mediator of cell death) respectively [14,15]. These evidences suggest the importance of autophagy and ubiquitin ligases in regulation of DDR and cell fate determination after genotoxic stress.

In human genome > 1000 E3 Ligases have been postulated and many of them remain uncharacterized [16]. RING E3 Ligase constitutes the biggest family of E3 ligases [16,17]. TRIM family proteins comprise the largest subgroup of RING E3 ligases and play diverse role in regulation of cellular homeostasis [18,19]. The reports from our lab and others show that TRIMs regulate autophagy and play crucial role in cell survival and death during different stress conditions including genotoxic stress [20–24]. We and others previously reported that TRIM8; a potential oncogene positively regulates TNF-α induced NF-κB pathway and clonogenic potential of cancer cells [25,26]. NF-κB is pleotropic transcriptional regulator of many key genes involved in various functions including autophagy which plays important role at various step of tumorigenesis [27]. Here we demonstrate TRIM8, as a novel regulator of autophagy and DDR cross talk. TRIM8 regulates lysosomal biogenesis and autophagy flux which inhibits caspase activity and promotes cell survival during genotoxic stress condition.

2. Materials and methods

2.1. Cells and reagent

HEK293 and HeLa cells were grown at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Thermo Fisher Scientific, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Gibco, Thermo Fisher Scientific, USA). TRIM8 in pCDNA-3 was provided by Dr. Walther H Mothes (Section of Microbial Pathogenesis, Yale School of Medicine, USA) [28]. HA-TRIM8 and HA-TRIM8ΔR in pCGN-HA was provided by Dr. S Hatakeyama (Department of Biochemistry, Hokkaido University Graduate School of Medicine) [29].

GFP-LC3 was provided by Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan) [30], mCherry-GFP-LC3, mCherryGFP-p62 and GFP-p62 by Dr. Terje Johansen (Dept. of Biochemistry, Institute of Medical Biology, University of Tromsø) [31]. shRNA for TRIM8, and control were provided by Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain). PX459 V2.0 was provided by Dr. Feng Zhang (McGovern Institute for Brain Research, MIT, USA) [32]. The primary antibodies used were: Anti-HA-HRP, Anti-FLAG-HRP and Anti-LC3 (Sigma, USA), rabbit polyclonal against β-Actin (Abcam, USA), rabbit polyclonal against XIAP, LAMP1 and caspase-3 (Cell signaling technology, USA). Secondary antibodies HRP-conjugated anti-rabbit and anti-mouse antibodies (Jackson ImmunoResearch, USA) were used. Rotenone, Wortmannin, MG132, Etoposide, Ammonium Chloride, Bafilomycin A1, M2 FLAG-Affinity Gel and EZview™ Red Anti-HA Affinity Gel were purchased from Sigma-Aldrich, USA. G418 was purchased from Gibco, Invitrogen, USA. For transfection FuGENE HD (Roche, Germany) or Lipofectamine® 2000 (Invitrogen, USA) were used. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) reagent was purchased from MP Biomedicals, USA.

2.2. Generation of TRIM8-sgRNA clones

TRIM8-sgRNA clones were generated as described by Ran et al. 2013 [32]. Two guide RNAs targeting the first exon of TRIM8 were designed using CRISPR design tool [32] for generation of two clones targeting TRIM8. sg-RNA-top and sg-RNA-bottom were synthesized as described earlier for both guide RNAs [32]. Synthesized oligos were annealed and cloned into *Bbs*I -linearized pSpCa9(BB)-2A-Puro (PX459) V2.0 vector. TRIM8-sgRNA-1 and TRIM8-sgRNA-2 clone were transformed into competent Stbl3 *E. coli* strain and transformants were screened by colony PCR using U6 sequencing primer and antisense sgRNA. Positive clones were finally confirmed by Sanger sequencing. Empty vector PX459 V2.0 or TRIM8-sgRNA1 & 2 were transfected in HeLa and MCF7 cells using lipofectamine 2000. After 48 h of transfection, RNA was isolated and cDNA was synthesized. TRIM8 specific primers were used to check the transcript levels of TRIM8 in both empty vector and TRIM8-sgRNA1 & 2 transfected cells. TRIM8-sgRNA guide sequences and colony PCR primers are listed in the Supplementary Table 1.

2.3. Generation of stable cell lines

To study the role of TRIM8 in regulation of autophagy, HEK293-LC3-GFP was generated as described previously [24].

2.4. Autophagy assay by fluorescent microscopy

Autophagy was monitored using HEK293-GFP-LC3 stable cell line [24]. HEK293-GFP-LC3 cells were seeded at density of 1.5×10^5 cells per well in 24 well plate and transfected with respective constructs using FuGENE HD Transfection Reagent (Roche, Germany). After 24 h of transfection, cells were monitored for GFP-LC3 puncta using IX81 fluorescent microscope (Olympus, Japan). Images obtained were used for counting of LC3 puncta. Numbers of puncta per cell were counted in minimum 100 cells.

Similarly to assess autophagy flux using fluorescent microscopy, mCherry-GFP-LC3 and mCherry-GFP-p62 constructs were co-transfected with indicated constructs. After 24 h of transfection numbers of red and green puncta per cell were counted in minimum 100 cells and graph plotted for average number of red puncta per cell.

2.5. Quantitative analysis of gene expression

Total RNA was isolated using RNAiso Plus Reagent (Takara, JAPAN) and was reverse transcribed to synthesize cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. Real time PCR was performed using either by SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara, Japan) or iTaq™ universal SYBR® Green supermix as per the manufacturer's instruction.

Specific primers for the genes are listed in the Supplementary Table 2.

2.6. Cell death and viability assay

The cellular proliferation was analyzed by MTT assay. HeLa and HEK293 cells were seeded in 96-well plate at a density of 2×10^4 cells/well. The cells were transfected with indicated vectors using Lipofectamine® 2000 (Invitrogen, USA). After 24 h of transfection, cells were treated with or without etoposide for indicated time. After treatment 20 µl MTT (5 mg/ml) was added to each well and incubated for 1.5 h. MTT containing media was replaced by MTT solubilization agent DMSO (SRL, India) to dissolve the precipitate of purple colored formazan and colour intensity was monitored using colorimetric microplate reader (BioTek Instruments, Inc. USA) at 620 nm wavelength.

The cell death and viability was monitored by Trypan blue dye exclusion assay as described previously [33].

2.7. Western blotting

To check autophagy by western blotting, HEK293, MCF7 and HeLa cells were seeded at a density of 2.5×10^5 per well in 12 well plate and TRIM8/TRIM8-shRNA/TRIM8-sgRNA was transfected using standard calcium phosphate transfection method or Lipofectamine® 2000 (Invitrogen, USA) [34]. To analyze XIAP and Caspase-3 levels, HEK293 and HeLa cells were seeded at a density of 4.5×10^5 per well in 6 well plate. The cells were transfected using either calcium phosphate transfection method or Lipofectamine® 2000 (Invitrogen, USA). After 24 h of transfection, cells were treated with indicated chemicals. After treatment, cells were harvested, washed with ice cold PBS and lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris–Cl, 5 mM EDTA, 1% NP40, 1% Glycerol and 1× protease inhibitor cocktail (SIGMA, USA). Protein concentration was determined by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, USA) and equal amount of proteins were resolved on 12.5% SDS-PAGE. Proteins were electro-blotted on PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) at 110 V for 1 h at 4 °C. Following the transfer, the membrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 h at room temperature. The membrane was incubated overnight with specific primary antibody. After incubation membrane was washed three times with TBS-T (TBS containing 0.1% Tween-20) and incubated with a secondary antibody at room temperature for 1 h. The membrane was washed three times with TBS-T and signal visualized by using Western BloT Chemiluminescent Substrates (Takara) or Luminata Forte Western HRP substrate (EMD Millipore) by exposing to X-ray film.

2.8. Immunoprecipitation

To study the protein interactions, immunoprecipitation experiments were performed. Briefly, HEK293 cells were plated at a density of 2×10^6 per 90-mm-diameter dish and transfected with HA-TRIM8 using calcium phosphate transfection method [34]. After 36 h of transfection, cells were treated with indicated chemicals and incubated for 12 h. After treatment, cells were harvested, washed with ice cold PBS (Gibco, Thermo Fisher Scientific, USA) and lysed in immunoprecipitation buffer (100 mM NaCl, 50 mM Tris–HCl, 0.1% NP40 containing 1 mM PMSF). Cell lysate was incubated with EZview™ Red Anti-HA Affinity Gel (Sigma, USA) on roller shaker overnight at 4 °C. The gel beads were washed four times with IP buffer, resuspended in 5× SDS-PAGE sample buffers and separated on 12% SDS-PAGE and analyzed by western blotting using specific antibodies.

For co-immunoprecipitation, FLAG-XIAP and HA-TRIM8 were transfected in 60-mm dish as indicated. After transfection and treatment cells were lysed in FLAG IP buffer (150 mM NaCl, 50 mM Tris–HCl, 1% Triton X-100 containing 1 mM PMSF). Cell lysates were incubated with M2 FLAG-Affinity Gel (Sigma, USA) and further processing was done as mentioned above.

2.9. Caspase-3 luciferase assay

Caspase 3/7 activation assay was performed using CaspaseGlo® 3/7 Assay Systems (Promega, USA) according to manufacturer instructions. Briefly, indicated constructs were transfected in white bottom 96 well plate using Lipofectamine® 2000 transfection reagent (Invitrogen, USA) by forward transfection method. DNA:transfection reagent mixture were dispensed into 96 well plate and thereafter 20,000 cells per well were plated. After 24 h of transfection, cells were treated with etoposide (100 μM) for indicated time. The caspase substrate was added to each well, incubated for 1 h at room temperature and luminescence was measured using luminometer (Berthold Technologies, Germany).

2.10. Statistical analysis

Data are shown as mean ± SEM for n observations. Comparisons of groups were performed using student *t*-test for repeated measurements to determine the levels of significance for each group. The experiments were repeated minimum three times independently and probability values of $p < 0.05$ were considered as statistically significant. The data was normalized as maximum value was considered as 100% and 0 as 0% for all data set.

3. Results

3.1. TRIM8 regulates autophagic flux

The emerging evidences suggest that NF-κB regulate autophagy [35–37]. We had previously shown that TRIM8 positively regulate TNF-α induced NF-κB [25,26], whereas its implication in autophagy and DDR is still not clear. To investigate role of TRIM8 in regulation of autophagy, HEK293-GFP-LC3 stable cell line was transfected with TRIM8 and GFP-LC3 puncta were monitored by fluorescent microscopy. TRIM8 transfected cells show increased number of GFP-LC3 puncta indicating increased autophagosome formation (Fig. 1A & B).

We further checked the role of TRIM8 on autophagy flux by microscopy using tandem construct of mCherry-GFP-LC3 and mCherry-GFP-p62 fluorescent reporters as described previously [31,38]. The yellow puncta (red and green merge) indicate autophagosomes whereas red puncta indicate autophagosomes fused with lysosomes also called as autophagolysosomes [38]. TRIM8 was co-transfected with mCherry-GFP-LC3 and mCherry-GFP-p62 fluorescent reporter constructs. The expression of TRIM8 significantly increased the number of p62 red puncta (Fig. 1D and E). Similarly, number of LC3 red puncta (Fig. 1F) significantly increased in TRIM8 co-transfected cells. Autophagy flux was further confirmed using LC3 western blotting [39]. TRIM8 was transfected in HEK293 and MX1 (p53 wild type cells) cells and treated with Bafilomycin-A1 (inhibitor of autophagosome-lysosome fusion) and analyzed by western blotting using LC3 antibody which detects both LC3-I and LC3-II (PE-conjugated). The level of 16 kDa band corresponding to LC3-II (conjugated form) increased in TRIM8 transfected Bafilomycin-A1 treated cells compared to control (Fig. 1C, Supplementary Fig. 1A). We also knocked down TRIM8 using sgRNA in MCF7 cells (p53 wild type) and treated with Bafilomycin-A1. Western blotting showed decrease in LC3-II levels in TRIM8 knock down MCF7 cells (Supplementary Fig. 1B). p62/SQSTM1 level was also monitored in the same blot. The level of 62 kDa band corresponding to p62/SQSTM1 decreased in untreated TRIM8 knock down cells as compared to control indicating possible transcriptional regulation of cellular p62/SQSTM1 levels trough TRIM8. The reduced level of p62/SQSTM1 in Bafilomycin-A1 treated TRIM8 knock down cells compared to control was observed (Supplementary Fig. 1B). To further check the effect of TRIM8 on p62/SQSTM1 expression we expressed TRIM8 in HEK293 cells and checked p62/SQSTM1 levels by western blotting. The expression of TRIM8 increased the levels of p62/SQSTM1 levels as compared to vector transfected cells (Supplementary Fig. 1C). Similarly knock down of TRIM8 in MCF7 cells reduced the mRNA levels of p62/SQSTM1 (Supplementary Fig. 1D). We further confirmed our observation by expressing TRIM8 in HEK293 cells and treated with protein translation inhibitor; Cycloheximide (CHX). The levels of p62/SQSTM1 increased levels in TRIM8 transfected cells however decreased after 4 h of CHX treatment. This suggests that TRIM8 transcriptionally regulates p62/SQSTM1 and its turnover is enhanced probably through autophagy flux. It also suggests the role of p62 in DNA damage beyond autophagy at least in genotoxic stress conditions (Supplementary Fig. 1E). Together these results also confirm that TRIM8 positively regulates autophagy flux both in cells having functional or mutated p53.

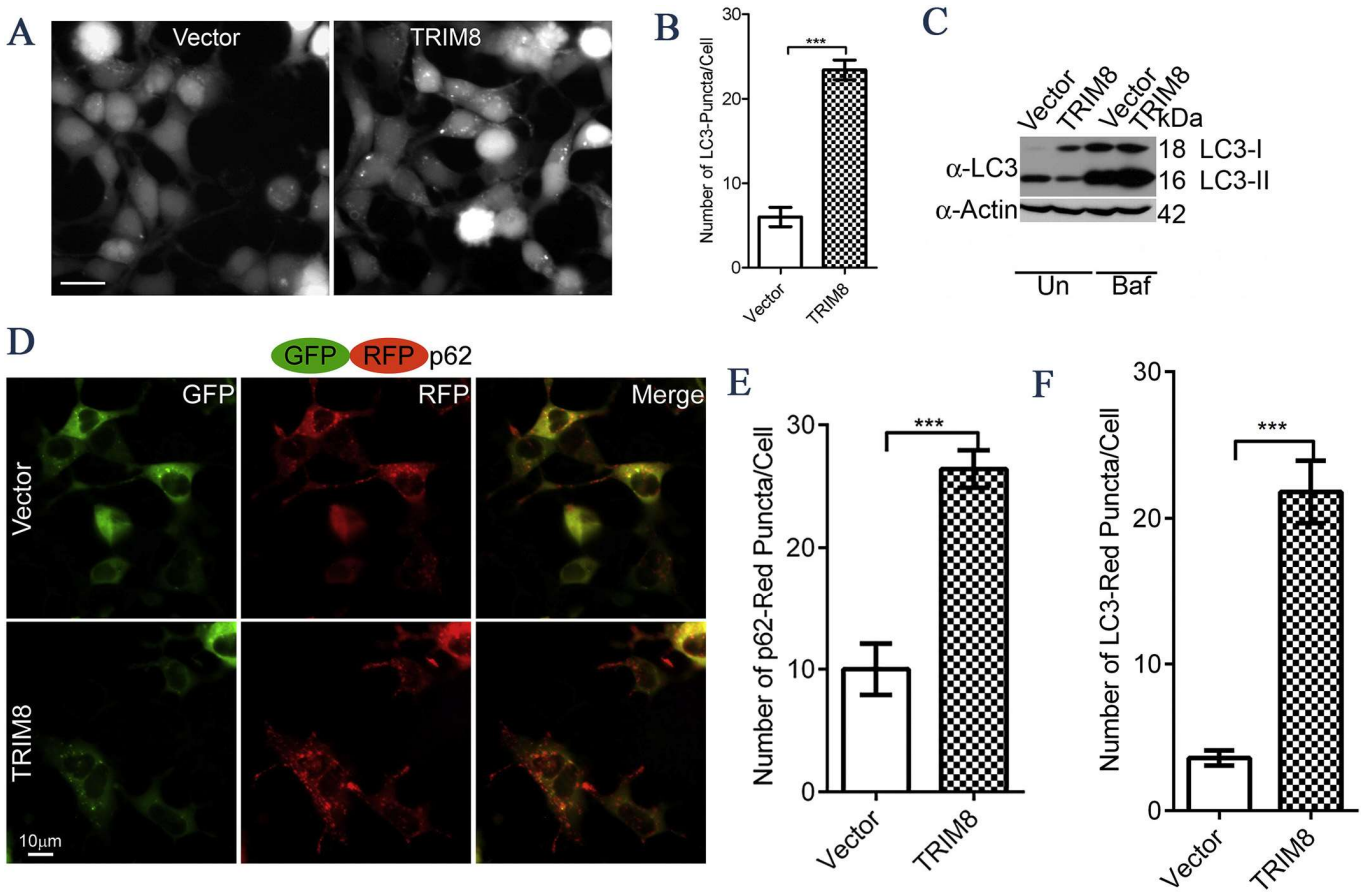

Fig. 1. TRIM8 regulates autophagic flux. (A) The expression of TRIM8 induces autophagy. HEK293-GFP-LC3 stable cell line was transfected with TRIM8 and vector, cells were observed under fluorescent microscope after 24 h of transfection. The scale bar represents 20 μM. (B) Quantification of TRIM8 induced autophagic puncta. The numbers of puncta per cell were counted and graph was plotted for numbers of GFP-LC3 puncta per cell of representative Fig. 1A. Asterisk (*) indicates number of puncta statistically significant from vector; p value < 0.05, SEM of minimum three independent experiments (C) TRIM8 increases autophagic flux. TRIM8 was transfected in HEK293 cells, treated with 250 nM Bafilomycin-A1 for 10 h and blotted with anti-LC3 antibody to detect the protein level of the same. (D) Vector or TRIM8 were co-transfected with mCherry-GFP-p62, cells were observed under fluorescent microscope for red and yellow puncta after 24 h of transfection. (E) Quantification of TRIM8 induced red puncta in mCherry-GFP-p62 co-transfected cells. The numbers of p62 puncta per cell were counted and graph was plotted for numbers of mCherry-p62 puncta per cell. (F) Quantification of TRIM8 induced red puncta in mCherry-GFP-LC3 co-transfected cells. The numbers of LC3 puncta per cell were counted and graph was plotted for numbers of mCherry-LC3 puncta per cell. Asterisk (***) indicates number of puncta statistically significant from vector; p value < 0.001, SEM of minimum three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. RING domain of TRIM8 is required for autophagy

TRIM8 is a multi-domain protein and distinct domains may play unique role in regulation of TRIM8 mediated cellular processes [17,24,40]. The RING domain of TRIM family proteins is known for their E3 ligase activity [23,41–43] therefore we analyzed if the RING domain is required for TRIM8 mediated autophagy. HEK293-GFP-LC3 stable cell line was co-transfected with TRIM8 (full length) or TRIM8ΔR (RING domain deleted). We observed the number of puncta per cell increased in TRIM8 transfected cells as compared to control (Fig. 2A) however number significantly decreased in TRIM8ΔR transfected cells as compared to FL-TRIM8 transfected cells (Fig. 2A).

We checked whether the RING domain is required for TRIM8 mediated autophagic flux. Vector, FL-TRIM8 and TRIM8ΔR were expressed in HEK293 cells and treated with or without NH_4Cl (Lysotropic agent) and Bafilomycin-A1. Western blotting showed that levels of 16 kDa band corresponding to LC3-II increased in FL-TRIM8 transfected cells treated with NH_4Cl and Bafilomycin-A1 as compared to vector control (Fig. 2B), whereas TRIM8ΔR showed no difference compared to control (Fig. 2B). We further confirmed the role of RING domain in autophagy flux by fluorescent microscopy. Both TRIM8 and TRIM8ΔR were co-transfected with mCherry-GFP-LC3 and red/yellow puncta were monitored. The number of red puncta decreased in TRIM8ΔR compared to FL-TRIM8 transfected cells indicating the reduced autophagic flux (Fig. 2C). These results demonstrated that RING domain of TRIM8 is required for autophagy induction and its flux.

3.3. TRIM8 stabilizes during genotoxic stress and modulates autophagy

TRIMs are known to homo and hetero oligomerize with other TRIMs, and either auto-ubiquitinates or their binding partner due to their inherent E3 Ligase activity [41,42] and regulate their turnover in different patho-physiological conditions [24]. To check stability, we expressed HA tagged TRIM8 (HA-TRIM8) and treated the cells with genotoxic stress inducer, Etoposide; mitochondrial complex-I inhibitor, Rotenone; Ubiquitin Proteasome system (UPS) inhibitor, MG132; Lysotropic agent Ammonium Chloride (NH_4Cl), and inhibitor of autophagy Wortamannin (PI3-kinase inhibitor) (Fig. 3A). The western blot showed low level of 62 kDa band corresponding to TRIM8 in normal conditions suggesting its high turnover. The level of TRIM8 significantly increased in the presence of MG132 and wortmannin treatment indicating that its turnover is mediated by both autophagy and UPS (Fig. 3A). Interestingly we observed high level of 62 kDa band corresponding to TRIM8 in etoposide treated cells as compared to

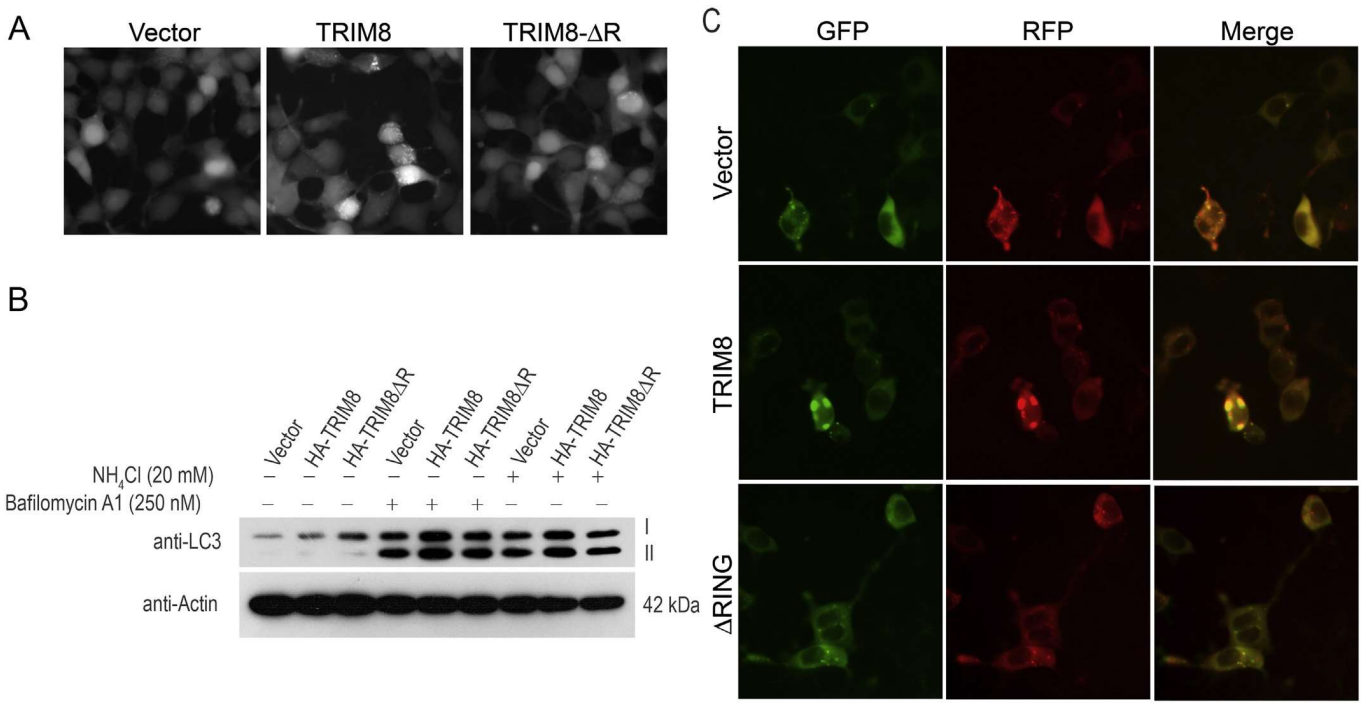

Fig. 2. RING domain of TRIM8 is required for autophagy. (A) HEK293-GFP-LC3 stable cell line was transfected with TRIM8/TRIM8ΔR and vector, cells were observed under fluorescent microscope after 24 h of transfection. (B) Vector, TRIM8 or TRIM8ΔR were transfected in HEK293 cells, treated with 250 nM Bafilomycin-A1 for 10 h or with 20 mM NH_4Cl for 4 h. Western blotting was performed to detect the levels of LC3I and II. (C) TRIM8 or TRIM8ΔR were co-transfected with mCherry-GFP-LC3, cells were observed under fluorescent microscope for red and yellow puncta after 24 h of transfection. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

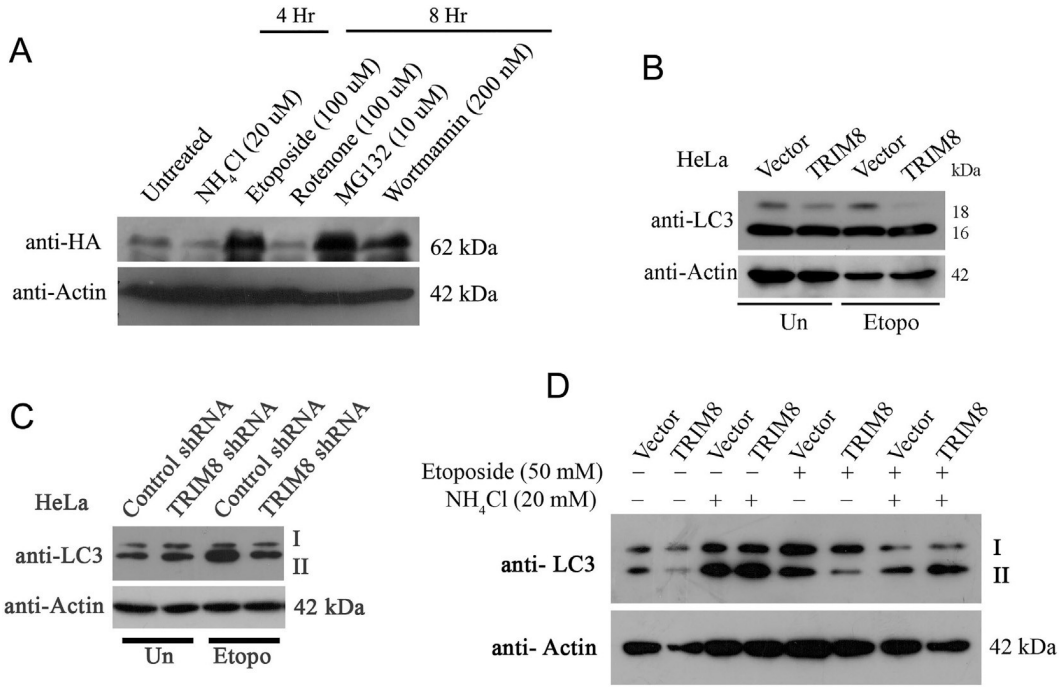

Fig. 3. TRIM8 stabilizes during genotoxic stress and regulates autophagy. (A) TRIM8 was transfected in HEK293 cells and was treated as indicated. Western blotting was performed and blot was probed with HA to monitor TRIM8 levels. (B)TRIM8 induces autophagy during genotoxic stress. TRIM8 was transfected in HeLa cells and were treated with 50 μM etoposide for 8 h. Western blotting was performed to detect the levels of LC3I and II. (C) TRIM8 knockdown decreased autophagy during genotoxic stress condition. TRIM8-shRNA was transfected in HeLa cells, treated with 50 μM etoposide for 8 h and western blotting was performed to monitor LC3 levels. (D) TRIM8 was transfected in HEK293 cells. After 24 h of transfection cells were treated with etoposide (8 h) in presence or absence of NH_4Cl (3 h).

control indicating stabilization of TRIM8 during genotoxic stress (Fig. 3A). The level of TRIM8 was unaltered in cells treated with NH_4Cl and rotenone suggesting the stabilization of TRIM8 during etoposide treatment is specific and may play important role during DNA damage response (Fig. 3A). Previously TRIM8 has been shown as direct p53 target gene [44] hence we also monitored its transcriptional regulation during genotoxic stress. HEK293 (p53 negative) cells were treated with etoposide (Supplementary Fig. 2A) and TRIM8 expression was analyzed using qRT-PCR. TRIM8 level increased in presence of etoposide (Supplementary Fig. 2A). The mRNA level of TRIM8 also increased in presence of etoposide in p53 positive MCF7 cells (data not shown).

E3 ligases are known to regulate their own stability hence we checked whether TRIM8 can regulate its own stability. FL-TRIM8 and TRIM8ΔR was expressed in HEK293 cells and its level were analyzed in presence of proteasome inhibitor MG132. The band of 62 kDa corresponding to full length TRIM8 was only observed in MG132 treated cells whereas del RING form of TRIM8 was observed in both untreated and MG132 cells. This indicates that RING domain is essential for its own turnover (Supplementary Fig. 2B). HA tagged full length TRIM8 and TRIM8ΔR was transfected in HEK293 cells and treated with MG132 and immunoprecipitation was performed. Specific higher molecular weight conjugate was predominantly observed in FL-TRIM8, as compared ΔR transfected cells (Supplementary Fig. 2C). *Re*-probing of the blot with ubiquitin specific antibody showed enhanced level of ubiquitinated proteins in FL-TRIM8 as compared to TRIM8ΔR pull down lanes. (Supplementary Fig. 2C).

Genotoxic stress induced autophagy has crucial role to play in cell survival and death [5]. We speculated that TRIM8 stabilization during genotoxic stress may have role in autophagy. To further analyze, HeLa cells were transfected with TRIM8, treated with etoposide and autophagy was monitored by LC3 blotting at early time points. Western blotting showed increased LC3-II/LC3-I ratio in TRIM8 transfected etoposide treated cells as compared to vector transfected cells (Fig. 3B) suggesting TRIM8 enhanced the rate of autophagy during genotoxic stress. The shRNA mediated knock down of TRIM8 reduced the LC3-II/LC3-I ratio (Fig. 3C) in etoposide treated cells as compared to vector transfected cells.

To further confirm this hypothesis, we further investigated whether TRIM8 modulates autophagy flux during genotoxic stress. LC3II levels increased in TRIM8 transfected cells treated with etoposide and NH_4Cl as compared to vector indicating enhanced autophagy flux in presence of TRIM8 (Fig. 3D). These result shows that TRIM8 regulates autophagy and promotes autophagy flux during genotoxic stress.

3.4. TRIM8 promotes lysosomal biogenesis

Initiation of lysosomal biogenesis is observed during autophagy, which is regulated by Transcription factor EB (TFEB) [45,46]. The increased level of TRIM8 mediated autophagic flux suggests that TRIM8 may also regulate lysosomal biogenesis. TRIM8 was knocked down in HeLa and MCF7 cells using CRISPR/Cas-9 vector expressing guide RNA targeting TRIM8 (TRIM8-sgRNA). The transcript levels of key lysosomal proteins; lysosomal protein transmembrane 4 alpha (LAPTM4A) and ATPase H+ transporting V0 subunit d1 (ATP6V0D1) was monitored. LAPTM4A is resident protein of mammalian lysosomes and plays role in small molecule transport across endosomal and lysosomal membrane [47]. ATP6V0D1 is a component of vacuolar ATPase (V-ATPase) complex that mediates acidification of eukaryotic intracellular organelles. They both are structural constituents of lysosome.

The expression analysis showed marked reduction in levels of LAPTM4A and ATP6V0D1 in both TRIM8 sgRNA transfected HeLa and MCF7 indicating reduced lysosomal biogenesis (Fig. 4A and B). We further confirmed the effect of TRIM8 on lysosomal biogenesis by expressing TRIM8 in HEK293 cells. TRIM8 transfection increased the transcript levels of lysosomal associated membrane protein 1 (LAMP1) (Fig. 4C). The increased level 42 kDa band corresponding to non-glycosylated LAMP1 in TRIM8 transfected cells compared to vector transfected cells (Fig. 4D) was also observed. The blot also indicated increase in band around 110 kDa which indicates glycosylated LAMP1 (recruited to lysosomes) (Fig. 4D) in TRIM8 transfected cells.

We further analyzed if TRIM8 regulates lysosomal biogenesis during genotoxic stress. HEK293 cells transfected with either TRIM8 and treated with etoposide and the levels of glycosylated LAMP1 was monitored. The glycosylated LAMP1 levels in vector transfected cells increased in presence of etoposide. The level of LAMP1 further increased in TRIM8 transfected cells as compared to vector (Fig. 4E). These results suggest that TRIM8 promotes lysosomal biogenesis.

3.5. TRIM8 protects cells from genotoxic stress induced cell death

Autophagy plays vital role in cell survival during genotoxic stress induced cell death [5–7]. Therefore we further analyzed if TRIM8 regulates genotoxic stress induced cell death. To investigate the role of TRIM8 in genotoxic stress induced cell death, HEK293 cells were transfected with TRIM8 and cell viability was analyzed by MTT assay [48]. Interestingly, the expression of TRIM8 increased cellular viability in the presence of etoposide as compared to vector transfected cells (Fig. 5A). It was further confirmed by knocking down TRIM8 using shRNA in HeLa cells. TRIM8-shRNA transfected showed reduced viability in etoposide treated cells as compared to control (Fig. 5B) further confirming the cytoprotective role of TRIM8 during genotoxic stress induced cell death.

Genotoxic agent etoposide is known to activate apoptotic cascade including main executioner caspase-3 to induce cell death [4,49]. We first monitored the activation of Casapse-8 & -9 however no effect was observed. Interestingly, the western blotting showed that the level of 17/19 kDa band corresponding to cleaved subunit of caspase-3 decreased in TRIM8 transfected and etoposide treated cell as compared to control (Fig. 5C). Caspase-3 enzymatic activity was also monitored by cleavage of luminogenic DVED substrate. Caspase-3 activity significantly increased in vector transfected and etoposide treated cells at 16 h as compared to untreated cells. The expression of TRIM8 cells showed significantly low caspase-3 activity in etoposide treated cells (Fig. 5D). Together these results show that TRIM8 protects cells from genotoxic stress induced cell death by inhibiting caspase-3 activation.

3.6. TRIM8 mediated autophagy promotes the degradation of cleaved subunit of caspase-3

Autophagy plays cytoprotective role during genotoxic stress induced cell death [50–52]. Therefore we further explored the role of TRIM8 mediated autophagy in genotoxic stress induced cell death. HEK293 cells were transfected with TRIM8 and treated with etoposide in presence of wortmannin (inhibitor of phosphatidylinositol 3-kinase). TRIM8 expression increased cell viability in etoposide treated cell compared to vector transfected (Fig. 6A). Interestingly, viability reduced in etoposide-wortmannin co-treated cells as compared to only etoposide treated cells (Fig. 6A). This shows that inhibition of autophagy negates the cytoprotective effect of TRIM8 during genotoxic stress induced cell death.

We confirmed the same by monitoring caspase-3 activation using western blotting. The level of 17/19 kDa band corresponding to cleaved subunit of caspase-3 decreased in TRIM8 transfected etoposide treated cells as compared to control however its level increased in presence of wortmannin in TRIM8 transfected etoposide treated cells (Fig. 6B) indicating cleaved subunit is available for caspase-3 activation during autophagy inhibition. Hence we hypothesized that TRIM8 mediated autophagy may regulate cleaved subunit of caspase-3 levels through autophagy. To further confirm this hypothesis, the level of Caspase-3 was monitored by western blotting by enhancing the autophagy by rapamycin. Western blotting showed increased level of 17/19 kDa bands corresponding to cleaved subunit of caspase-3 in vector

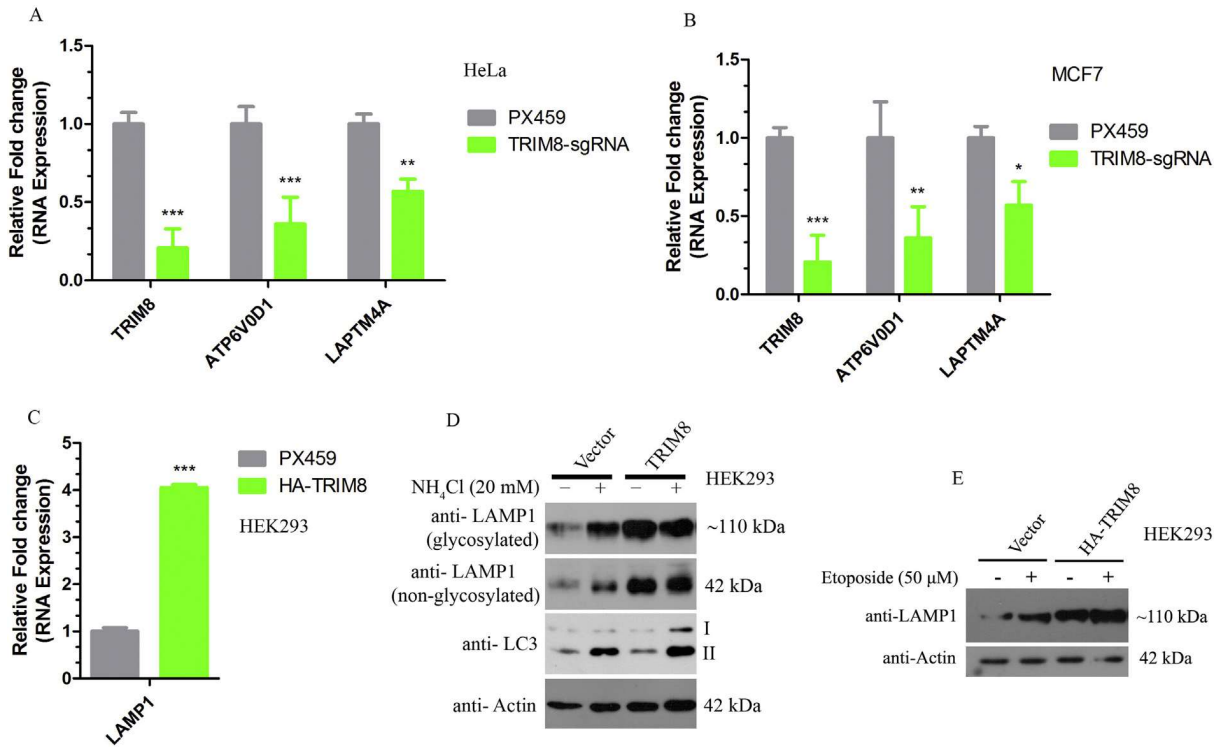

Fig. 4. TRIM8 promotes lysosomal biogenesis. (A) HeLa and (B) MCF7 cells were transfected with PX459 or TRIM8-sgRNA. cDNA was synthesized from RNA isolated from cells after 48 h of transfection. Transcript levels of TRIM8, LAPTM4A and ATP6V0D1 were monitored by real time PCR using specific primers. (C) TRIM8 increases LAMP1 expression. HEK293 cells were transfected with vector or TRIM8 and expression of LAMP1 was monitored by real time PCR by using specific primers. (D) Vector or TRIM8 were transfected in HEK293 cells and treated with 20 mM NH_4Cl for 3 h. Western blotting was performed to check levels of glycosylated and non-glycosylate LAMP1 protein by anti-LAMP1 antibody. (E) HEK293 cells were transfected with vector or TRIM8 and treated with 50 µM etoposide for 8 h. Western blotting was performed to analyze LAMP1 levels. Asterisk (*), (**) and (***) indicates fold change statistically significant from vector; p value < 0.05, < 0.01 and < 0.001(respectively), SEM of minimum three independent experiments.

transfected rapamycin-etoposide co-treated cells compared to only etoposide treated cells (Fig. 6C). The expression of TRIM8 reduced the levels of 17/19 kDa band representing active caspase-3 levels in rapamycin-etoposide co-treated cells compared to vector transfected cells (Fig. 6C).

We further confirmed the effect of TRIM8 on cleaved caspase-3 levels by knocking down TRIM8 in HeLa cells using 2 sgRNA targeting TRIM8 (Supplementary Fig. 1D). We observed increased level of cleaved subunit of caspase-3 in cells transfected with TRIM8-sgRNA and treated with etoposide (Fig. 6D). The level further increased in TRIM8-sgRNA transfected cells treated with etoposide in presence of wortmannin. To further confirm the cleaved caspase-3 degradation by TRIM8 mediated autophagy we performed western blotting in presence of autophagy inhibitors (NH_4Cl and Bafilomycin A1) and UPS inhibitor (MG132). The level of 17/19 kDa corresponding to cleaved caspase-3 subunits was more in TRIM8 transfected cells co-treated NH_4Cl and Bafilomycin A1 whereas no difference was observed in MG132 co-treated cells (Fig. 6E). These results suggest that TRIM8 mediated autophagy promotes degradation of cleaved subunit of caspase-3.

3.7. TRIM8 stabilizes XIAP during genotoxic stress and inhibits caspase-3 activation

Inhibitor of apoptosis proteins (IAPs) are well known E3 ligases for their role in regulation of apoptotic pathways during genotoxic stress [53,54]. X-Linked Inhibitor of Apoptosis (XIAP) has been identified as an autophagy regulator. XIAP activates NF-κB signaling to enhance Beclin-1 expression which is essential for autophagy [55,56]. We further monitored the XIAP and its role in TRIM8 mediated regulation of genotoxic stress induced cell death. TRIM8 was transfected in HEK293 cells and treated with etoposide for 16 h and monitored XIAP levels. The level of 53 kDa band corresponding to XIAP decreased in etoposide treated cells compared to control and whereas 17/19 kDa band of cleaved subunit of caspase-3 increased (Fig. 7A) in vector transfected cells. We observed no difference in XIAP levels in untreated cells transfected with either vector or TRIM8. XIAP level increased in TRIM8 transfected as compared to vector transfected and etoposide treated cells, suggesting TRIM8 mediated XIAP stabilization during genotoxic stress conditions (Fig. 7A). Interestingly, reduced levels of 17/19 kDa band corresponding to cleaved caspase-3 subunits (Fig. 7A) was observed in TRIM8 transfected cells treated with etoposide as compared to control. Furthermore the stabilization of XIAP and caspase-3 cleavage was monitored at different time point in presence of etoposide. The western blotting showed no difference in the level of 17/19 kDa band corresponding to cleaved caspase-3 subunits at 8 and 16 h of etoposide treatment in vector and TRIM8 transfected cells however the levels reduced at 24 h (Fig. 7B) in TRIM8 transfected cells. The endogenous level of XIAP level increased in TRIM8 transfected cells at 16 h compared to control whereas no difference was observed at other time points (Fig. 7B). The result suggests that XIAP stabilization precedes caspase-3 activation in TRIM8 transfected cells during genotoxic stress.

XIAP is known to directly interact with caspase-3 via linker/BIR2 domain to inhibit its activity [57–59]. We further analyzed if TRIM8 form a part of this complex by monitoring its interaction with XIAP and Caspase-3 by immunoprecipitation. HEK293 cells were transfected with vector or TRIM8 and treated with etoposide in presence/absence of MG132 and NH_4Cl. The 55 kDa band corresponding to XIAP was observed in HA-TRIM8 pull down (Fig. 7C). The increased level of XIAP in pulldown was observed in the cells treated with etoposide as compared to untreated cells. As XIAP is known to interact with caspase-3 and inhibits its activation, therefore we checked if caspase-3 is also part of TRIM8 and XIAP complex. The blot showed 35 kDa band corresponding

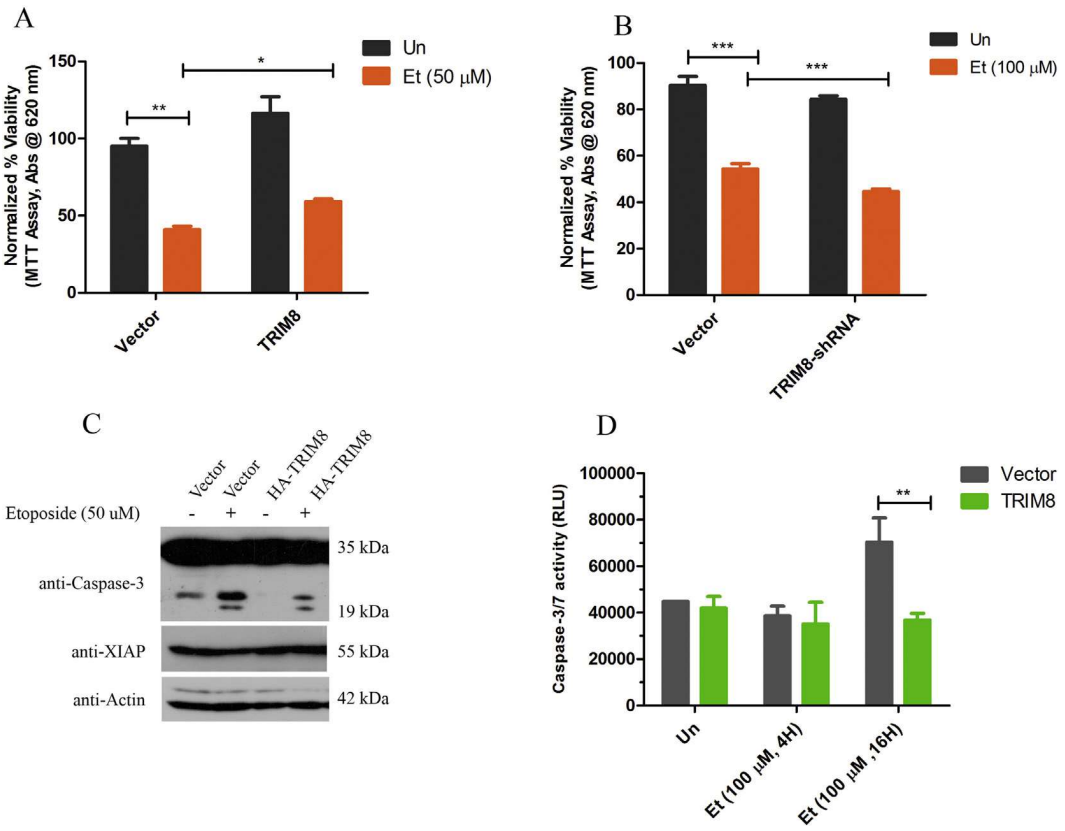

Fig. 5. TRIM8 inhibits caspase-3 activity and protects from genotoxic stress induced cell death. (A) HEK293 cells were transfected with vector or TRIM8. Cells were treated with etoposide for 24 h and MTT assay was performed to check cellular viability. (B) TRIM8 was knocked down in HeLa cells by transfecting TRIM8-shRNA. Cells were treated with etoposide for 24 h and MTT assay was performed to check % cell viability. Asterisk (*), (**), (***) indicates normalized % viability statistically significant from vector; p value < 0.05, < 0.01 and < 0.001(respectively), SEM of minimum three independent experiments. (C) TRIM8 inhibits caspase-3 activity. HEK293 cells were transfected with vector or TRIM8 and treated with etoposide for 24 h. Western blotting was performed to check the levels of cleaved caspase-3 using specific antibody that detects both pro and cleaved caspase-3. (D) caspase-3 activity was measured using CaspaseGlo® 3/7 Assay Systems. HEK293 cells were transfected with vector or TRIM8 and treated with etoposide for indicated time. Caspase-3 activity was measured by following manufacturers' protocol. Asterisk (**) indicates caspase-3/7 activity statistically significant from vector; p value < 0.01, SEM of minimum three independent experiments.

to pro-caspase-3 in HA-TRIM8 pull down. This result indicates that TRIM8-XIAP and pro-caspase-3 may form a complex during genotoxic stress (Fig. 7C). Further, this interaction was confirmed by reverse-IP by Flag-XIAP pull down. HEK293 cells were co-transfected with FLAG-XIAP and either vector, HA-TRIM8 or HA-TRIM8ΔR, cells were treated with etoposide and XIAP-IP was performed. The band corresponding to both full length and TRIM8ΔR was observed in the pull down further confirming the interaction of TRIM8 and XIAP (Fig. 7D). It also showed that RING domain of TRIM is not required for its interaction with XIAP (Fig. 7D). The 37 kDa band corresponding to pro-caspase-3 was observed in pull down in TRIM8 or TRIM8ΔR transfected cells. The level of pro-caspase-3 band increased in etoposide treated cells compared to its control (Fig. 7D). These observations confirm that TRIM8 appears in complex with XIAP and caspase-3 which prevent its activation in presence of etoposide.

4. Discussion

Autophagy is emerging as major response during genotoxic stress and determines the cell fate depending upon the level of damage [60–62]. The molecular regulators of cross talk of autophagy and cell death during genotoxic stress are not well understood. In the present study we identified TRIM8 as a novel regulator of lysosome biogenesis and autophagy flux during genotoxic stress conditions which protects cells from apoptosis.

The reports from our group and others show that TRIM is sub class of RING family of E3 Ligases which forms unique and dynamic subcellular structures in different stress conditions and infections [17,22–24,43,63]. The CC domain of TRIMs help to homo- and heterodimerize and activate E3 Ligase activity to regulate their turnover either through conventional UPS or autophagy pathway [18,22,42,64]. The results in this study further suggest that TRIM8 may form higher molecular weight complexes regulating its own ubiquitination and turnover in basal conditions. The evidences here suggest that TRIM8 turnover is high and is regulated both by UPS and autophagy pathway whereas it is stabilized during genotoxic stress. Interestingly, p53 mediated transcriptional upregulation of TRIM8 had also been observed during UV radiation leading to stabilization of p53 and reduction of cell proliferation [44,65]. This suggests that beyond transcriptional upregulation of TRIM8, the role of either pathway (UPS or autophagy) in regulating the turnover of TRIM8 is essential for its function in normal cellular function however its implication in different patho-physiological conditions needs to be further studied.

During DNA damage NF-κB is activated leading to increased levels of TNF-α and persistent DNA damage can later lead to activation of caspase-8 induced cell death [66]. We and other previously reported that TRIM8 positively regulates TNF-α induced NF-κB pathway [25,26]. Recent evidences strongly suggest that NF-κB activation regulates autophagy which plays essential role in DDR [36,62,67], hence we postulated the role of TRIM8 in regulation of autophagy. The evidences here suggest that TRIM8 potentiate autophagy during genotoxic stress condition as TRIM8 expressing cells show increased number of

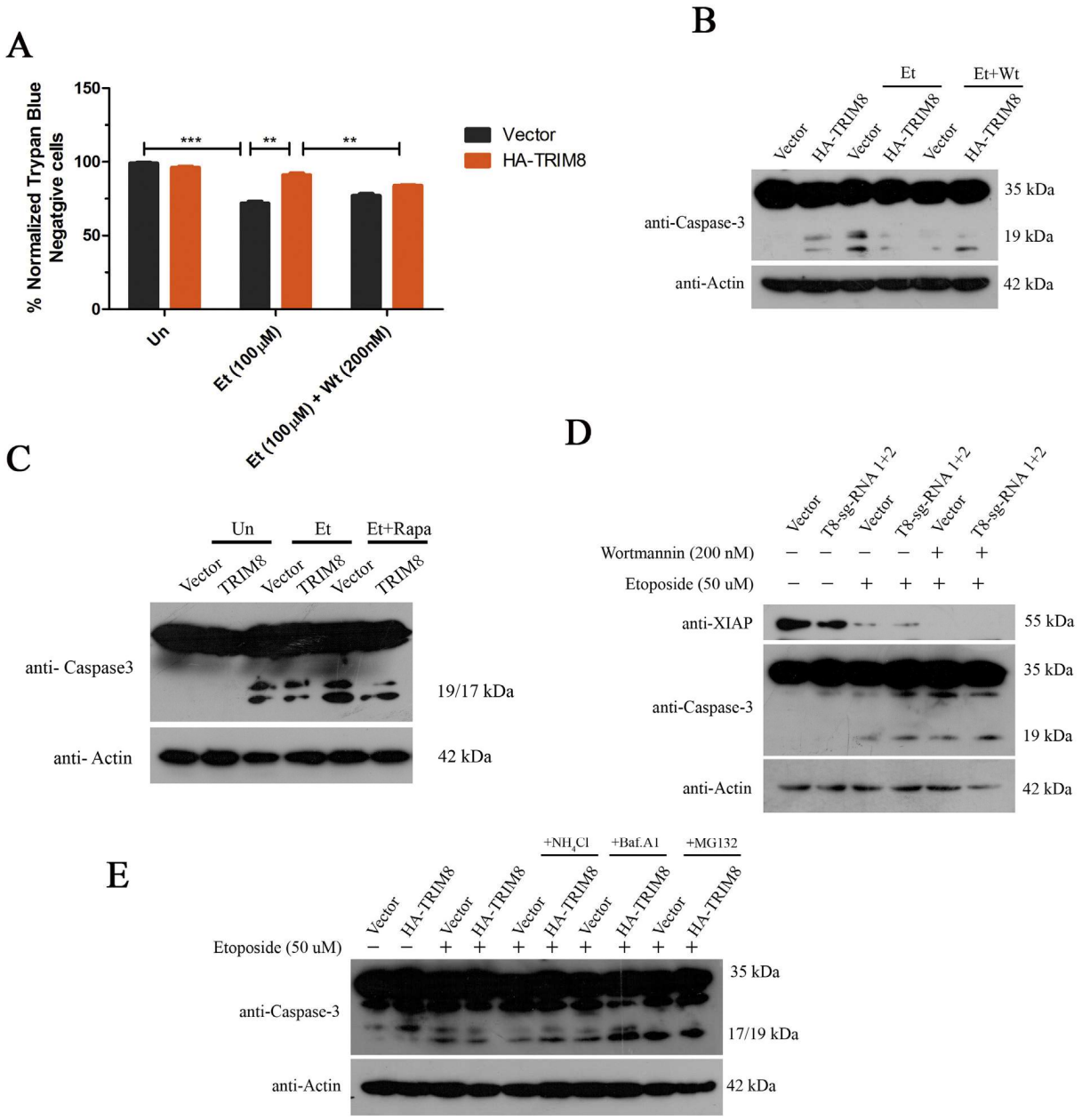

Fig. 6. TRIM8 mediated autophagy is required for cell survival during genotoxic stress. (A) HEK293 cells were transfected with Vector or TRIM8. Cells were treated with etoposide and wortmannin as indicated for 24 h. Trypan blue dye exclusion assay was performed to check cell viability. Asterisk (**), (***) indicates normalized % trypan blue negative cells statistically significant from vector; p value < 0.01 and < 0.0001 (respectively), SEM of minimum three independent experiments. (B) Autophagy inhibition by wortmannin increases level of cleaved subunit of caspase-3 in TRIM8 expressing cells. HEK293 cells were transfected with vector or TRIM8 and treated etoposide and wortmannin for 24 h as indicated. Cleaved caspase-3 levels were monitored by western blotting using caspase-3 specific antibody. (C) TRIM8 reduces cleaved caspase-3 levels and is enhanced in presence of rapamycin. HEK293 cells were transfected with vector or TRIM8 and treated with etoposide in presence and absence of rapamycin for 24 h. Cleaved caspase-3 levels were monitored by western blotting using caspase-3 specific antibody. (D) TRIM8 knockdown sensitizes genotoxic stress induced cell death when autophagy is inhibited. TRIM8 was knocked down in HeLa cells using TRIM8-sgRNA 1 & 2. Cells were treated with etoposide in presence or absence of wortmannin and western blotting was performed to check the levels of cleaved caspase-3. (E) TRIM8 mediated autophagy is required for degradation of cleaved caspase-3. HEK293 cells were transfected with TRIM8 and treated with etoposide 24 h in presence of NH_4Cl (20 mM, 4 h), Bafilomycin A1 (25 nM, 8 h) or MG132 (10 μM, 10 h). Cleaved caspase-3 levels were monitored by western blotting using caspase-3 specific antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

autophagy puncta, LC3-II levels. The induction of TRIM8 mediated autophagy was observed in cells having functional p53 (MCF-7 and MX1) and non-functional p53 (HEK-293 and HeLa) suggesting its role in autophagy is independent of p53 regulated mechanisms. Further, TRIM8 also regulates lysosomal biogenesis by activating the transcription of genes of lysosomes which are required for its function and autophagy flux. This may be one of the survival mechanisms of cancer cells under genotoxic insults, where nuclear DNA released during DNA damage conditions may be transported to the lysosome through autophagy to alleviate cytotoxicity. This hypothesis is further supported by previous observation where autophagy plays essential role in degradation of nuclear DNA during DNA damage conditions [11]. Moreover reports suggest that autophagy deficient cells accumulate damaged DNA whereas autophagy competent cells can repair DNA damage hence helps in survival [11,31,51,61]. This needs to be further investigated during genotoxic stress conditions. Interestingly, TRIM8

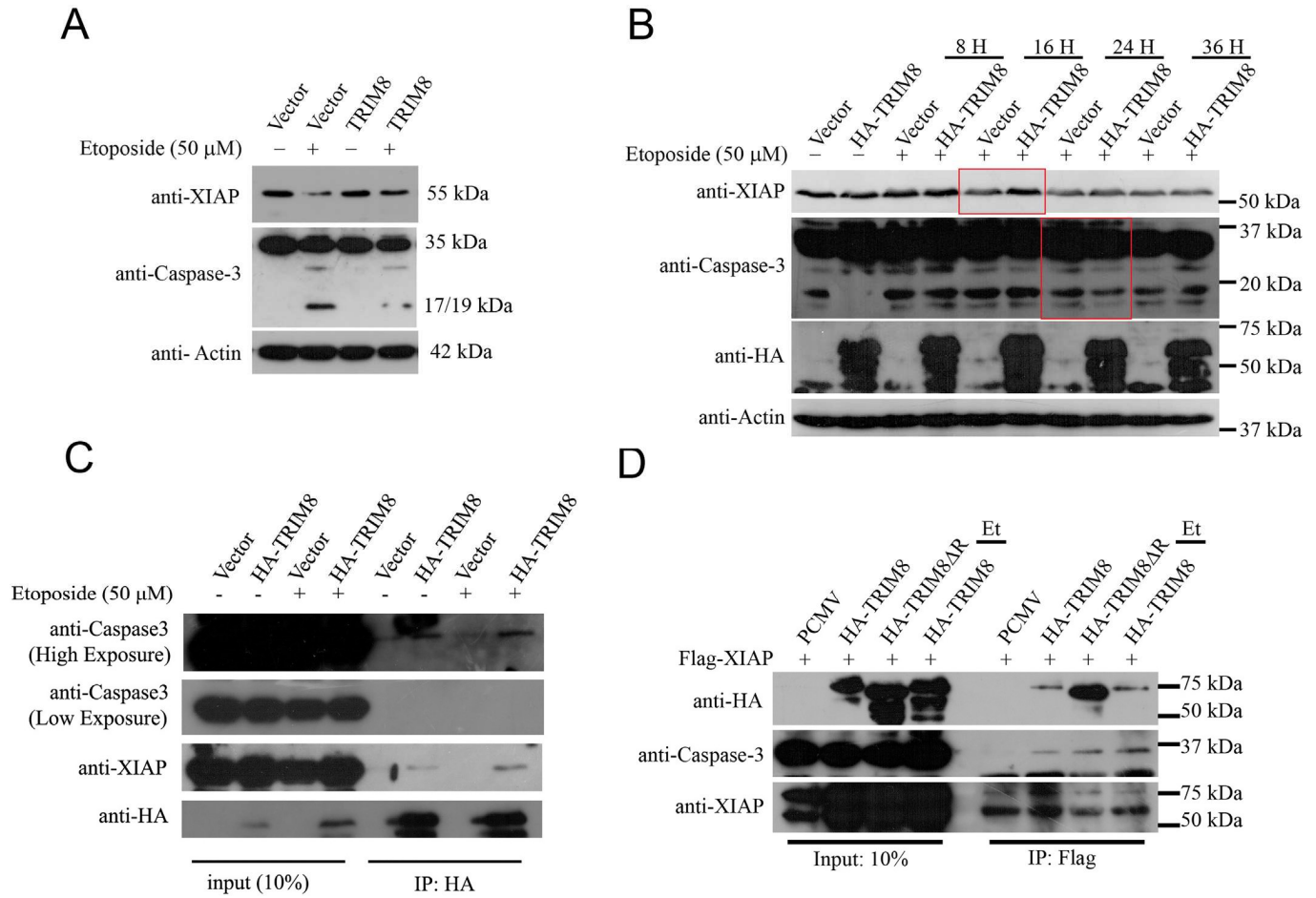

Fig. 7. TRIM8 interacts with XIAP and caspase-3 and stabilizes XIAP. (A) HEK293 cells were transfected with Vector or TRIM8 and treated with etoposide for 24 h. Western blotting was performed to check the levels of XIAP and cleaved caspase-3 by using specific antibodies. (B) HEK293 was transfected with vector or TRIM8 and treated with etoposide for indicated time. Western blotting was performed to check the levels of XIAP and cleaved caspase-3 by using specific antibodies. (C) Immunoprecipitation was performed to check interaction of TRIM8, XIAP and caspase-3. HA tagged TRIM8 was transfected in HEK293 cells, post transfection cells were treated with etoposide for 24 h in presence of MG132 (10 μM, 10 h) and wortmannin (20 mM, 4 h). HA immunoprecipitation and western blotting was performed. Indicated antibodies were used to detect interaction. (D) Co-IP was performed to re-confirm interaction of TRIM8, XIAP and caspase-3. Vector, FALG-XIAP, HA-TRIM8 and HA-TRIM8ΔR were transfected as indicated. Cells were treated with MG132 (10 μM, 10 h) in presence and absence of etoposide (50 μM, 10 h). FLAG immunoprecipitation and western blotting was performed. Indicated antibodies were used to detect interaction.

transcriptionally regulates p62 which has pleiotropic functions like selection of cargo during autophagy, restriction of inflammation by promoting mitophagy [68] and DNA damage induced inflammation and senescence [69] hence oncogenesis. These evidences highlight the complex relationship of TRIM8 mediated regulation of p62 and possible role beyond autophagy and implication in tumorigenesis.

The evidences here suggest that autophagy regulate the crosstalk of DDR and cell death during genotoxic stress condition. The expression of TRIM8 enhanced the cellular viability under genotoxic stress whereas knock down sensitized the cells to death. Autophagy has been shown to regulate Caspase-8 turnover in normal conditions [23,70]. During persistent genotoxic stress conditions, Caspase-8 is activated leading to cell death. In our experiments we consistently observed that expression of TRIM8 showed no effect on Casapse-8 activation or turnover during genotoxic stress however regulates the activation of critical executioner caspase-3. The reduced level of cleaved caspase-3 subunit in TRIM8 transfected cells suggests that TRIM8 mediated autophagy may regulate the degradation of cleaved subunit of caspase-3. Our hypothesis was further supported as cleaved subunit of caspase-3 levels were rescued in presence of inhibitors of autophagy (NH_4Cl and Bafilomycin A1). Interestingly enhancing the autophagy flux through rapamycin also decreased the level of activated caspase-3 further supporting our hypothesis. These results strongly suggest that TRIM8 regulated autophagy facilitate the degradation of cleaved subunit of caspase-3 preventing caspase activation and downstream cleavage cascade even during genotoxic stress conditions.

XIAP is a major regulator of cell death and autophagy [55]. It directly binds to caspase-3, -7 and -9 and inhibits their proteolytic activity [58,71]. Therefore cellular XIAP levels during genotoxic stress may determine the cellular fate. In the current study, TRIM8 knock down reduced the XIAP levels, whereas its enhanced expression stabilizes XIAP during genotoxic stress conditions. XIAP also strongly activates NF-κB via BIR (Baculovirus Inhibitor of apoptosis protein Repeat) domain mediated dimerization and binding to TGF-beta activated kinase 1 (MAP3K7) binding protein 1 (TAB1) [56]. XIAP mediated NF-κB activation also induced expression of genes involved in autophagy like Beclin-1 [55]. Hence, TRIM8 mediated XIAP stabilization may have at least two important outcomes during tumorigenesis. Firstly, activation of NF-κB, leading to expression of genes involved in autophagy, metabolism, and proliferation. Secondly, TRIM8 mediated stabilized XIAP forming multiprotein complex which sequester caspase-3 and prevents its cleavage and activation. This is one of the novel mechanisms for preventing cell death during genotoxic stress conditions and radiation therapy. This needs to be further validated in several in vivo cancer models.

In summary the current study describes TRIM8, as a novel regulator

of DDR-autophagy cross-talk which play cytoprotective role during genotoxic stress induced cell death. The evidences here further support our previous report suggesting TRIM8 as potential oncogene [25]. This however needs to be critically evaluated as TRIM8 may belong to family of double edged sword genes having both tumor suppressor and oncogenic functions. Previously, it had been observed that TRIM8 stabilized p53 by binding and preventing its degradation through mdm2 during ionizing radiation condition [44]. The loss of heterozygosity is observed in glioblastoma [72,73] however TRIM8 level is maintained through STAT3 suggesting its oncogenic functions [74]. Similarly in another report TRIM8 had been shown to enhance STAT3 signaling and promote src mediated anchorage independent growth, characteristic of tumor cells [29]. TRIM8 may further provide tumor cells survival advantage by enhancing the rate of autophagy and caspase inhibition during chemotherapy and radiation therapy hence may play crucial role in drug resistance as observed here. The understanding of TRIM8 mediated regulation of autophagy, lysosomal biogenesis and its cross talk with cell death will help to potentiate the effect of anti neoplastic drugs targeting DNA damage.

5. Conclusions

The current study provides several evidences supporting the cytoprotecive role of TRIM8 during genotoxic stress. TRIM8 enhances autophagy flux through lysosomal biogenesis and autophagy mediated degradation of cleaved subunit of Casaspe-3 during genotoxic stress conditions. The stabilization of TRIM8 during genotoxic stress conditions provides survival advantage to cancer cells specifically undergoing chemo or radiation therapy.

Conflict of interest

The authors declare no conflict of interest.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellsig.2018.04.003.

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Enforced lysosomal biogenesis rescues erythromycin- and clindamycin-induced mitochondria-mediated cell death in human cells

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Abstract

Antibiotics are the front-line treatment against many bacterial infectious diseases in human. The excessive and long-term use of antibiotics in human cause several side effects. It is important to understand the underlying molecular mechanisms of action of antibiotics in the host cell to avoid the side effects due to the prevalent uses. In the current study, we investigated the crosstalk between mitochondria and lysosomes in the presence of widely used antibiotics: erythromycin (ERM) and clindamycin (CLDM), which target the 50S subunit of bacterial ribosomes. We report here that both ERM and CLDM induced caspase activation and cell death in several different human cell lines. The activity of the mitochondrial respiratory chain was compromised in the presence of ERM and CLDM leading to bioenergetic crisis and generation of reactive oxygen species. Antibiotics treatment impaired autophagy flux and lysosome numbers, resulting in decreased removal of damaged mitochondria through mitophagy, hence accumulation of defective mitochondria. We further show that over-expression of transcription factor EB (TFEB) increased the lysosome number, restored mitochondrial function and rescued ERM- and CLDM-induced cell death. These studies indicate that antibiotics alter mitochondria and lysosome interactions leading to apoptotsis and may develop a novel approach for targeting inter-organelle crosstalk to limit deleterious antibiotic-induced side effects.

Keywords Antibiotics · Side effects · Mitochondria · Lysosome · Autophagy

Introduction

Antibiotics are considered as one of the more important discoveries of modern medicine by preventing large-scale morbidity and mortality associated with microbial infectious diseases. However, it has been observed that certain populations are more prone to antibiotic's side effects [1]. In particular, severe side effects in infants, children, and aging

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FMRpolyG alters mitochondrial transcripts level and respiratory chain complex assembly in Fragile X associated tremor/ataxia syndrome [FXTAS]

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ABSTRACT

Fragile X-associated tremor/ataxia syndrome (FXTAS) is an inherited neurodegenerative disorder caused by an expansion of 55 to 200 CGG repeats (premutation) in *FMR1*. These CGG repeats are Repeat Associated non-ATG (RAN) translated into a small and pathogenic protein, FMRpolyG. The cellular and molecular mechanisms of FMRpolyG toxicity are unclear. Various mitochondrial dysfunctions have been observed in FXTAS patients and animal models. However, the causes of these mitochondrial alterations are not well understood. In the current study, we investigated interaction of FMRpolyG with mitochondria and its role in modulating mitochondrial functions. Beside nuclear inclusions, FMRpolyG also formed small cytosolic aggregates that interact with mitochondria both in cell and mouse model of FXTAS. Importantly, expression of FMRpolyG reduces ATP levels, mitochondrial transmembrane potential, mitochondrial supercomplexes assemblies and activities and expression of mitochondrial DNA encoded transcripts in cell and animal model of FXTAS, as well as in FXTAS patient brain tissues. Overall, these results suggest that FMRpolyG alters mitochondrial functions, bioenergetics and initiates cell death. The further study in this direction will help to establish the role of mitochondria in FXTAS conditions.

1. Introduction

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a neurodegenerative disorder characterized by progressive intention tremor (parkinsonism), gait ataxia and cognitive decline [1]. In addition, neuropathy, thyroid dysfunctions, hypertension, immune dysfunction and cardiac arrhythmias are also observed in some patients of FXTAS [2]. FXTAS affects 1 in ~3000 male and 1 in ~5000 female and disease symptoms get more pronounced with the age [3]. The Fragile X mental retardation (*FMR1)* gene, located on the q-arm of the chromosome X, encodes for the FMRP-RNA binding protein, which is involved in regulation of transport and local translation of mRNAs in brain and is essential to synaptic plasticity and neuronal development [4–6]. An expansion of CGG trinucleotide repeats within the 5′-UTR of *FMR1* causes different neuropathological conditions based on the number of CGG repeats.

Firstly, expansions exceeding 200 CGG repeats, which are called full mutations, are the main cause of Fragile X syndrome (FXS), a neurodevelopmental disease characterized by intellectual disability and autism. Expansions over 200 CGG repeats lead to hypermethylation and silencing of the *FMR1* promoter. Hence, the FMRP protein encoded by *FMR1* is absent, which ultimately results in alterations of the brain synaptic plasticity [7]. Secondly, Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is caused by presence of 55 to 200 CGG repeats, which is named premutation [8]. The prevalence of the CGG premutation carrier varies among populations but is estimated to range between 1 in 110 to 250 in females and 1 in 260 to 800 in males [9,10]. At the histopathological level, FXTAS is characterized by neuronal cell loss and presence of large ubiquitin-positive intranuclear inclusions in both neurons and astrocytes [11]. Rare ubiquitin positive inclusions are also observed in non-CNS organs like kidney and thyroid [12]. At the molecular level and in strict contrast to Fragile-X, the CGG premutation expansion does not inhibit but promotes *FMR1* expression in FXTAS, resulting in 2 to 8 folds higher levels of *FMR1* mRNA in FXTAS patients compared to control individuals [13,14]. Importantly, expression of mutant RNA containing the CGG premutation is pathogenic both in cell and animal models [15–20]. Studies in the last decade have identified two main mechanisms of how CGG repeats expression can be toxic for

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NLRX1 regulates TNF-α-induced mitochondria-lysosomal crosstalk to maintain the invasive and metastatic potential of breast cancer cells

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ABSTRACT

An increased level of proinflammatory cytokines, including TNF-α in tumor microenvironment regulates the bioenergetic capacity, immune evasion and survival of cancer cells. Emerging evidences suggest that mitochondrial immune signaling proteins modulates mitochondrial bioenergetic capacity, in addition to the regulation of innate immune response. The optimal oxidative phosphorylation (OxPhos) capacity is required for the maintenance of functional lysosomes and autophagy flux. NLRX1, a mitochondrial NOD family receptor protein, regulates mitochondrial function during apoptosis and tissue injury. However, its role in regulation of mitochondrial and lysosomal function to modulate autophagy flux during inflammatory conditions is not understood. In the current study, we investigated the role of NLRX1 in modulating TNF-α induced autophagy flux and mitochondrial turnover and its implication in regulating the invasive and metastatic capability of breast cancer cells. Expression analyses of clinical breast cancer samples and meta-analysis of multiple public databases revealed that NLRX1 expression is significantly increased in basal-like and metastatic breast carcinoma as compared to non-basal-like and primary breast cancer. Depletion of NLRX1 expression in triple-negative breast cancer cells, altered the organization and activity of OxPhos complexes in presence of TNF-α. NLRX1 depletion further impaired lysosomal function and hence the turnover of damaged mitochondria through mitophagy in presence of TNF-α. Importantly, loss of NLRX1 decreased OxPhos-dependent cell proliferation and migration ability of triple-negative breast cancer cells in presence of TNF-α. These evidences suggest an essential role of NLRX1 in maintaining the crosstalk of mitochondrial metabolism and lysosomal function to regulate invasion and metastasis capability of breast cancer cells.

1. Introduction

Breast cancer is the second most common malignancy diagnosed among women worldwide and a leading cause of mortality in women population [1]. The heterogeneity of breast cancer makes them a challenging solid tumor to diagnose and treat. A primary or early breast tumor, characterized by the luminal-like subtype and presence of estrogen receptor (ER), progesterone receptor (PR) and/or HER2 status, is potentially curable. The surgical removal of the tumor combined with hormonal and systemic therapy is considered to be the best treatment option [2]. In contrast, basal-like subtype and triple-negative (ER, PR and HER2, negative) metastatic breast cancer is currently considered as incurable with a long-term survival rate of < 5% [3]. The study of the cellular pathways involved in differential regulation of the invasive phenotype of triple negative compared to ER/PR positive tumors would help to understand the intratumoral heterogeneity and clonal evolution of the metastatic breast cancer cells.

Chronic inflammation, a hallmark of solid tumor, is intricately associated with initiation and progression of many cancer types, including breast cancer [4]. The specific role of inflammation in

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NLRX1 resides in mitochondrial RNA granules and regulates mitochondrial RNA processing and bioenergetic adaptation

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ABSTRACT

The role of mitochondria is emerging in regulation of innate immunity, inflammation and cell death beyond its primary role in energy metabolism. Mitochondria act as molecular platform for immune adaptor protein complexes, which participate in innate immune signaling. The mitochondrial localized immune adaptors are widely expressed in non-immune cells, however their role in regulation of mitochondrial function and metabolic adaption is not well understood. NLRX1, a member of NOD family receptor proteins, localizes to mitochondria and is a negative regulator of anti-viral signaling. However, the submitochondrial localization of NLRX1 and its implication in regulation of mitochondrial functions remains elusive. Here, we confirm that NLRX1 translocates to mitochondrial matrix and associates with mitochondrial FASTKD5 (Fas-activated serine-threonine kinase family protein-5), a bonafide component of mitochondrial RNA granules (MRGs). The association of NLRX1 with FASTKD5 negatively regulates the processing of mitochondrial genome encoded transcripts for key components of complex-I and complex-IV, to modulate its activity and supercomplexes formation. The evidences, here, suggest an important role of NLRX1 in regulating the post-transcriptional processing of mitochondrial RNA, which may have an important implication in bioenergetic adaptation during metabolic stress, oncogenic transformation and innate immunity.

1. Introduction

ATP generation through oxidative phosphorylation (OxPhos) is one of the major function of mitochondria, besides its additional important roles in numerous biosynthetic reactions, maintaining intracellular ion homeostasis, apoptosis and innate immune signaling [1]. Mitochondrial outer membrane and mitochondrial contact sites serve as molecular platform for the assembly of dynamic signaling complexes that forms during viral infection [2]. Mitochondrial outer membrane proteins namely, MAVS and STING act as adaptors for the downstream activation of anti-viral signaling [3, 4]. NLRX1 (Nod-Like Receptor (NLR) protein family member) negatively regulates innate immune responses during viral infections. Recent reports from our lab and others have shown that mitochondrial immune signaling proteins are widely expressed in non-immune cells and their levels are altered during tumor progression [5, 6]. Similarly, the role of NLRX1 in controlling mitochondrial metabolic functions and apoptosis during inflammatory condition and tissue injury is emerging [7, 8]. However, its sub-mitochondrial localization and molecular mechanism(s) of regulating mitochondrial function is not well understood.

The OxPhos system, embedded in inner mitochondrial membrane, is composed of five multiprotein complexes forming the mitochondrial respiratory chain (MRC). The MRC complexes are assembled from nearly 100 protein subunits, of which approximately 90 subunits are encoded by nuclear genes and imported into mitochondria. The remaining 13 subunits are encoded by mitochondrial DNA (mtDNA) [9]. In addition, mitochondrial genome encodes 22 tRNAs and 12S and 16S mt-rRNAs that are essential for the synthesis of mitochondria-encoded proteins. As all protein factors involved in the expression of mitochondrial genome are nuclear encoded, a strict coordination between nuclear and mitochondrial gene expression programs is necessary, especially during physiological responses requiring changes in energy demands, such as conditions of altered carbon sources [10]. The regulatory mechanisms of mitochondrial gene expression and its

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Systemic Analysis of miRNAs in PD Stress Condition: miR-5701 Modulates Mitochondrial–Lysosomal Cross Talk to Regulate Neuronal Death

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Abstract Parkinson's disease (PD) is complex neurological disorder and is prevalent in the elderly population. This is primarily due to loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) region of the brain. The modulators of the selective loss of dopaminergic neurons in PD are still not well understood. The small non-coding RNAs specifically miRNAs fine-tune the protein levels by post-transcriptional gene regulation. The role of miRNAs in PD pathogenesis is still not well characterized. In the current study, we identified the miRNA expression pattern in 6-OHDA-induced PD stress condition in SH-SY5Y, dopaminergic neuronal cell line. The targets of top 5 miRNAs both up- and down regulated were analyzed by using StarBase. The putative pathways of identified miRNAs included neurotrophin signaling, neuronal processes, mTOR, and cell death. The level of miR-5701 was significantly downregulated in the presence of 6-OHDA. The putative targets of miR-5701 miRNA include genes involved in lysosomal biogenesis and mitochondrial quality control. The transfection of miR-5701 mimic decreased the transcript level of VCP, LAPTM4A, and ATP6V0D1. The expression of miR-5701 mimic induces mitochondrial dysfunction, defect in autophagy flux, and further sensitizes SH-SY5Y cells to 6-OHDA-induced cell death. To our knowledge, the evidence in the current study demonstrated the dysregulation of specific pattern of miRNAs in PD stress conditions. We further characterized the role of miR-5701, a novel miRNA, as a potential regulator of the mitochondrial and lysosomal function determining the fate of neurons which has important implication in the pathogenesis of PD.

Keywords Autophagy flux · Lysosome · miRNA · Parkinson's disease · Mitochondria

Introduction

Parkinson's disease (PD) is a second most common neurodegenerative movement disorder in elderly population. This is clinically characterized by resting tremor, rigidity, bradykinesia, and postural instability due to preferential loss of dopamine-producing neurons in the *substantia nigra pars compacta* region of the midbrain [1]. The other pathological hallmark of PD is the presence of intracytoplasmic proteinaceous deposits termed as Lewy bodies (LBs) and dystrophic neurites (Lewy neurites) in surviving neurons. These aggregates consist of fibrillar α-synuclein, molecular chaperones, ubiquitin, and neurofilaments [2]. The mechanisms of further progression and selective loss of dopaminergic neurons in PD had been a focus of research for the last several years; however, it is still not well understood. There is no effective therapy, and dopamine (DA) supplementation only provides symptomatic relief. It is important to indentify the modulators of selective neuronal loss in PD to find the next generation of therapeutic strategies.

Now there are established evidences suggesting mitochondrial dysfunction is one of the major causative factors of PD [3–5]. The first evidence of association of mitochondrial dysfunction with PD came from the observation of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPTP), which causes symptoms of PD during drug abuse and produces severe

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hsa-miR-4485 regulates mitochondrial functions and inhibits the tumorigenicity of breast cancer cells

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Abstract The modulation of mitochondrial functions is important for maintaining cellular homeostasis. Mitochondria essentially depend on the import of RNAs and proteins encoded by the nuclear genome. MicroRNAs encoded in the nucleus can translocate to mitochondria and target the genome, affecting mitochondrial function. Here, we analyzed the role of miR-4485 in the regulation of mitochondrial functions. We showed that miR-4485 translocated to mitochondria where its levels varied in response to different stress conditions. A direct binding of miR-4485 to mitochondrial 16S rRNA was demonstrated. MiR-4485 regulated the processing of pre-rRNA at the 16S rRNA-ND1 junction and the translation of downstream transcripts. MiR-4485 modulated mitochondrial complex I activity, the production of ATP, ROS levels, caspase-3/7 activation, and apoptosis. Transfection of a miR-4485 mimic downregulated the expression of regulatory glycolytic pathway genes and reduced the clonogenic ability of breast cancer cells. Ectopic expression of miR-4485 in MDA-MB-231 breast carcinoma cells decreased the tumorigenicity in a nude mouse xenograft model. Furthermore, levels of both precursor and mature miR-4485 are decreased in tumor tissue of breast cancer patients. We conclude that the mitochondria-targeted miR-4485 may act as a tumor suppressor in breast carcinoma cells by negatively regulating mitochondrial RNA processing and mitochondrial functions.

Keywords Mitochondria · miR-4485 · Breast cancer · Tumor suppressors · RNA processing · Mouse xenograft

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Introduction

Mitochondria are indispensable for energy production, lipid and carbohydrate metabolism, redox regulation, calcium signaling, and cell death. Mitochondria have also been implicated in the regulation of innate immunity, inflammation, and antiviral signaling [1, 2]. Mitochondrial dysfunction is associated with numerous pathologies, including metabolic and neurodegenerative disorders, cardiomyopathies, cancer, and aging [3, 4]. Reprogramming of mitochondrial functions is one of the major hallmarks of tumor cell metabolism [5, 6]. To cope with growing bioenergetic demands of rapid proliferation, cancer cells can switch from an efficient but slow mitochondrial respiration to the less efficient but rapid aerobic glycolysis [7–10]. Some of the key intermediates, such as citrate and glycerol, are redirected from the Krebs cycle to meet increased demands of tumor cells in macromolecular synthesis [11, 12]. Although mechanisms of metabolic reprogramming in rapidly dividing cancer cells are being extensively studied, many of the processes remain elusive.

Proteomics studies have revealed that the human mitochondrion contains more than a thousand distinct

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MITA modulated autophagy flux promotes cell death in breast cancer cells

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ABSTRACT

The crosstalk between inflammation and autophagy is an emerging phenomenon observed during tumorigenesis. Activation of NF-κB and IRF3 plays a key role in the regulation of cytokines that are involved in tumor growth and progression. The genes of innate immunity are known to regulate the master transcription factors like NF-κB and IRF3. Innate immunity pathways at the same time regulate the genes of the autophagy pathway which are essential for tumor cell metabolism. In the current study, we studied the role of MITA (Mediator of IRF3 Activation), a regulator of innate immunity, in the regulation of autophagy and its implication in cell death of breast cancer cells. Here, we report that MITA inhibits the fusion of autophagosome with lysosome as evident from different autophagy flux assays. The expression of MITA induces the translocation of p62 and NDP52 to mitochondria which further recruits LC3 for autophagosome formation. The expression of MITA decreased mitochondrial number and enhances mitochondrial ROS by increasing complex-I activity. The enhancement of autophagy flux with rapamycin or TFEB expression normalized MITA induced cell death. The evidences clearly show that MITA regulates autophagy flux and modulates mitochondrial turnover through mitophagy.

1. Introduction

The tumor microenvironment is complex milieu having the cells of different origin, including immune cells [1,2]. The tumor cells show increased levels of definite pattern of cytokines which probably helps the tumor cells to reprogramme gene expression pattern and metabolism for their survival [3,4]. Interestingly, the origin of the increased level of cytokines is attributed to the immune cells that are recruited to the tumor microenvironment. The role of the tumor cells themselves in regulation of inflammation is not yet clear and needs to be established in order to modulate different metabolic and signaling pathways to inhibit tumor cells proliferation and metastasis.

NF-κB and IFNs are key regulators of distinct set of anti- and pro-inflammatory cytokines. The regulators of these pathways are critical in different innate immune pathways. It is observed that during the cellular transformation, the genes regulating antitumorigenic cytokines are lost from the tumor cells. MITA, for example, is downregulated or functionally inactivated in different types of cancer including breast cancer, acute myeloid leukemia and prostate cancer [5–7]. Interestingly, the adaptor proteins like MAVS and MITA, antiviral signaling proteins, are localized on the mitochondria and ER-mitochondria contact site respectively [8,9]. During viral infection, RNA and DNA viruses are recognized by distinct proteins like RIG1 and cyclic GMP-AMP synthase (cGAS) respectively which further interact with MITA [10,11]. The interaction recruits downstream signaling proteins and activate NF-κB and IFN that induces antiviral response. Moreover, the evidences also suggest that these proteins regulate mitochondrial functions during infection [12]. The specific localization of these proteins on mitochondria suggests their role beyond innate immunity in metabolism under normal physiological conditions.

It is observed that critical innate immunity pathways (NF-κB and IFN) are also involved in the regulation the expression of the genes involved in autophagy [13–15]. NF-κB regulates the expression of p62 that is essential for the regulation of selective elimination of defective mitochondria called as mitophagy [13]. The increased level of autophagy is important for the tumor cell metabolism and adaptation for increased rate of cell division [16,17]. The selective elimination of the defective organelles via autophagy is important for the cellular homeostasis [18–20]. The degradation of mitochondria may down regulate the levels of several mitochondrial and mitochondrial associated membrane resident proteins like MAVS and MITA hence downregulating and maintaining inflammation in physiological limits.

Autophagy (macroautophagy) is a sequential process of degradation of cytoplasmic material as well as organelles through lysosomes. The first step involves the formation of autophagophore membrane which encloses the portion of cytoplasm to form autophagosome [21]. The outer membrane of autophagosomes fuses with lysosomes and forms autophagolysosomes [22,23]. The lysosomal enzymes degrade the enclosed cytoplasmic material and inner membrane of autophagosome [24]. Defect in autophagy leads to several diseased conditions including

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Original contribution

TRIM4; a novel mitochondrial interacting RING E3 ligase, sensitizes the cells to hydrogen peroxide (H_2O_2) induced cell death

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ABSTRACT

The emerging evidences suggest that posttranslational modification of target protein by ubiquitin (Ub) not only regulate its turnover through ubiquitin proteasome system (UPS) but is a critical regulator of various signaling pathways. During ubiquitination, E3 ligase recognizes the target protein and determines the topology of ubiquitin chains. In current study, we studied the role of TRIM4, a member of the TRIM/RBCC protein family of RING E3 ligase, in regulation of hydrogen peroxide (H_2O_2) induced cell death. TRIM4 is expressed differentially in human tissues and expressed in most of the analyzed human cancer cell lines. The subcellular localization studies showed that TRIM4 forms distinct cytoplasmic speckle like structures which transiently interacts with mitochondria. The expression of TRIM4 induces mitochondrial aggregation and increased level of mitochondrial ROS in the presence of H_2O_2. It sensitizes the cells to H_2O_2 induced death whereas knockdown reversed the effect. TRIM4 potentiates the loss of mitochondrial transmembrane potential and cytochrome c release in the presence of H_2O_2. The analysis of TRIM4 interacting proteins showed its interaction with peroxiredoxin 1 (PRX1), including other proteins involved in regulation of mitochondrial and redox homeostasis. TRIM4 interaction with PRX1 is critical for the regulation of H_2O_2 induced cell death. Collectively, the evidences in the current study suggest the role of TRIM4 in regulation of oxidative stress induced cell death.

1. Introduction

The studies in the last two decades suggest that beside metabolism, mitochondria plays crucial role in other cellular processes like cell death, inflammation and differentiation [1–3]. The regulation of mitochondrial function is required for cellular homeostasis and its dysregulation had been implicated in various pathological conditions like neurodegeneration, ageing, inflammation, infection and cancer [4–6]. The understanding of the regulation of mitochondrial functions is important to modulate its function in associated pathological condition.

Mitochondria are one of the primary sites of the production of reactive oxygen species (ROS) during physiological and pathological conditions [7,8]. The regulated level of ROS plays critical role in different cellular processes like cell cycle, proliferation, differentiation, migration [9–11]; however, its excess leads to the activation of cell death pathways [12,13]. The physiological level of ROS is maintained by redox reactions and activity of several antioxidant enzymes like glutathione peroxidases (GPX), thioredoxins (TRX) and peroxiredoxins (PRX) [14–16]. PRXs are member of low molecular weight peroxidases, involved in regulation of redox signaling [16]. PRX scavenge low concentrations of H_2O_2, hence acts as modulator of H_2O_2 signaling [16,17]. The regulation of different antioxidant enzymes and their selective role in oxidative stress induced cell death is less understood.

The emerging evidences suggest that ubiquitin mediated posttranslational modifications plays critical role in the regulation of redox pathways [18,19]. The ubiquitin E3 ligases are terminal protein during ubiquitination and provide specificity to this process as it recognizes the substrate and transfer Ub moiety to the target [20]. Ubiquitin E3 ligase, E6AP, regulates the cellular response during oxidative stress condition by modulating the turnover of PRX1 [21]. The role of specific E3 ligase, their recruitment to mitochondria and regulation of redox signaling, cell death during oxidative stress is less understood.

TRIM proteins are members of RING family of ubiquitin E3

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