Title: TRIM32 regulates mitochondrial mediated ROS levels and sensitizes the oxidative stress induced cell death

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A B S T R A C T

Emerging evidence suggests that ubiquitin mediated post translational modification is a critical regulatory process involved in diverse cellular pathways including cell death. During ubiquitination, E3 ligases recognize target proteins and determine the topology of ubiquitin chains. Recruitment of E3 ligases to targets proteins under stress conditions including oxidative stress and their implication in cell death have not been systemically explored. In the present study, we characterized the role of TRIM32 as an E3 ligase in regulation of oxidative stress induced cell death. TRIM32 is ubiquitously expressed in cell lines of different origin and form cytoplasmic speckle like structures that transiently interact with mitochondria under oxidative stress conditions. The ectopic expression of TRIM32 sensitizes cell death induced by oxidative stress whereas TRIM32 knockdown shows a protective effect. The turnover of

TRIM32 is enhanced during oxidative stress and its expression induces ROS generation, loss of mitochondrial transmembrane potential and decrease in complex-I activity. The pro-apoptotic effect was rescued by pan-caspase inhibitor or antioxidant treatment. E3 ligase activity of TRIM32 is essential for oxidative stress induced apoptotic cell death. Furthermore, TRIM32 decreases X-linked inhibitor of apoptosis (XIAP) level and overexpression of XIAP rescued cells from TRIM32 mediated oxidative stress and cell death. Overall, the results of this study provide the first evidence supporting the role of TRIM32 in regulating oxidative stress induced cell death, which has implications in numerous pathological conditions including cancer and neurodegeneration.

Keywords: Cell death Mitochondria, Oxidative stress ubiquitination TRIM32, XIAP

1. Introduction

Mitochondria are dynamic organelles and are implicated in various cellular functions including metabolism, cell death, inflammation, and immunity apart from its role in bioenergetics to maintain cellular homeostasis [1–3]. The equilibrium of the healthy mitochondrial net- work is maintained in the cells through the dynamic process of fusion and fission. The stressed or damaged mitochondria are labeled with ubiquitin (Ub) and selectively degraded through the process known as mitophagy in order to remove them from the healthy network [4]. Any defect in mitophagy or in the fusion and fission process leads to accu-mulation of defective mitochondria, resulting in the production of ex- cessive ROS and initiates cell death [5]. This phenomenon has been observed in many pathological conditions including neurodegeneration and autoimmune diseases [6,7].

Ub is versatile molecule that can form different types of Ub chains of different topology on the target proteins through seven conserved lysine residues [8,9]. The presence of atypical Ub chains on the target proteins regulate their stability and impart unique functional outcomes. The reported evidence suggests that ubiquitination during oxidative stress is initiated on the mitochondria which regulates mitophagy and cell death [10]. It was observed that K63 linked ubiquitination is initiated through Bre1 ubiquitin ligase during

oxidative stress that determines cell sur- vival [11]. The process of ubiquitination is achieved by the sequential action of three enzymes: E1 (Ub activating enzyme), E2 (Ub con- jugating enzyme), E3 (Ub ligases). The E3 ligases are terminal proteins in ubiquitination process and provide pathway specificity as they re- cognize the substrate and initiate the transfer of Ub. Interestingly, the human genome contains more than 1000 E3 Ligases and their functions are not well understood. Moreover, the role of specific E3 Ligases, their recruitment to mitochondria and regulation of cell death pathways during oxidative environment have not been well studied [12].

There are three major families of E3 Ligases of which the RING (really interesting new gene) family constitutes the most members [13,14]. Tripartite motif (TRIM) family proteins are a subfamily of RING E3 Ligases and characterized by the presence of conserved RBCC domain structures, which include the RING finger, one or two B-box motifs, a coiled-coil region and a variable c-terminal domain. There are more than 76 TRIM proteins and their role is associated with the reg- ulation of the innate immune response during viral infections [15,16]. Recent reports suggest that TRIMs play multiple roles beyond immune responses including cell survival, stem cell maintenance, miRNA bio- genesis, embryogenesis, and transcriptional regulation [17,18]. How- ever, the role of TRIMs in pathophysiological processes has only re- cently begun to emerge and remains uncharacterized.

We have systematically investigated the role of TRIMs in the reg- ulation of cell death and mitochondrial function [13,14,19]. In this study, we report that TRIM32 turnover increases during oxidative stress, translocates to the mitochondria, induces mitochondrial ag- gregation, and sensitizes the cells to H_2O_2 induced death. In addition, we found that TRIM32 regulates the turnover of XIAP which is im- portant for the regulation of apoptotic cell death under oxidative stress conditions.

2. Materials and method

2.1. Cells and reagents

HEK293 cells were grown as described previously [14]. Full length TRIM32 was cloned into

pAc.GFP·N1 (Clontech, Takara, Japan). The primary antibodies used in the study were β-actin (Abcam, USA), Caspase-3, AIF, XIAP (Cell Signaling Technology, Inc., USA), Ub (Santa Cruz, USA), TRIM32 (GeneTex, USA), and anti-HA peroxidase (Roche, Germany). HRP-conjugated anti-mouse and anti-rabbit antibodies (Thermo Scientific, USA) were used as secondary antibodies. Rotenone, H_2O_2, N-acetyl-cysteine (NAC), Trypan blue, Decylubiquinone, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sodium Pyruvate, and Propidium Iodide (PI) were purchased from Sigma-Al- drich, USA. z-VAD.fmk from Invivogen (USA), Tetramethylrhodamine, methyl ester (TMRM) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) kit from Invitrogen (USA).

2.2. Constructs

Full length TRIM32 was provided by Dr. Walther Mothes (Section of Microbial Pathogenesis, Yale University School of Medicine, USA. HA- TRIM32, HA-TRIM32-(65–653), were provided by Prof. Shigetsugu Hatakeyama (Department of Biochemistry, Hokkaido University Graduate School of Medicine, Japan). pDsRed2-mito Vector (hereafter MTRFP) used for the visualization of mitochondria using fluorescent microscopy was purchased from Clontech, Takara, (Japan). This vector encodes a fusion protein DsRed2 with mitochondrial targeting sequence of human cytochrome c oxidase subunit VIII. For construction of GFP- tag constructs, full-length coding regions of the TRIM32 (NM_012210) was PCR amplified with PfuUltra high-fidelity DNA polymerase (Stratagene, USA) and cloned into pAc.GFP·N1 (Clontech, Takara, Japan). Conformation of TRIM32-GFP cloned was verified by colony PCR, restriction digestion. shRNA for TRIM32 and control cloned in pSM2c vector were previously reported by Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain).

2.3. Generation of stable cell lines

The stable cell line HEK293-MTRFP was generated to study the co- localization of TRIM32 with mitochondria. Method for the generation of above stable cell line was described previously [20].

2.4. Analysis of subcellular localization of TRIM32 by confocal microscopy

For cellular localization of TRIM32-GFP, HEK293-MTRFP cells were seeded at density of 1.5×10^5 cells per well in 24 well plate containing glass cover slip and TRIM32-GFP was transfected by using standard calcium phosphate transfection method [21]. After 24 h of transfection, the cells were treated with indicated chemicals and monitored using Leica TCS-SP5-II confocal microscope (Germany) for co-localization of TRIM32 with mitochondria.

2.5. Cell death assay

The cell viability was analyzed by Trypan blue and MTT assay. HEK293 cells were plated at the density of 1.5×10^5 cells/per well in 24 well plate and indicated plasmid were transfected by using standard calcium phosphate transfection method. After 24 h of transfection the cells were treated with indicated chemical and incubated for 24 h, further the cells were stained with trypan blue. Minimum 100 cells per view were counted and at least four views were selected. The average trypan blue cells per experimental set were plotted. In MTT assay, HEK293 cells were plated at the density of 0.3×10^5 cells/ well in 48 well plate and indicated plasmid were transfected by using standard calcium phosphate transfection method. After 24 h of transfection the cells were treated with indicated chemical. MTT assay was performed as described previously [22]. Caspase3/7 activation assay was per- formed using Caspase-Glo® 3/7 Assay (Promega, USA), according to manufacturer protocol.

2.6. Western blotting

To study the protein turnover and ubiquitination, HEK293 cells were seeded at density of 2.5×10^5/well in 12 well plate and trans- fected using standard calcium phosphate transfection method. After 48 h of transfection cells were treated with reagents as indicted in the figures. After the treatment, cells were

harvested and rinsed with ice cold PBS and lysed in NP40 lysis buffer (150 mM NaCl, 50 mM Tris-Cl, 1% NP40, 1 mM PMSF). Protein concentration was determined by Bradford assay and equal protein loaded on 10% SDS-PAGE. Protein was electro blotted on PVDF membrane at 100 V for one hour 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 PBS) for 1 h at room temperature. The membrane was incubated overnight with spe- cific primary antibody. After incubation, the membrane was washed three times for 10 min with PBS-T (PBS containing 0.1% Tween 20) and incubated with a secondary antibody at room temperature for 1 h. The membrane was again washed three times with PBS-T and protein bands on the membrane was then visualized by using EZ-ECL chemilumines- cence detection kit for HRP (Biological Industries, Israel) by exposing to X-ray film.

2.7. Analysis of reactive oxygen species and mitochondrial membrane potential

3. ROS was analyzed by microscopy and spectrofluorometry using MitoSOX as mitochondria ROS indicator and CM-H$_2$DCFDA for total cellular ROS respectively. Mitochondrial membrane potential ($\Delta\Psi$) was analyzed by TMRM staining using spectrofluorometry. Briefly, HEK293 cells were transfected in 24 well plate with indicated constructs and treated with indicated chemicals. Both assays were performed as de- scribed previously [23,24].

3.1. Analysis of complex-I activity

Complex-I activity was determined by BN-PAGE (Blue-Native Page) and Spectro-Photometry. HEK293 cells were transfected and treated as indicated in the figures and BN-PAGE was performed as described previously [25].

3.2. Statistical analysis

3.3. Data are shown as mean ± SEM for all the observations. Comparisons of groups were performed using student *t*-test for repeated measurements to determine the levels of significance for each group.

Each experiment has been repeated independently and probability va- lues of $p < 0.05$ were considered as statistically significant.

4. Results

4.1. TRIM32 sensitizes oxidative stress induced cell death

In the current study, we investigated the role of TRIM32 in regulation of cell death and survival under oxidative stress conditions. HEK293 cells were transfected with TRIM32-GFP and treated with different stress inducing agents including TNFα+cycloheximide (CHX) (inflammatory stress), tunicamycin (ER stress), Etoposide (DNA da- mage), H_2O_2 and rotenone (oxidative stress), and TRIM32-GFP ex- pression confirmed by microscopy (Fig. S1A). TRIM32-GFP over- expression had no effect on cell death in the presence of TNFα+CHX, Etoposide and tunicamycin, but showed significantly increased cell death in presence of H_2O_2 and rotenone relative to vector transfected cells (Fig. 1A). TRIM32 induced oxidative stress cell death was further conformed in SKNSH, U87MG and MDAMB-231. An increased cells death was observed in TRIM32 overexpressing cells during oxidative stress (H_2O_2 and rotenone) with different severity in different cell line compared to control (Fig. S1 B, C, D). Further, TRIM32-GFP expressing cells showed evidence of apoptotic morphology within 24 h of the oxidative stress (H_2O_2 and rotenone) as compared to control (Fig. S1 A). TRIM32 induced cell death during oxidative stress was also confirmed by propidium iodide staining (Fig. S1 E). The knockdown of TRIM32 rescued oxidative stress induced cell death as indicated by a decrease in trypan blue positive cells (Fig. 1B). The knockdown of TRIM32 in HEK293 cells was validated by RT-PCR (Fig. 1B).

TRIM32 induced apoptotic cell death was confirmed by monitoring caspase activation. TRIM32 overexpression resulted in increased levels of the 18 kDa band corresponding to the cleaved subunit of caspase-3 as compared to vector in the presence of both H_2O_2 and rotenone (Fig. 1C). Caspase-3/7 activity was also monitored by luciferase assay and TRIM32 expressing cells showed significantly higher caspase-3/7 activity in the presence of oxidative stress as compared to vector transfected cells whereas

TRIM32 knockdown cells showed decreased activity (Fig. 1D). Collectively, these data strongly suggest that TRIM32 sensitizes cells to oxidative stress induced apoptosis via caspase activity.

4.2. E3 ligase activity of TRIM32 is essential for oxidative stress induced cell death

TRIM32 is a novel ubiquitin E3 ligase, however its cellular function during oxidative stress induced cell death is not well understood. The *in-silico* analysis showed that TRIM32 is a multi-domain protein, having an N-terminal RING (20 to 65 amino acids), B-box (103 to 133 amino acids), coiled coil (138 to 197 amino acids) and C-terminal NHL domain (360 to 648 amino acids) (Fig. 2A). The RT-PCR analysis demonstrated that TRIM32 is expressed highly in HEK293, A549, SH-SY5Y, T47D, HBL100 compare to Hela, HepG2, MCF7 and MDA-MB-231 cell line (Fig.S2 A). TRIM32 has characteristic N-terminal RING domain, which imparts E3 Ligase activity of RING E3 proteins hence, E3 ligase activity of TRIM32 was analyzed. HEK293 cells were transfected with TRIM32- GFP and vector, treated with H_2O_2 and rotenone in presence and absence of MG132, to block proteasomal degradation of ubiquitinated proteins. Western blotting showed that TRIM32 overexpression in- creased the levels of ubiquitinated proteins in the presence of MG132 as compared to vector control (Fig. 2B). Similarly, increased ubiquitinated proteins were observed in rotenone and H_2O_2 treatment in presence of MG132 in TRIM32-GFP transfected cells as compared to vector trans- fected cells (Fig. 2B). This suggests that TRIM32 contains a functional RING domain and exhibits E3 Ligase activity, which has important implications in oxidative stress induced cell death. To characterize the role of E3 ligase activity in oxidative stress induced cell death, full length TRIM32 (FL-TRIM32), TRIM32-ΔR, and Vector were transfected, and cell death analyzed by MTT assay following to rotenone and H_2O_2 treatment. As observed above, the expression of FL-TRIM32 sensitized the cells to oxidative stress induced cell death as compared to vector. Interestingly the deletion of the RING domain showed no significant effect on cell death in the presence of either H_2O_2 or rotenone (Fig. 2C) compared to vector control. Because TRIM family proteins can auto-ubiquitinate and regulate self-turnover during normal cellular condi- tions and stabilized during stressed conditions, we monitored its levels during oxidative stress conditions. Western blotting showed the levels of the 72 kDa band corresponding to endogenous TRIM32

decreased in the presence of H_2O_2 and rotenone (Fig. 2D); however, no significant differences were observed at the mRNA level (Fig. S2 B). A prior study showed that K63 linked ubiquitination increased during the H_2O_2 in- duced oxidative stress which probably acts as a rescue mechanism during oxidative stress [26]. Hence, we analyzed the effect of TRIM32 on K63 linked ubiquitination. TRIM32-GFP was co-transfected with HA- Ub-K63 in HEK293 and treated with H_2O_2 with/without lysosomal and proteasomal inhibitor (NH4CL and MG132 respectively) (Fig. 2E). The western blotting shows that K63 link ubiquitination significantly in- creased in H_2O_2 treated cells in both the presence and absence of ly- sosomal and proteasomal inhibition in vector control cells. However, the turnover of K63 linked ubiquitination significantly increased in

 TRIM32 over-expressed cells in the presence of H_2O_2 and rotenone, which was blocked in the presence of lysosomal and proteasomal in- hibitor (Fig. 2E). This suggests that TRIM32 modulates K63 mediated ubiquitination during oxidative stress and enhances the turnover of the ubiquitinated proteins.

1.1. TRIM32 induces loss of transmembrane potential and enhances mitochondrial ROS production during oxidative stress conditions

The dysregulation of mitochondrial function is a primary cause of oxidative stress induced cell death, hence, we monitored cellular ROS, mitochondrial ROS and mitochondrial membrane potential in TRIM32 expressing cells. Total ROS was analyzed by the oxidant-sensitive dye, CM-H_2DCFDA in TRIM32 over-expression condition during the oxida- tive stress (H_2O_2 and Rotenone). As expected, ROS increased in the oxidative stress conditions as compared to untreated vector transfected cells. Interestingly, expression of TRIM32 further enhanced rotenone and H_2O_2 induced ROS level (Fig. 3A). The generation of mitochondrial ROS was monitored by MitoSOX staining. The dye permeates live cells where it is selectively targeted to the mitochondria, oxidized by su- peroxide and produces red fluorescence. The number of MitoSOX po- sitive cells significantly increased during oxidative stress (H_2O_2 and rotenone) in TRIM32 transfected cells as compared to vector (Fig. 3B). The generation of ROS may lead to the loss of mitochondrial membrane

potential ($\Delta\Psi$), hence TMRM staining was performed in the presence of TRIM32 during the oxidative stress to measure its effect on mitochon- drial membrane potential. The fluorescence intensity significantly decreased in the TRIM32 over-expressing cells in the presence of oxidative stress (H_2O_2 and rotenone) (Fig. 3C). To further confirm the role of mitochondrial mediated pathways in induction of cell death, experiments were performed in the presence of caspase inhibitor or anti- oxidants. The vector and TRIM32 transfected cells were treated with a pan-caspase inhibitor (zVAD.fmk) or *N*-acetyl cysteine (NAC) in the presence or absence of oxidative stress inducer (H_2O_2 and rotenone). Treatment with zVAD.fmk or NAC rescued TRIM32 mediated oxidative stress (H_2O_2 and rotenone) induced cell death as evidenced by trypan blue staining (Fig. 3D). To further study the role of TRIM32 in oxidative stress induced cell death, we monitored cell death in presence of so- dium pyruvate, which specifically quenches hydrogen peroxide (H_2O_2) and inhibits cell death pathways [19]. TRIM32 mediated oxidative stress induced cell death was significantly reversed in presence of so- dium pyruvate (Fig. S3). These data suggest that TRIM32 may regulate mitochondrial function and further enhances oxidative stress condi- tions.

1.2. TRIM32 translocate to mitochondria and regulates complex-I activity during oxidative stress conditions

TRIMs form unique complexes of higher order molecules and can dynamically associate with subcellular organellar [27] hence we monitored the subcellular localization of TRIM32 and its translocation during oxidative stress conditions. A HEK293-MTRFP stable cell line was transfected with TRIM32-GFP and observed using confocal mi- croscopy. Under normal conditions, TRIM32 showed punctuate staining and no or partial co-localization with mitochondria (Fig. 4A). Inter- estingly, in the presence of H_2O_2 and rotenone, TRIM32 showed larger size puncta and co-localized with mitochondria (Fig. 4A, S4). This was further conformed by measuring endogenous TRIM32 level during the oxidative stress in mitochondrial fractionation by western blotting (Fig. 4B). Immunoblotting with a TRIM32 antibody showed increased levels of TRIM32 in mitochondrial fraction under both oxidative stress conditions (H_2O_2 and rotenone) as compared to control. These ob- servations indicate that TRIM32 translocates to mitochondria and in- duces mitochondrial aggregation under oxidative stress.

The expression of TRIM32 increased levels of ROS suggesting the alteration of mitochondria function. The mitochondrial complex-I is the entry point of electrons in the electron transport chain (ETC) [28] and its dysregulation causes leakage of electrons, which is one of the pri- mary sources of ROS. Therefore, we analyzed role of TRIM32 in reg- ulation of complex-I activity. The expression of TRIM32 showed de- creased super-complex and complex I activity (Fig. 4C) as observed by In-gel activity staining. These observations strongly suggest that TRIM32 localizes to mitochondria and regulate the complex-I activity during oxidative stress conditions.

1.3. TRIM32 mediated turnover of XIAP regulates ROS and cell death

XIAP belongs to the IAP family and binds caspase family members mainly, CASP3 and CASP7 in mammalian cells and inhibits caspase activity under normal conditions. A previous study reports that TRIM32 regulates XIAP degradation, which may have important implications in cell death and survival [33]. To study the role of XIAP in TRIM32 regulated oxidative stress induced cell death, we monitored XIAP levels in the presence of TRIM32. The expression of TRIM32 decreased en- dogenous levels of XIAP as compared to vector control (Fig. 5A). To check the role of XIAP in TRIM32 induced ROS and cell death during the oxidative stress, XIAP was co-expressed with TRIM32 and cellular ROS levels were analyzed in presence of H_2O_2. Interestingly, the co- expression of XIAP rescued TRIM32 induced ROS production in the presence of H_2O_2 (Fig. 5B). Similarly, the co-expression of XIAP de- creased TRIM32 induced caspase 3/7 activation and cell death (Fig. 5C). The observations indicate that TRIM32 increases the XIAP turn-over and XIAP overexpression can rescue TRIM32 mediated H_2O_2 induced ROS levels and cell death (Fig. 5D).

2. Discussion

The posttranslational modification of proteins by ubiquitin is an adaptive response under different types of stress conditions including DNA damage, hypoxia, infection and inflammation and oxidative stress [29].

TRIMs, members of the RING E3 Ligase family, play essential role in the regulation of stress pathways [30]. The intrinsic properties of TRIMs are known to form oligomeric complexes by interacting with self or different subgroups of TRIMs, hence TRIMs may act synergistically in different pathophysiological stress conditions [30,31]. The selective recruitment of specific E3 Ligases under oxidative stress conditions is not well understood. In the current study we describe the role of TRIM32 in regulating oxidative stress induced cell death.

We observed that RNA levels of TRIM32 are similar in different stress conditions; however, protein levels are regulated by its turnover through UPS (ubiquitin proteasome system) and autophagy according to the inherent nature of this family of proteins. TRIM32 turnover has been reported by other groups in muscular dystrophy condition where TRIM32 has been observed to serve as a substrate of autophagy [32]. The evidence presented in the current study suggests that TRIM32 specifically sensitizes cells to oxidative stress specifically in the pre- sence of rotenone, complex-I inhibitor and H_2O_2. It enhances caspase activity probably by activating intrinsic pathway of apoptosis [32]. A recent study also demonstrated the apoptotic potential of TRIM32 [33] in the presence of TNF; however, our study showed no significant dif- ferences and may be due to the different experimental conditions be- tween the two studies. The role of TRIM32 in regulating oxidative stress seems to be important in different patho-physiological conditions. It had been reported that TRIM32 regulates mitochondrial function, membrane potential and ROS levels in lung cancers [34], and TRIM32 inhibition by siRNA decreases the generation of ROS in hippocampal neurons [35].

TRIM32 is multi-domain protein having an N-terminal RING do- main, B Box, CC Domain and C terminal NHL domain [32]. The ex- periment here suggests that E3 Ligase activity is essential for the apoptotic property in oxidative stress conditions. Recent evidence has supported the role of E3 ligases in inhibition of redox homeostasis and mitochondrial bioenergetics in cardiomyocytes [36]. Emerging evi- dence suggests that atypical ubiquitin like chains are formed during stress conditions including oxidative stress [37]. Interestingly, the turnover of TRIM32 increases during oxidative stress and enforced expression of TRIM32 increases ubiquitination in oxidative stress sug- gest that ubiquitination events may play an important role in regulation of cell death. In the presence of H_2O_2, it has been observed that total

cellular K63 linked ubiquitination increases, which is important for modulating polysome assembly regulated by Rad6, Bre1 and Ubp2 act as an oxidative stress induced adaptive response in a yeast model system [11]. Consistent with this observation, we found that TRIM32 enhances the total ubiquitination and increased turnover of K63 linked ubiquitination. This evidence suggests that levels of TRIM32 are maintained in normal physiological conditions and its turnover increase in oxidative stress conditions and decrease K63-linked ubiquitination hence may enhance survival mechanism. This is further supported by a recent study where it has been shown that TRIM32 can synthesize unanchored K63-linked ubiquitin chains which activates ULK1, an au- tophagy regulating protein critical for cellular homeostasis [38].

Mitochondria are a major source of ROS generation and our ob- servation that TRIM32 mediated the regulation of oxidative stress in- duced cell death suggests its implication in mitochondrial function. Our cellular localization experiment suggests that TRIM32 forms typical cytoplasmic speckle-like structures, similar to other TRIM members [39] in normal conditions. Interestingly, during oxidative stress, TRIM32 co-localizes with mitochondria, which may regulate complex-I activity. Mitochondrial complex-I is the major site of electron entry in ETC and an observed decrease in complex-I activity may reflect electron leakage and increased mitochondrial ROS [40]. The MitoSOX staining and CM-H$_2$DCFDA staining in TRIM32 expressing cells showing high levels of ROS seem to support this speculation. A prior high throughput proteomics study analyzing the interacting proteins of TRIM32 included several proteins of mitochondrial origin further supporting our hy- pothesis [41]. In addition, TRIM32 and its interacting proteins such as FASTKD5 and DDX family of proteins may regulate mitochondrial RNA processing, which determines the level of mitochondrial DNA encoded transcripts and proteins hence determining the OXPHOS capacity [42]. Finally, evidence form our current study suggests that TRIM32 ac- tivates the caspase mediated pathway of apoptosis in the presence of oxidative stress conditions. IAPs including XIAP regulate the caspase-3 mediated pathway [43] and a recent study showed that TRIM32 en- hances XIAP turnover during TNF mediated apoptosis [32]. TRIM32 is thought to sensitize TNF induced apoptosis by antagonizing XIAP via direct ubiquitination [33]. Interestingly, XIAP is also known to trans- locate to mitochondria and regulate the threshold of apoptosis by mediating SMAC (second mitochondria-derived activator of caspases) turnover through the endo lysosomal pathway in mitochondria [44]. Our data showed

that TRIM32 regulates the levels of XIAP, therefore the interaction between XIAP and TRIM32 may regulate the XIAP turnover under normal cellular conditions. The decreased level of TRIM32 may enhance the level of XIAP, which is also known to act as an antioxidant by regulating the expression of several antioxidant genes including SOD1, SOD2, NQO1, HO-1, and Txn2. It also has been re- ported that XIAP regulates NF-kB and modulates the antioxidant genes. XIAP (−/−) MEFs showed increased levels of ROS and increased cell death in the presence of oxidative stress conditions [45]. Further E3 ligase activity of XIAP lowers the mitochondrial apoptotic potential via intra-mitochondrial degradation of its inhibitor SMAC [44] and hence may modulate the intrinsic pathway of apoptosis. These evidences suggest that downregulation of TRIM32 and increased level of XIAP is cellular adaptive response during oxidative stress conditions. The role of TRIM32 in ubiquitination of different apoptotic proteins, including the interaction with XIAP, formation of unanchored ubiquitin chain, and its role in mitochondrial function need to be further investigated. Regardless, our data demonstrate that E3 ligase activity of TRIM32 modulates super complex assembly and ROS levels during the oxidative stress. This suggest important role of TRIM32 in many patho- physio- logical conditions like neurodegeneration and tumorigenesis where mitochondrial role had been established.

Authors contribution

R.S. and P.P. conceptualized and designed the experiments, wrote manuscript. D.G. helped with mitochondrial experiments. A.S. helped with manuscript editing and arrangement. M.R., K.S. assisted with the different experiments.

Declaration of Competing Interest

The authors declare no competing interests.

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References

[1] A. Chatterjee, S. Dasgupta, D. Sidransky, Mitochondrial Subversion in Cancer, vol. 4, no. May (2011), pp. 638–655.

[2] S.M. Cloonan, A.M.K. Choi, Mitochondria: commanders of innate immunity and disease ? Curr Opin Immunol 24 (1) (2012) 32–40.

[3] S. Dimauro, C. Garone, Seminars in Fetal & Neonatal Medicine Metabolic disorders of fetal life : Glycogenoses and mitochondrial defects of the mitochondrial re- spiratory chain, Semin. Fetal Neonatal Med. 16 (4) (2011) 181–189.

[4] G. Ashrafi, T.L. Schwarz, The pathways of mitophagy for quality control and clearance of mitochondria, Cell Death Differ. 20 (1) (2013) 31–42.

[5] M. Frank, et al., Mitophagy is triggered by mild oxidative stress in a mitochondrial fission dependent manner, Biochim. Biophys. Acta, Mol. Cell Res. 1823 (12) (2012) 2297–2310.

[6] M. Martinez-Vicente, Neuronal mitophagy in neurodegenerative diseases, Front. Mol. Neurosci. 10 (March) (2017) 1–13.

[7] Y. Xu, J. Shen, Z. Ran, Emerging views of mitophagy in immunity and autoimmune diseases, Autophagy 16 (1) (2020) 3–17.

[8] S.H. Lecker, A.L. Goldberg, W.E. Mitch, Protein degradation by the ubiquitin-pro- teasome pathway in normal and disease states, J. Am. Soc. Nephrol. 17 (7) (2006) 1807–1819.

[9] W. Li, Y. Ye, Polyubiquitin chains: functions, structures, and mechanisms, Cell. Mol. Life Sci. 65 (15) (2008) 2397–2406.

[10] A. Kubli, D.A. Gustafsson, Mitochondria and mitophagy: the yin and yang of cell death control, Circ. Res. 111 (9) (2012) 1208–1221 vol. 111, no. 9, pp. 1208–21, 2012.

[11] J.A. King, R.A. Barkley, S. Barrett, K63 polyubiquitination is a new modulator of the oxidative stress response Gustavo, Nat. Struct. Mol. Biol. 22 (2) (2015) 116–123.

[12] M. Červinka, The role of mitochondria in apoptosis induced in vitro, Gen. Physiol. Biophys. 18 (SPEC. ISS) (1999) 33–40.

[13] D. Tomar, et al., TRIM13 regulates caspase-8 ubiquitination, translocation to au- tophagosomes and activation during ER stress induced cell death, Biochim. Biophys. Acta, Mol. Cell Res. 1833 (12) (2013) 3134–3144.

[14] M. Roy, et al., TRIM8 regulated autophagy modulates the level of cleaved Caspase-3 subunit to inhibit genotoxic stress induced cell death, Cell. Signal. 48 (April) (2018) 1–12.

[15] P.D. Uchil, et al., TRIM protein-mediated regulation of inflammatory and innate immune signaling and its association with antiretroviral activity, J. Virol. 87 (1) (2013) 257–272.

[16] Z. Zhou, et al., TRIM14 is a mitochondrial adaptor that facilitates retinoic acid- inducible gene-I-like receptor-mediatedinnate immune response, Proc. Natl. Acad. Sci. U. S. A. 111 (2) (2014) 245–254.

[17] A.M. Jaworska, N.A. Wlodarczyk, A. Mackiewicz, P. Czerwinska, The role of TRIM family proteins in the regulation of cancer stem cell self-renewal, Stem Cells 38 (2) (2020) 165–173.

[18] S.H. Lee, et al., The ubiquitin ligase human TRIM71 regulates let-7 microRNA biogenesis via modulation of Lin28B protein, Biochim. Biophys. Acta Gene Regul. Mech. 1839 (5) (2014) 374–386.

[19] D. Tomar, et al., TRIM4; a novel mitochondrial interacting RING E3 ligase, sensi- tizes the cells to hydrogen peroxide (H2O2) induced cell death, Free Radic. Biol. Med. 89 (2015) 1036–1048.

[20] L. Sripada, D. Tomar, P. Prajapati, R. Singh, A.K. Singh, Systematic Analysis of Small RNAs Associated with Human Mitochondria by Deep Sequencing : Detailed Analysis of Mitochondrial Associated miRNA, vol. 7, (2012) no. 9.

[21] Calcium phosphate-mediated transfection of eukaryotic cells, Nat. Methods 2 (4) (2005) 319–320.

[22] P. Prajapati, L. Sripada, K. Singh, K. Bhatelia, R. Singh, R. Singh, Biochimica et Biophysica Acta TNF- α regulates miRNA targeting mitochondrial complex-I and induces cell death in dopaminergic cells, BBA - Mol. Basis Dis. 1852 (3) (2015) 451–461.

[23] K. Singh, et al., NLRX1 acts as tumor suppressor by regulating TNF-α induced apoptosis and metabolism in cancer cells, Biochim. Biophys. Acta, Mol. Cell Res. 1853 (5) (2015) 1073–1086.

D. Gohel, et al., FMRpolyG alters mitochondrial transcripts level and respiratory chain complex assembly in fragile X associated tremor/ataxia syndrome [FXTAS], Biochim. Biophys. Acta Mol. basis Dis. 1865 (6) (2019) 1379–1388.

[24] D. Gohel, L. Sripada, P. Prajapati, K. Singh, M. Roy, BBA - molecular basis of disease FMRpolyG alters mitochondrial transcripts level and respiratory chain complex assembly in fragile X associated tremor / ataxia syndrome [FXTAS], BBA - Mol. Basis Dis. 1865 (6) (2019) 1379–1388.

[25] G.M. Silva, D. Finley, C. Vogel, K63 polyubiquitination is a new modulator of the oxidative stress response, Nat. Struct. Mol. Biol. 22 (2) (2015) 116–123.

[26] M.G. Koliopoulos, D. Esposito, E. Christodoulou, I.A. Taylor, K. Rittinger, Functional role of TRIM E3 ligase oligomerization and regulation of catalytic ac- tivity, EMBO J. 35 (11) (2016) 1204–1218.

[27] L. Sharma, J. Lu, Y. Bai, Mitochondrial respiratory complex I: structure, function and implication in human diseases, Curr. Med. Chem. 16 (10) (2009) 1266–1277.

[28] X. Jacq, M. Kemp, N.M.B. Martin, S.P. Jackson, Deubiquitylating enzymes and DNA damage response pathways, Cell Biochem. Biophys. 67 (1) (2013) 25–43.

[29] D. Tomar, R. Singh, TRIM family proteins: emerging class of RING E3 ligases as regulator of NF-κB pathway, Biol. Cell. 107 (1) (2015) 22–40.

[30] G. Meroni, TRIM/RBCC Proteins, Google Books, (2012).

[31] E. Lazzari, G. Meroni, TRIM32 ubiquitin E3 ligase, one enzyme for several pathologies: from muscular dystrophy to tumours, Int. J. Biochem. Cell Biol. 79 (2016) 469–477.

[32] Y.S. Ryu, et al., TRIM32 protein sensitizes cells to tumor necrosis factor (TNFα)- induced apoptosis via its RING domain-dependent E3 ligase activity against X- linked inhibitor of apoptosis (XIAP), J. Biol. Chem. 286 (29) (2011) 25729–25738.

[33] Y. Du, et al., TRIM32 overexpression improves chemoresistance through regulation of mitochondrial function in non-small-cell lung cancers, Onco. Targets. Ther. 11 (2018) 7841–7852.

[34] L. Wei, et al., Knockdown of TRIM32 protects hippocampal neurons from oxy- gen–glucose deprivation-induced injury, Neurochem. Res. 44 (9) (2019) 2182–2189.

[35] L. Hauck, et al., Cardiac-specific ablation of the E3 ubiquitin ligase Mdm2 leads to oxidative stress, broad mitochondrial deficiency and early death, vol. 12, (2017) no. 12.

[36] F. Shang, A. Taylor, Ubiquitin-proteasome pathway and cellular responses to oxi- dative stress, Free Radic. Biol. Med. 51 (1) (2011) 5–16.

[37] M. Di Rienzo, et al., Autophagy induction in atrophic muscle cells requires ULK1 activation by TRIM32 through unanchored K63-linked polyubiquitin chains, Sci. Adv. 5 (5) (2019) 1–15.

[38] K.S. Overå, et al., TRIM32, but not its muscular dystrophy-associated mutant, po- sitively regulates and is targeted to autophagic degradation by p62/SQSTM1, J. Cell Sci. 132 (23) (2019).

[39] A. Devin, et al., Mitochondria: Ultrastructure, dynamics, biogenesis and main functions, Mitochondria Obes. Type 2 Diabetes Compr. Rev. Mitochondrial Funct. Involv. Metab. Dis. (2019) 3–32.

[40] J. Zhang, M.M. Hu, Y.Y. Wang, H.B. Shu, TRIM32 protein modulates type I inter- feron induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination, J. Biol. Chem. 287 (34) (2012) 28646–28655.

[41] S. Nicklas, S. Okawa, A. Hillje, L. Gonz, A. Sol, J.C. Schwamborn, The RNA helicase DDX6 regulates cell-fate specification in neural stem cells via miRNAs, vol. 43, (2015), pp. 2638–2654 no. 5.

[42] J. Berthelet, L. Dubrez, Regulation of apoptosis by inhibitors of apoptosis (IAPs), Cells 2 (1) (2013) 163–187.

[43] A. Hamacher-Brady, S.C. Choe, J. Krijnse-Locker, N.R. Brady, Intramitochondrial recruitment of endolysosomes mediates Smac degradation and constitutes a novel intrinsic apoptosis antagonizing function of XIAP E3 ligase, Cell Death Differ. 21 (12) (2014) 1862–1876.

[44] U. Resch, Y.M. Schichl, S. Sattler, R. de Martin, XIAP regulates intracellular ROS by enhancing antioxidant gene expression, Biochem. Biophys. Res. Commun. 375 (1) (2008) 156–161.
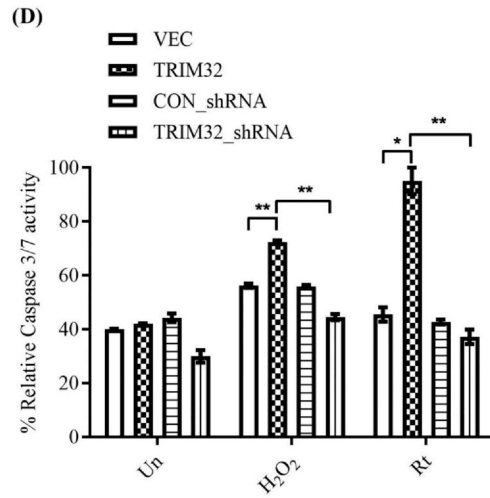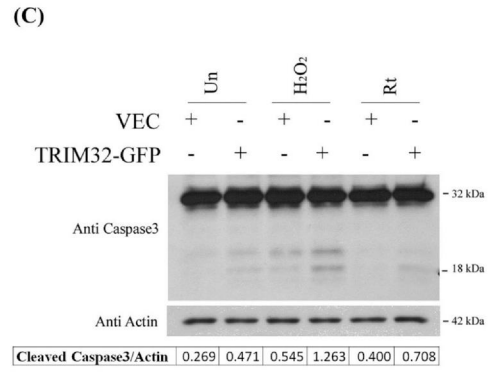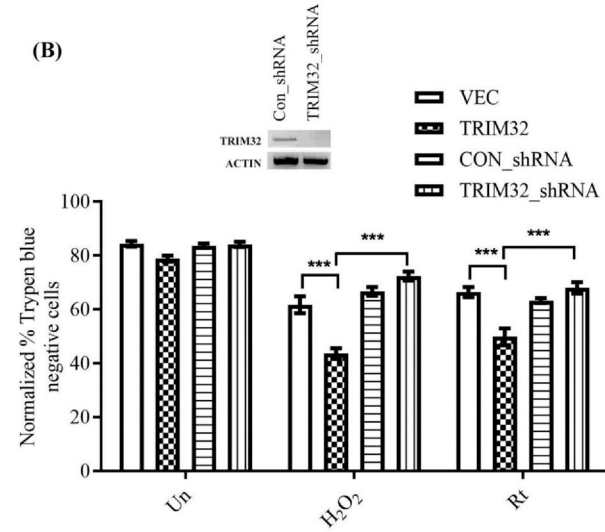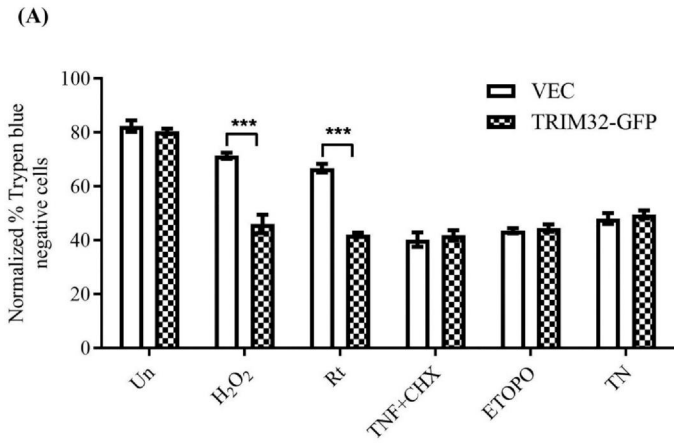
Figure Legends:

Fig. 1. TRIM32 sensitizes oxidative stress induced cell death. (**A**) HEK293 cells were transfected with vector and TRIM32-GFP and treatments of H_2O_2 (100 μM), rotenone (1 μM), TNF (20 ng/ml), CHX (10 μg/ml) and etoposide (50 μM) was given after 24 h. Trypan blue staining was performed after 24 h of treatment and trypan blue negative cells were plotted after percentage normalization. (**B**) & (**D**) HEK293 cells were transfected with vector, TRIM32, control shRNA and TRIM32 shRNA and treated with H_2O_2 (100 μM) and rotenone (1 μM) after 24 h. Trypan blue negative cells were plotted after percentage normalization and relative caspase 3/7 activity was measured respectively after 24 h of treatment. (**C**) HEK293 cells were transfected with vector and TRIM32-GFP and treated with H_2O_2 (100 μM) and rotenone (1 μM) after 24 h. Western blotting was performed using caspase-3 and actin antibody after 24 h of treatment. Asterisk (*) indicates that p value (*$p < 0.5$, **$p < 0.01$, ***$p < 0.001$), for SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 2. E3 ligase activity of TRIM32 is essential for oxidative stress induced cell death. (**A**) Schematic representation of Full length TRIM32 (**B**) HEK293 cells were transfected with vector and TRIM32-GFP and treated with H_2O_2 (100 μM), rotenone (1 μM) and MG132 (5 μM) for 24 h. Western blotting was performed using antibody against ubiquitin and actin. (**C**) HEK293 cells were transfected with vector, FL-TRIM32 and TRIM32ΔR and treated with H2O2 (100 μM) and rotenone (1 μM) for 24 h and % cell survival was analyzed using MTT assay. (**D**) HEK-293 cells were treated with H_2O_2 (100 μM) and rotenone (1 μM) and western blotting was performed to monitor the level of TRIM32 (**E**) HEK293 cells were transfected with HA-K63 and TRIM32-GFP and treated with H_2O (100 μM)$_2$, MG132 (5 μM) and NH_4Cl (20 mM) for 24 h and western blotting was performed using antibody against HA. Asterisk (*) indicates that p value (*p < 0.5, **p < 0.01, ***p < 0.001), for SEM.

Fig. 3. *Taujap Ji, et al* TRIM32 induces loss of transmembrane potential and enhances mitochondrial ROS production during oxidative stress conditions. (**A**) & (**C**) HEK293 cells were transfected with vector and TRIM32-GFP and treated with H_2O_2 (100 μM) and rotenone (1 μM) after 24 h and % CM-H_2DCFDA fluorescence and TMRM was analyzed after 24 h, respectively. (**B**) HEK293 cells were transfected with GFP-vector and TRIM32-GFP and treated with H_2O_2 (100 μM) and rotenone (1 μM). After 24 h, cells were stained with MitoSOX red and florescent microscopy was performed (**D**) HEK293 cells were transfected with Vector and TRIM32 and treated with H_2O_2 (100 μM), rotenone (1 μM), NAC (10 mM) and zVAD.fmk (20 μM) after 24 h. % normalized trypan blue negative cells were analyzed after 24 h. Asterisk (*) indicates that p value (*$p < 0.5$, **$p < 0.01$, ***$p < 0.001$), for SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. TRIM32 translocate to mitochondria and regulates complex-I activity during oxidative stress conditions. (A) HEK293-MTRFP cells were transfected with vector, TRIM32-GFP and treated with H_2O_2 (100 μM) and rotenone (1 μM) after 24 h and confocal microscopy was performed. (**B**) HEK293 cells were treated with H_2O_2 (100 μM) and rotenone (1 μM). Mitochondrial and cytosol fractions were isolated and analyzed by western blotting using TRIM32 antibody. (**C**) HEK293 cells were transfected with vector and HA-TRIM32 and in gel staining was performed for mitochondrial complex-I using BN-PAGE (Blue-Native). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. TRIM32 mediated turnover of XIAP regulates ROS and cell death. (A) HEK293 cells were transfected with Vector and HA-TRIM32 and western blotting was performed using HA and XIAP antibody (**B**), (**C**) & (**D**) HEK293 cells were co-transfected with Vector, TRIM32 and XIAP as indicated in figure and treated with H_2O_2 (100 μM) after 24 h and % CM-H_2DCFDA, Relative caspase 3/7 and cell survival was measured respectively. Asterisk (*) indicates that *p* value (*$p < 0.5$,
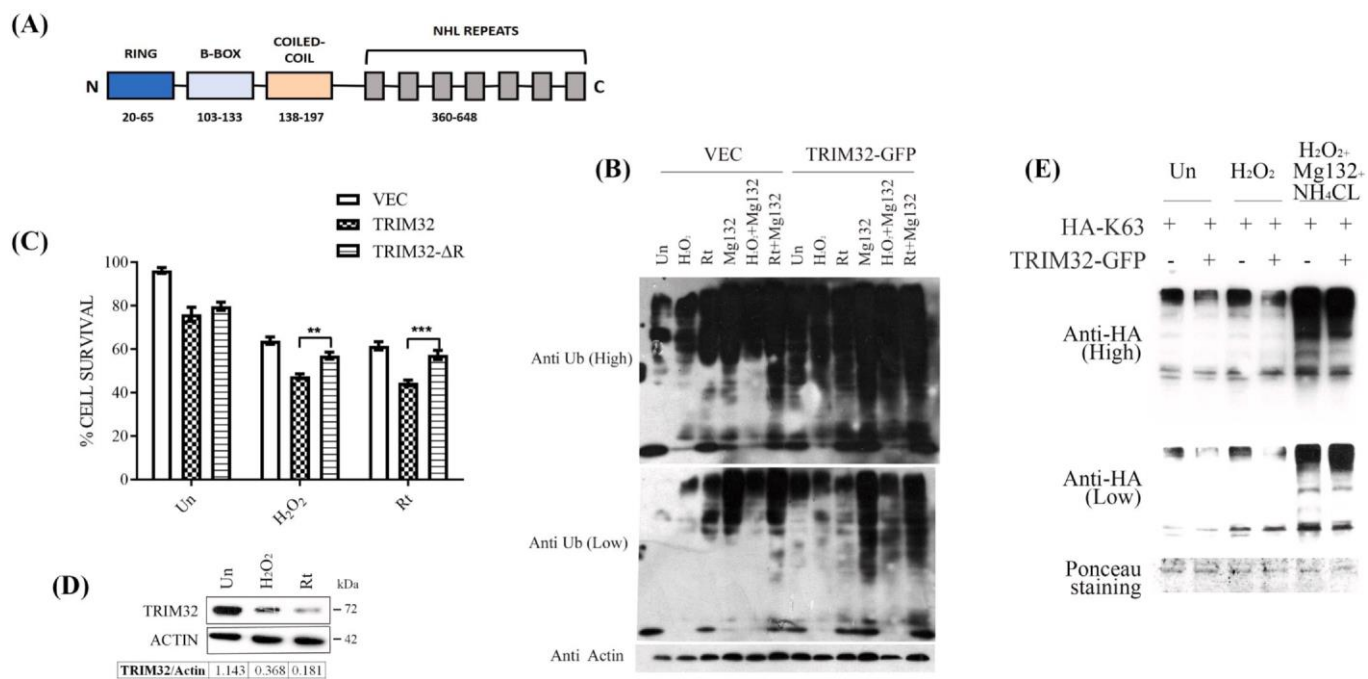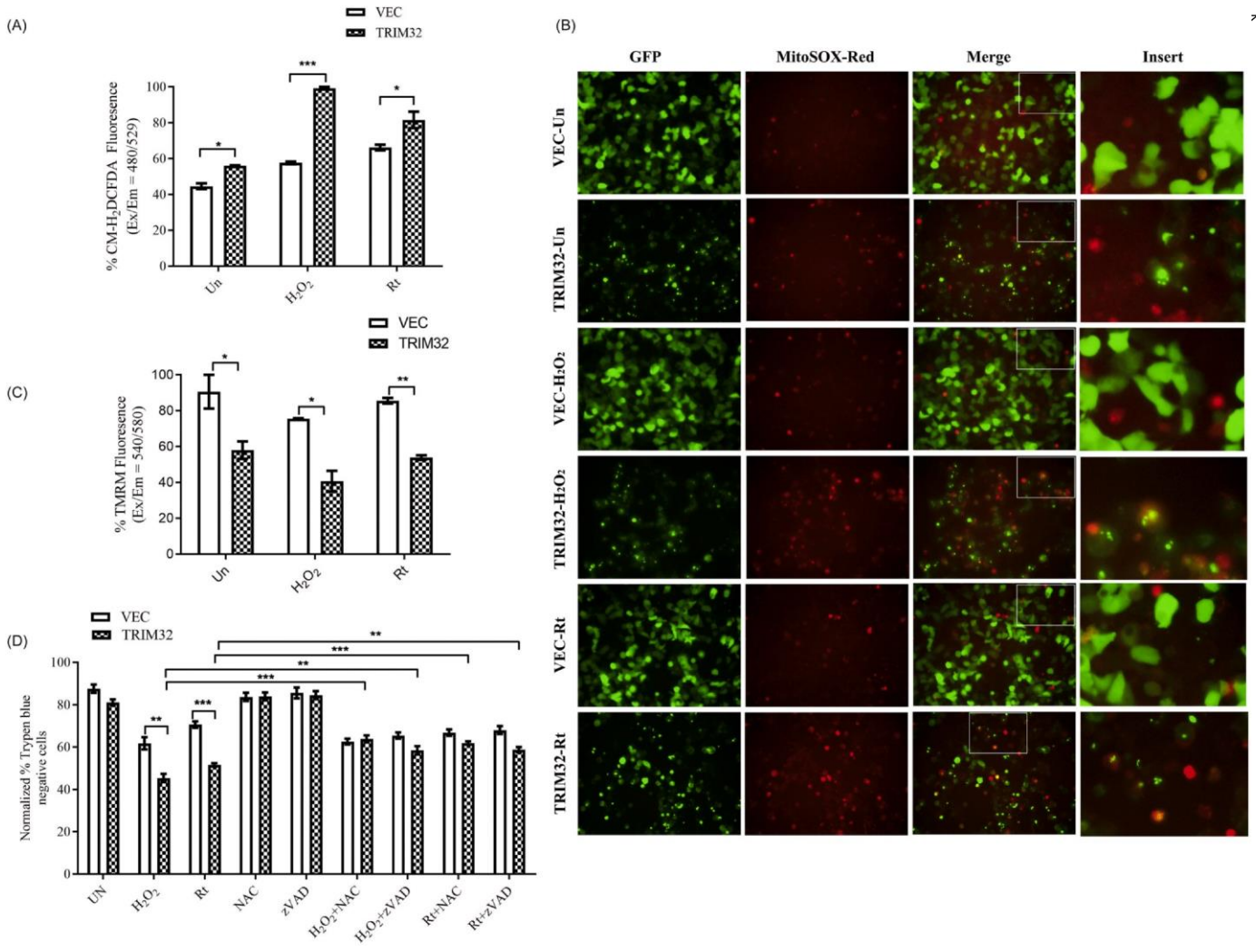
**$p < 0.01$, ***$p < 0.001$), for SEM.

Fig. 1.

(A)

Normalized % Trypen blue negative cells

100
80
60
40
20
0

VEC
TRIM32-GFP

*** ***

Un   H₂O₂   Rt   TNF+CHX   ETOPO   TN

(C)

	Un		H₂O₂		Rt	
VEC	+	−	+	−	+	−
TRIM32-GFP	−	+	−	+	−	+

Anti Caspase3 — 32 kDa
— 18 kDa

Anti Actin — 42 kDa

| Cleaved Caspase3/Actin | 0.269 | 0.471 | 0.545 | 1.263 | 0.400 | 0.708 |

(B)

Con_shRNA   TRIM32_shRNA

TRIM32
ACTIN

VEC
TRIM32
CON_shRNA
TRIM32_shRNA

Normalized % Trypen blue negative cells

100
80
60
40
20
0

*** ***   *** ***

Un   H₂O₂   Rt

(D)

VEC
TRIM32
CON_shRNA
TRIM32_shRNA

% Relative Caspase 3/7 activity

100
80
60
40
20
0

** **   * **

Un   H₂O₂   Rt

Fig. 2

(A)

(C)

(B)

(E)

(D)

| TRIM32/Actin | 1.143 | 0.368 | 0.181 |

Fig. 3.

(A)

(B)

(C)

(D)

(B) GFP | MitoSOX-Red | Merge | Insert

VEC-Un
TRIM32-Un
VEC-H₂O₂
TRIM32-H₂O₂
VEC-Rt
TRIM32-Rt

Fig. 4

(A) TRIM32-GFP | MT-RFP | Merge | Insert

Un
H₂O₂
Rt

(B)

	Un	H2O2	Rt	Un	H2O2	Rt	kDa
TRIM32							- 72
AIF							- 67
ACTIN							- 42

Mito   Cyto

| TRIM32/AIF | 0.8 | 0.9 | 1.7 | 13.0 | 3.5 | 3.8 |
| TRIM32/ACTIN | | | | 1.6 | 1.3 | 1.1 |

(C)

VEC | HA-TRIM32

I₂ + III₂   n1
I + III₂ + IV₃   n2
I + III₂ + IV₂   n3
I + III₂ + IV₁   n4
I + III₂ + II₀   n5
I

| Complex-I | 76.7 | 61.0 |
| Supper Complex | 140.8 | 103.9 |

Fig. 5

(A)

(B)

(C)

(D)