Chapter 8:

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

DNA damage response (DDR) is evolved to ensure genomic integrity and cell survival. DDR includes sensing of the DNA damage, recruitment of DNA repair proteins and repair. 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 (Roos and Kaina, 2006) however many other regulators still needs to be identified. Pathways regulating cell survival/death and DNA repair process coordinate to maintain cellular homeostasis (Erol, 2011; Roos and Kaina, 2013). Cancer cells exploit their compromised DDR; it helps them in avoiding cellular checkpoints and proliferate. Therapeutic intervention of cancer by radiation and chemotherapy targets the genomic DNA of malignant cells and exploits the DDR-apoptosis crosstalk to kill cancer cells (Day et al., 2009). Genotoxic stress caused by these therapeutic agents can lead to growth arrest or activation of death pathways in cells (Biton and Ashkenazi, 2011; Tenev et al., 2011). The cross talk of different cell survival and death pathways; their regulators during genotoxic stress in cancer cells remains to be investigated.

Autophagy induced by genotoxic agents have been implicated both in cell death and cell survival, hence needs more investigation (Polewska et al., 2013; Rodriguez-Rocha et al., 2011). Intricate crosstalk exists between autophagy and apoptosis and apoptosis acts as the last resort for cells in severe genotoxic stress (Tang et al., 2015). Autophagy eliminates defective organelles, aggregated or dysfunctional proteins and in turn regulates cellular homeostasis (Kundu and Thompson, 2008; Mizushima, 2007; Ryter et al., 2013). Autophagy is also essential for removal of extranuclear DNA produced by genotoxic stress that can lead to sterile inflammation. Interestingly, autophagy mediated clearance of exported damaged nuclear DNA is essential for cell survival. And the failure extranuclear DNA clearance leads to cytosolic DNA sensors like cGAS/STING mediated clearance of inflammatory pathways

(Lan et al., 2014). Ubiquitination is a critical Post translational modification which regulates both autophagy and DDR during DNA damage. DDR is initiated by histone ubiquitination at the double stranded breaks (DSB) by ubiquitin E3 ligases RNF8. Furthermore, sustained ubiquitination of histones H2A is promoted on preexisting ubiquitin chains of by E3 ligases like RNF168. Ubiquitination at DSB sites promotes nucleosome destabilization and provide docking signal for recruitment of damage responsive and repair proteins (Brinkmann et al., 2015; Feng and Chen, 2012). Interestingly, It had been observed that p62/SQSTM1 (an autophagy adaptor protein) accumulation induced by autophagy deficiency in cells shows sequestration of RNF168 and sensitizes tumor cell to radiation. TRIM2 and ubiquitin E3 ligases complex like $CRL4^{DDB2}$ regulate the levels of p53 and BIM (Bcl-2-interacting mediator of cell death) and play pivotal role in regulation of DDR (Thompson et al., 2011; Yang et al., 2016).

The largest subgroup of RING E3 ligases: TRIMs play various role in regulation of cellular homeostasis (Marin, 2012; Sardiello et al., 2008). Previous reports had shown that TRIMs regulate autophagy and NF-κB pathway thus may play crucial role in cell death and survival during different stress conditions including genotoxic stress (Kimura et al., 2016a; Kimura et al., 2015; Li et al., 2011; Mandell et al., 2014; Tomar et al., 2013a; Tomar et al., 2012a; Tomar et al., 2012b). Moreover, genotoxic stress is known to activate NF-κB but whether the regulators of NF-κB pathway also affect DDR is not well known. Therefore, the role of TRIM8 was further explored in DDR and autophagy.

8.1 TRIM8 regulates autophagic flux

The emerging evidences suggest that NF-κB regulate autophagy (Criollo et al., 2012; Djavaheri-Mergny et al., 2007; Ye et al., 2011). It had been shown that TRIM8 positively regulates TNF-α-induced NF-κB (Li et al., 2011; Tomar et al., 2012b), therefore, its role in autophagy and DDR was further investigated. TRIM8 was transfected in HEK293-GFP-LC3 stable cell line and LC3 puncta were monitored by fluorescent microscopy. TRIM8 transfection increased number of GFP-LC3 puncta suggesting increased autophagosome formation (Figure 8.1 (I) A & B).

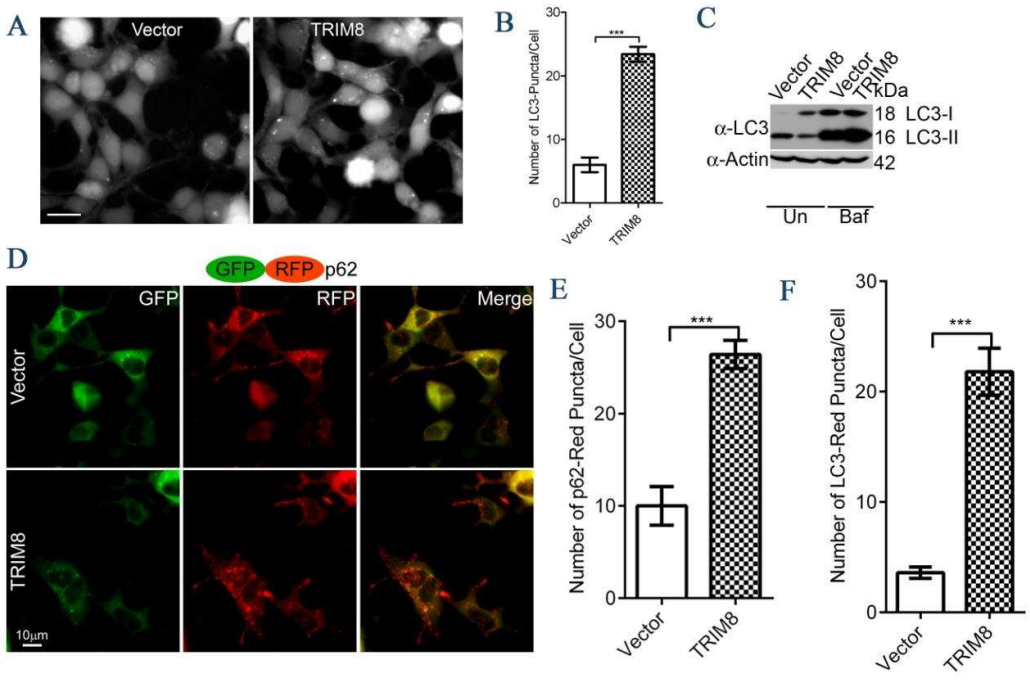

Figure 8.1 (I): TRIM8 regulates autophagic flux. *(A) The expression of TRIM8 induces autophagy. HEK293-GFP-LC3 stable cell line was transfected with TRIM8 and vector, cells were observed under fluorescent microscope after 24 h of transfection. The scale bar represents 20 μM. (B) Quantification of TRIM8 induced autophagic puncta. The graph was plotted for numbers of GFP-LC3 puncta per cell of representative Figure 8.1 (I) A. (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.

Further the role of TRIM8 on autophagy flux was monitored by microscopy using tandem construct of mCherry-GFP-LC3 and mCherryGFP-p62 fluorescent reporters as described previously (Kimura et al., 2007; Pankiv et al., 2007). The yellow puncta (red and green merge) were considered as autophagosomes whereas red puncta as autophagolysosomes (autophagosomes fused with lysosomes) (Kimura et al., 2007). TRIM8 was co-transfected with mCherryGFP-LC3 and mCherry-GFP-p62 fluorescent reporter constructs. The expression of TRIM8 significantly increased the number of p62 red puncta (Figure 8.1 (I) D and E). Similarly, number of LC3 red puncta (Figure 8.1 (I) F) significantly increased in TRIM8 co-transfected cells. Autophagy flux was further confirmed using LC3 western blotting (Chittaranjan et al., 2015).

TRIM8 was transfected in p53 wild type cells HEK293 and MX1 and treated with Bafilomycin-A1 (autophagosome-lysosome fusion inhibitor) and western blotting was performed to detect LC3 levels using 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 (Figure 8.1 (I) C, Figure 8.1 (II) A).

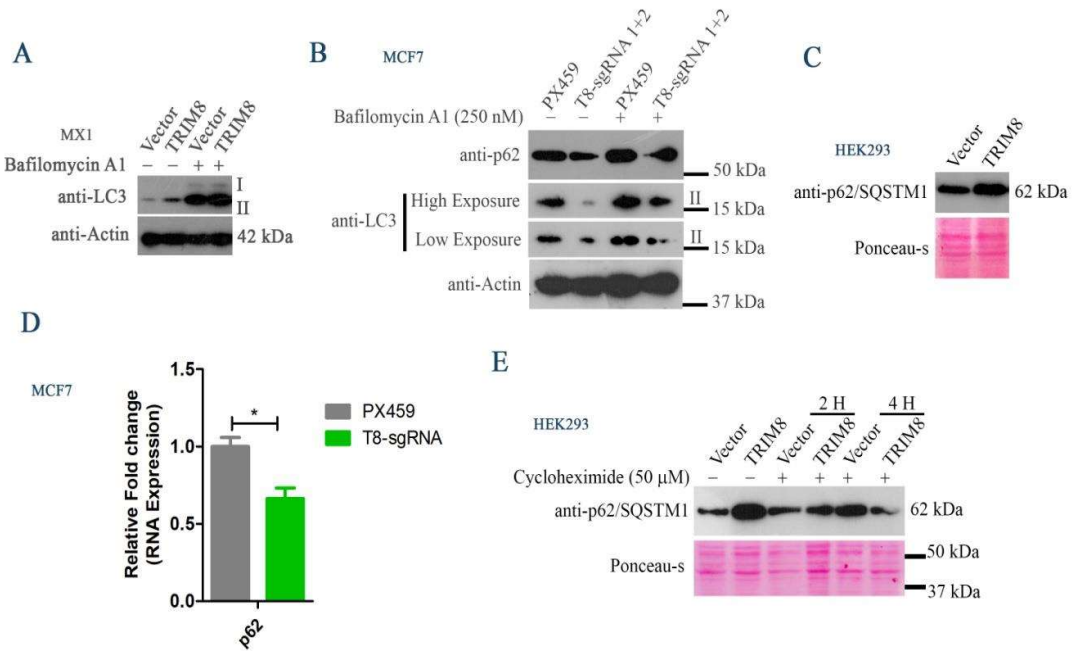

Figure 8.1 (II): TRIM8 promotes p62/SQSTM1 expression. *(A) MX1 cells were transfected with vector or TRIM8 and treated with 250 nM Bafilomycin A1 for 10 hours and blotted with anti-LC3 antibody to detect the protein level of the same. (B) PX459 or TRIM8-sgRNA were transfected in MCF7 cells and treated with 250 nM Bafilomycin A1 for 10 hours. Western blotting was performed to detect the levels of LC3 and p62/SQSTM1. (C) HEK293 cells were transfected with vector or TRIM8 and western blotting was performed to check the p62/SQSTM1 levels. (D) MCF7 cells were transfected with PX459 of TRIM8-sgRNA. cDNA was synthesized and q-PCR was performed to check the expression of p62/SQSTM1 mRNA levels. (E) Vector or TRIM8 was transfected in HEK293 cells and treated with 50 µM cycloheximide for 2 or 4 hours. Western blotting was performed to monitor p62/SQSTM1 levels. Asterisk (*) indicates fold change statistically significant from untreated; p value <0.05; SEM of minimum three independent experiments.*

Further, TRIM8 was knocked down using sgRNA in p53 wild type MCF7 cells and treated with Bafilomycin-A1. Decrease in LC3-II levels were observed in TRIM8 knocked down MCF7 cells (Figure 8.1 (II) B). The levels of p62/SQSTM1 levels were also checked in the same blot. The 62 kDa band corresponding to p62/SQSTM1 was decreased in untreated TRIM8 knock down cell suggesting possible transcriptional regulation of cellular p62/SQSTM1 levels by TRIM8. The reduced level of p62/SQSTM1 in BafilomycinA1 treated TRIM8 knock down cells compared to control was observed (Figure 8.1 (II) B). The effect of TRIM8 on p62/SQSTM1 expression was further investigated. TRIM8 was expressed in HEK293 cells and p62/SQSTM1 levels were checked by western blotting. Interestingly, TRIM8 increased p62/SQSTM1 levels as compared to vector transfected cells (Figure 8.1 (II) C). Also, TRIM8 knock down in MCF7 cells reduced p62/SQSTM1 mRNA levels (Figure 8.1 (II) D). Furthermore to confirm these observation TRIM8 was expressed in HEK293 cells and de novo protein synthesis was blocked using translation inhibitor; Cycloheximide (CHX). The levels of p62/SQSTM1 increased levels in TRIM8 transfected cells however decreased after 4 hrs of CHX treatment. These results suggest that TRIM8 transcriptionally upregulates p62/SQSTM1 and enhances its turnover probably through autophagy flux. It also suggests the role of p62 in DNA damage beyond autophagy at least in genotoxic stress conditions (Figure 8.1 (II) E). Together these results also confirm that TRIM8 is a positive regulator of autophagy flux both in cells with functional or mutated p53.

8.2 RING domain of TRIM8 is required for autophagy

Individual domains of TRIM8 may play distinctive role in regulation of TRIM8 associated cellular processes (Rajsbaum et al., 2014a; Reymond et al., 2001; Tomar et al., 2012a). RING domain of TRIM family proteins possess E3 ligase activity (Bell et al., 2012; Napolitano and Meroni, 2012; Tomar et al., 2013a; Tomar and Singh, 2014) therefore the role of RING domain in TRIM8 mediated

autophagy was checked. Either TRIM8 (full length) or TRIM8ΔR (RING domain deleted) constructs were transfected in HEK293-GFP-LC3 stable cell line. Interestingly increased number of GFP-LC3 puncta per cell was observed in TRIM8 transfected cells as compared to control (Figure 8.2 A) whereas TRIM8ΔR transfection failed to increase GFP-LC3 puncta compared to control transfected cells (Figure 8.2 A).

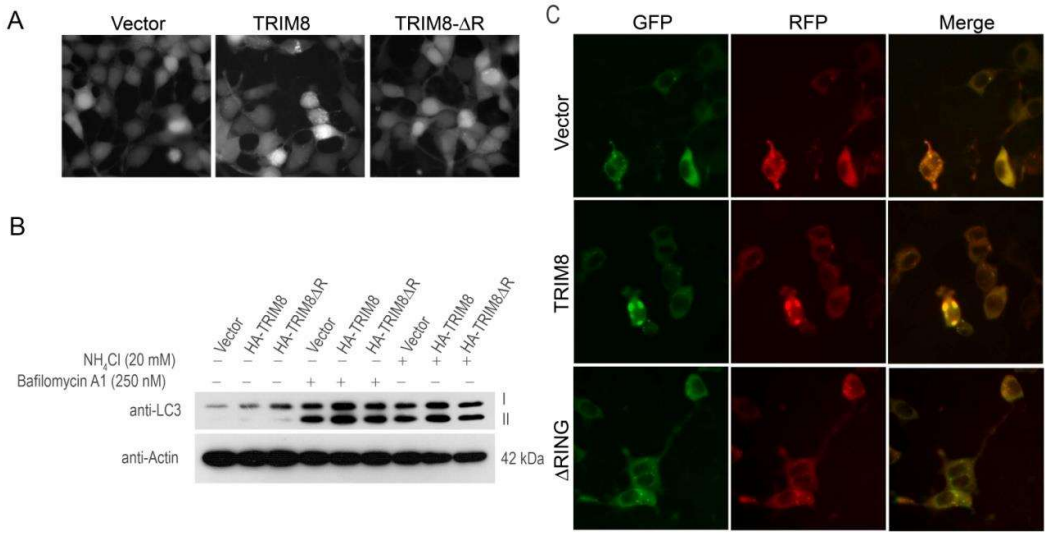

Figure 8.2: RING domain of TRIM8 is required for autophagy. *(A) HEK293-GFP-LC3 stable cell line was transfected with TRIM8/TRIM8ΔR and vector, cells were observed under fluorescent microscope after 24 hours of transfection. (B) Vector, TRIM8 or TRIM8ΔR were transfected in HEK293 cells, treated with 250 nM Bafilomycin A1 for 10 hours or with 20 mM NH4Cl for 4 hours. 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 hours of transfection.*

Further the contribution of the RING domain in TRIM8 mediated autophagic flux was checked. Vector, FL-TRIM8 and TRIM8ΔR were expressed in HEK293 cells and treated with or without Lysotropic agent, NH_4Cl or Bafilomycin-A1.

The levels of LC3 were monitored using western blotting. It was observed that the 16 kDa bands corresponding to LC3-II increased in FL-TRIM8 transfected cell treated with either NH_4Cl or Bafilomycin-A1, compared to control transfected cells (Figure 8.2 B). Transfection of TRIM8ΔR showed no difference in LC3-II levels compared to control (Figure 8.2 B). RING domain's role in autophagy flux was further monitored by fluorescent microscopy. TRIM8 or TRIM8ΔR were co-transfected with mCherry-GFP-LC3 and red/yellow puncta were monitored. Interestingly, decrease in red puncta was observed in TRIM8ΔR compared to FL-TRIM8 transfected cells (Figure 8.2 C). These results suggest that TRIM8'd RING domain is required for induction of autophagy and its flux.

8.3 TRIM8 stabilizes during genotoxic stress and modulates autophagy

TRIMs proteins can homo and hetero oligomerize with TRIM family members and promote ubiquitination of self or of their binding partner due to their intrinsic E3 Ligase activity (Bell et al., 2012; Napolitano and Meroni, 2012). They in turn regulate their turnover in diverse pathophysiological conditions (Tomar et al., 2012a). To check stability, HA tagged TRIM8 (HA-TRIM8) was expressed in HEK293 cells and treated with genotoxic stress inducer, Etoposide; Lysotropic agent Ammonium Chloride (NH_4Cl); mitochondrial complex-I inhibitor, Rotenone; Ubiquitin Proteasome system (UPS) inhibitor, MG132; and autophagy inhibitor and autophagy inhibitor Wortmannin (PI3-kinase inhibitor) (Figure 8.3 (I) A). The western blot showed low level of 62 kDa band corresponding to TRIM8 in normal conditions indicating its high turnover. Interestingly, the level of TRIM8 increased in the presence of MG132 and wortmannin treatment suggesting its turnover is mediated by both autophagy and UPS (Figure 8.3 A). Further, high level of 62 kDa band was also observed in etoposide treated cells, compared to control indicating stabilization of TRIM8 during genotoxic stress (Figure 8.3 A). The level of

TRIM8 was unaltered in NH_4Cl and rotenone treated cells suggesting that the stabilization of TRIM8 during etoposide treatment is specific and it may have important role during DNA damage response (Figure 8.3 A).

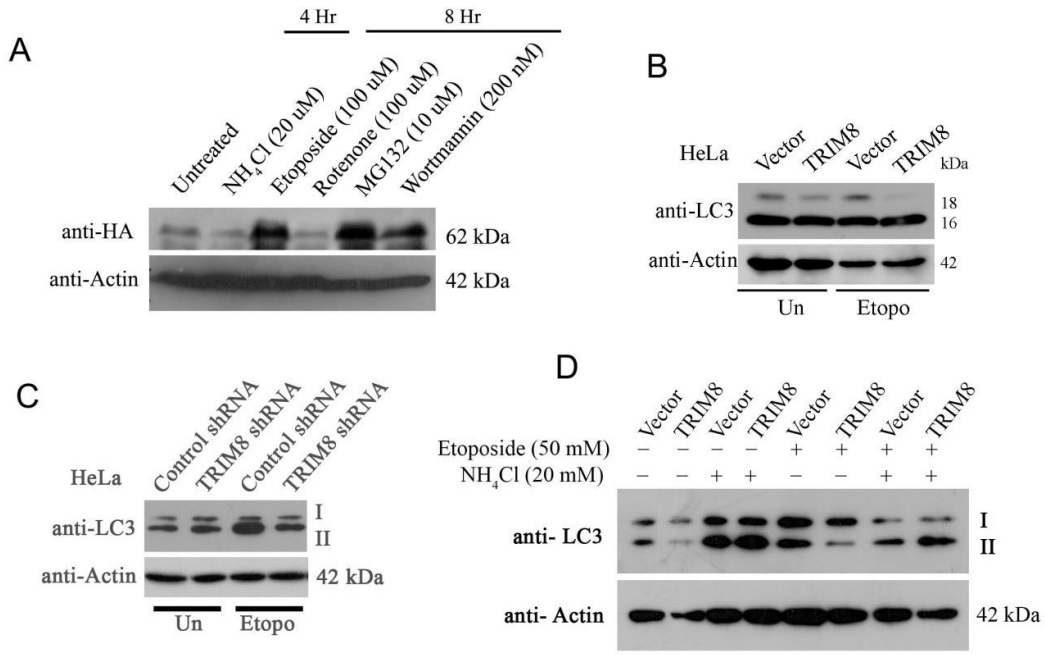

Figure 8.3 (I): TRIM8 stabilizes during genotoxic stress and regulates autophagy. *(A) TRIM8 was transfected in HEK293 cells and was treated as indicated. Western blotting was performed, and blot was probed with HA to monitor TRIM8 levels. (B)TRIM8 induces autophagy during genotoxic stress. TRIM8 was transfected in HeLa cells and were treated with 50 µM etoposide for 8 hours. 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 hours and western blotting was performed to monitor LC3 levels. (D) TRIM8 was transfected in HEK293 cells. After 24 hours of transfection cells were treated with etoposide (8 hours) in presence or absence of NH4Cl (3 hours).*

TRIM8 is also a direct target of p53 gene (Caratozzolo et al., 2012) hence its transcriptional regulation during genotoxic stress was monitored. p53 negative HEK293 cells were treated with etoposide (Figure 8.3 (II) A) and TRIM8 expression was checked using qRT-PCR. Interestingly, mRNA expression of TRIM8 was temporally increased in presence of etoposide (Figure 8.3 (II) A). Etoposide also increased TRIM8 expression in p53 positive MCF7 cells (data not shown). Suggesting etoposide induces TRIM8 expression is independent of p53.

E3 ligases including TRIMs are known to regulate their own turnover hence the same was checked for TRIM8. FL-TRIM8 and TRIM8ΔR was expressed in HEK293 cells and treated with proteasome inhibitor MG132 to check their turnover. 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 (Figure 8.3 (II) B). Possible self ubiquitination of TRIM8 was further checked by immunoprecipitation. HEK293 cells were transfected with control vector, HA-TRIM8 and TRIM8ΔR and treated with MG132. IP was performed using HA specific beads. Interestingly, specific higher molecular weight adducts were predominantly observed in FL-TRIM8, as compared ΔR transfected cells (Figure 8.3 (II) C). The blot also showed comparatively more ubiquitinated proteins in pull down of FL-TRIM8 as compared to TRIM8ΔR pull down lanes. (Figure 8.3 (II) C).

Autophagy induced by genotoxic stress plays crucial role in cell death and survival (Polewska et al., 2013). Therefore, the effect of TRIM8 stabilization in genotoxic stress induced autophagy was checked. Hence, HeLa cells were transfected with TRIM8, and treated with etoposide. Autophagy was monitored by LC3 blotting at early time points. Surprisingly, increased LC3-II/LC3-I ratio was observed in TRIM8 transfected etoposide treated cells as

compared to control transfected cells (Figure 8.3 (I) B). It was reconfirmed by TRIM8 knockdown using shRNA. Knockdown of TRIM8 significantly reduced the LC3-II/LC3-I ratio (Figure 8.3 (I) C) in etoposide treated cells as compared to vector transfected cells. Suggesting TRIM8 can control rate of autophagy during genotoxic stress.

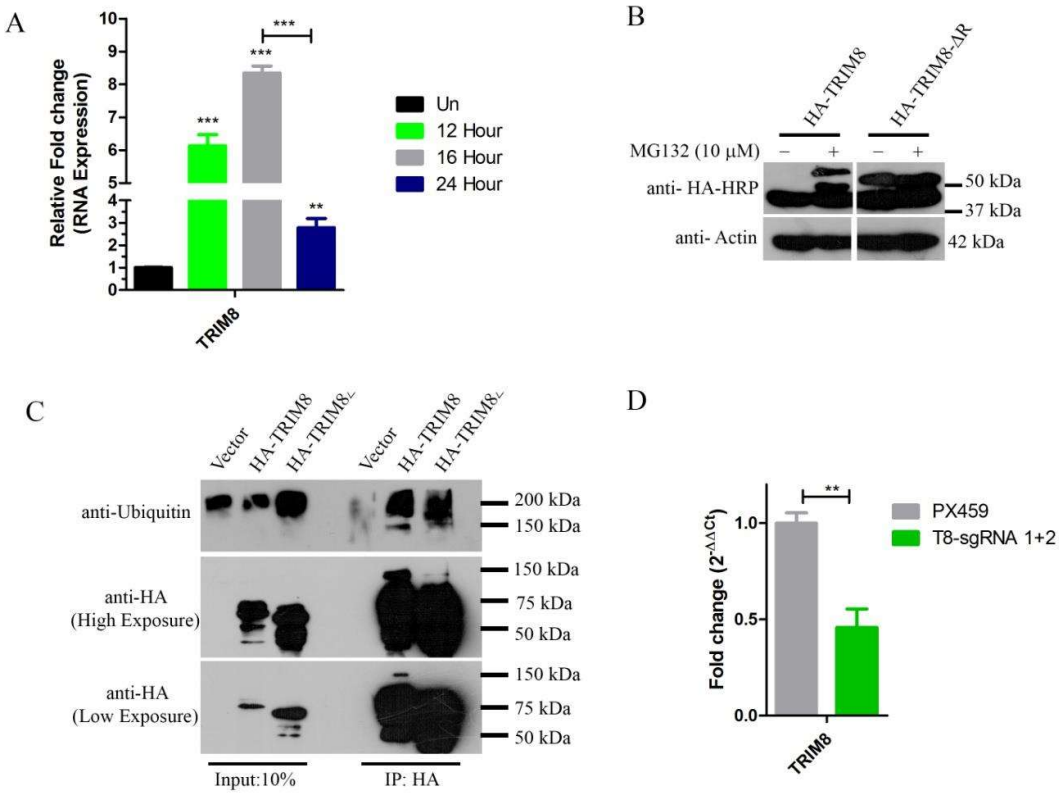

Figure 8.3 (II): Genotoxic stress enhances expression of TRIM8. *(A) HEK293 cells were treated with 50 μM etoposide for indicated time. cDNA was synthesized from isolated RNA and q-PCR analysis was performed to check expression of TRIM8 using specific primers. (B) HEK293 cells were transfected with HA-TRIM8 and HA-TRIM8ΔR. Cells were treated with or without MG132 as indicated. Western blotting was performed to check the expression of HA tagged proteins. (C) IP was performed to check the higher molecular weight conjugates on HA tagged TRIM8 and TRIM8ΔR. HEK293 cells were transfected with Vector, HA-TRIM8 and HA-TRIM8ΔR and treated*

with MG132 (10 µM) for 10 hours. HA-IP and western blotting was performed. (D) HEK293 cells were transfected with PX459 and TRIm8TRIM8-sgRNA 1&2. cDNA was synthesized from isolated RNA and q-PCR analysis was performed to check expression of TRIM8 using specific primers. Asterisk (**) & (***) indicates fold change statistically significant from untreated; p value <0.01, <0.001 respectively; SEM of minimum three independent experiments.

The observation was further confirmed by monitoring TRIM8's role in autophagy flux during genotoxic stress. Interestingly the LC3II levels increased in TRIM8 transfected cells co-treated with etoposide and NH_4Cl as compared to vector. Further suggesting enhanced autophagy flux in presence of TRIM8 during genotoxic stress (Figure 8.3 (I) D). These results confirmed that TRIM8 regulates autophagy and promotes autophagy flux during genotoxic stress.

8.4 TRIM8 promotes lysosomal biogenesis

Initiation of lysosomal biogenesis is observed during autophagy, which is regulated by master Transcription factor EB (TFEB) (Chua et al., 2014; Settembre et al., 2011). TRIM8 enhanced autophagic flux suggested its possible role in lysosomal biogenesis. Hence, the role of TRIM8 in lysosomal biogenesis was investigated. By using CRISPR/Cas-9 vector expressing guide RNA targeting TRIM8 (TRIM8-sgRNA), TRIM8 was knocked down in HeLa and MCF7 cells and the mRNA levels of structural lysosomal proteins; lysosomal protein transmembrane 4 alpha (LAPTM4A) and ATPase H+ transporting V0 subunit d1 (ATP6V0D1) was analyzed. LAPTM4A encodes for lysosomal resident protein involved in small molecule transport across endosomal and lysosomal membrane (Cabrita et al., 1999). ATP6V0D1 encoded protein is a component of vacuolar ATPase (V-ATPase) complex involved in acidification of eukaryotic intracellular organelles.

Interestingly the mRNA expression of LAPTM4A and ATP6V0D1 in TRIM8 sgRNA transfected HeLa and MCF7 was reduced (Figure 8.4 A & B). mRNA and protein expression of another lysosomal marker lysosomal associated membrane protein 1 (LAMP1) was checked to re-confirm the effect of TRIM8 on lysosomal biogenesis.

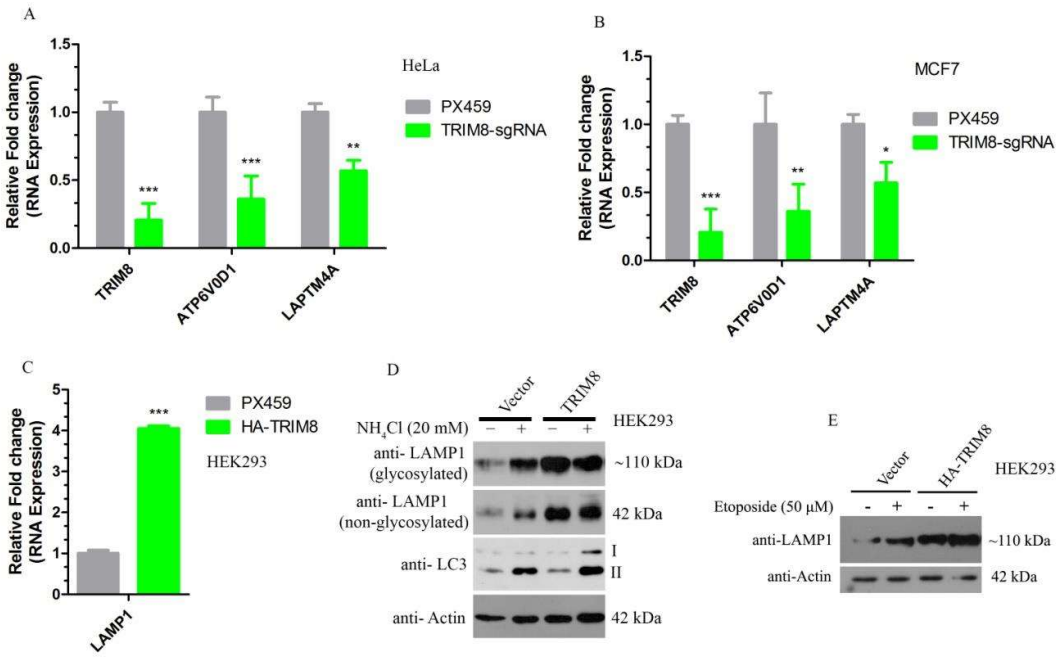

Figure 8.4: TRIM8 promotes lysosomal biogenesis. *(A) HeLa and (B) MCF7 cells were transfected with PX459 or TRIM8-sgRNA. cDNA was synthesized from RNA isolated from cells after 48 hours of transfection. Transcript levels of TRIM8, LAPTM4A and ATP6V0D1 were monitored by real time PCR using specific primers. (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 NH4Cl for 3 hours. 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 hours. Western blotting was performed to analyse 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.

Interestingly, LAMP1 mRNA levels were increased in TRIM8 transfected cells (Figure 8.4 C) and similar increase in 42 kDa band corresponding to non-glycosylated LAMP1 was observed in TRIM8 transfected cells compared to control transfected cells (Figure 8.4 D). The blot also showed increase in band around 110 kDa which is indicative of glycosylated LAMP1 (recruited to lysosomes) (Figure 8.4 D) in TRIM8 transfected cells.

Further TRIM8 regulated lysosomal biogenesis during genotoxic stress was analysed. TRIM8 or control vector was transfected in HEK293 cells and treated with etoposide. Western blotting was performed to check the levels of glycosylated LAMP1 which showed an increase in glycosylated LAMP1 levels in vector transfected etoposide treated cells. The level of LAMP1 further increased in etoposide treated TRIM8 transfected cells as compared to control (Figure 8.4 E). Together these results suggest that TRIM8 promotes lysosomal biogenesis which may result in enhanced autophagic flux.

8.5 TRIM8 protects cells from genotoxic stress induced cell death

Autophagy can help tolerate stress and promote cell survival during genotoxic stress (Polewska et al., 2013; Rodriguez-Rocha et al., 2011; Tang et al., 2015). Therefore, it was checked whether TRIM8 mediated autophagy affects genotoxic stress induced cell death. To investigate, control or TRIM8 transfected HEK293 cells were treated with etoposide and cell viability was analysed by MTT assay (Mosmann, 1983). It was observed that TRIM8 expression increased cellular viability of etoposide treated cells as compared to control transfected cells (Figure 8.5 A).

Conversely, TRIM8 was knocked down using shRNA in HeLa cells and cell viability was monitored. Reduced viability was observed in TRIM8 knockdown

etoposide treated cells as compared to control (Figure 8.5 B) further confirming that TRIM8 plays cytoprotective role during genotoxic stress. Etoposide activates apoptotic cascade including main executioner caspase-3 to induce cell death (Day et al., 2009; Jamil et al., 2015). Therefore, the effect of TRIM8 expression on caspase-3 activation was monitored. Interestingly, the levels of 17/19 kDa band corresponding to cleaved subunit of caspase-3 was found reduced in TRIM8 transfected, etoposide treated cell as compared to control (Figure 8.5 C).

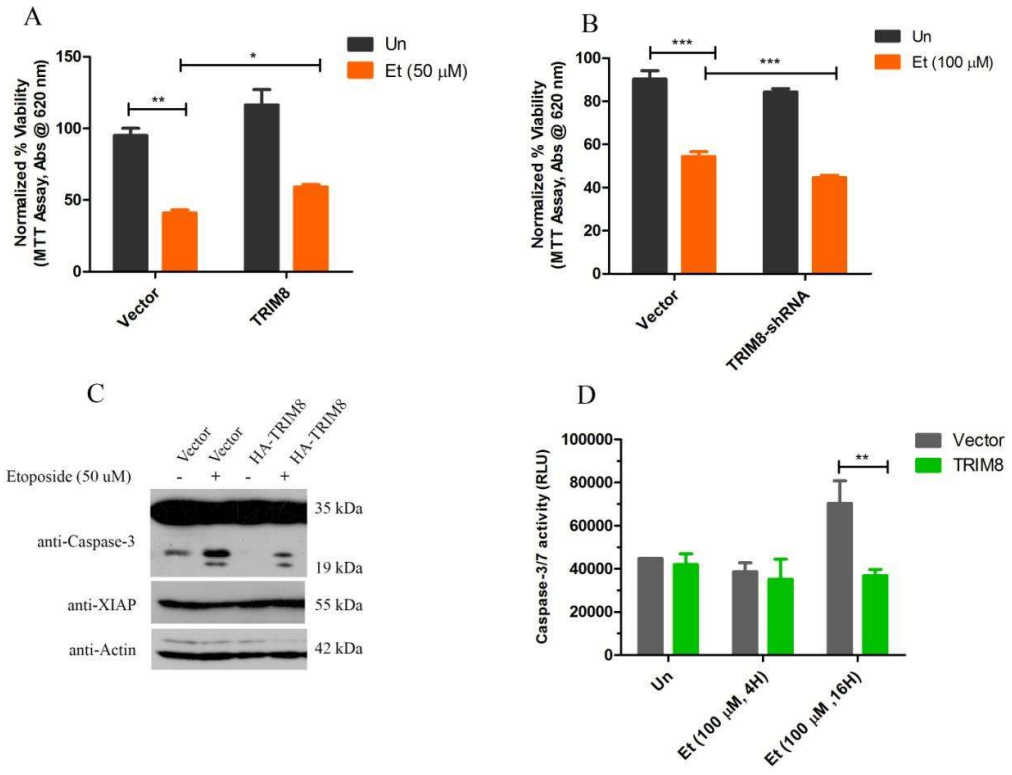

Figure 8.5: TRIM8 inhibits caspase-3 activity and protects from genotoxic stress induced cell death. *(A) HEK293 cells were transfected with vector or TRIM8. Cells were treated with etoposide for 24 hours. MTT assay was performed to check cellular viability. (B) TRIM8 was knocked down in HeLa cells by transfecting TRIM8-shRNA. Cells were treated with etoposide for 24 hours 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 hours. 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.

Since the cytoprotective effects of TRIM8 also corroborated with reduced caspase-3 cleavage, the caspase-3 enzymatic activity was also monitored by cleavage of luminogenic DVED substrate. The assay showed significantly increased caspase-3 activity in etoposide treated control transfected cells compared to untreated. Whereas the expression of TRIM8 significantly lowered caspase-3 activity in etoposide treated cells compared to control transfected cells (Figure 8.5 D). Together these results confirm that TRIM8 is cyto-protective during genotoxic stress induced cell death by inhibiting caspase-3 activation.

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

Autophagy can be cytoprotective during genotoxic stress induced cell death (Levine and Abrams, 2008; Qiang et al., 2013; Tasdemir et al., 2008). Therefore, the role of TRIM8 mediated autophagy in genotoxic stress induced cell death was further monitored. HEK293 cells were transfected with TRIM8 or control and treated with etoposide in presence or absence of wortmannin (inhibitor of phosphatidylinositol 3-kinase). Increased cell viability in etoposide treated cell

was observed in TRIM8 transfected cells compared to vector transfected cells (Figure 8.6 A). Interestingly, TRIM8 transfected etoposide-wortmannin co-treated cells as showed reduced viability compared to etoposide alone treated cells (Figure 8.6 A). Suggesting, autophagy inhibition negates TRIM8 induced cell viability during genotoxic stress.

It was re-confirmed by checking caspase-3 activation using western blotting. The level of 17/19 kDa band equivalent to cleaved subunit of caspase-3 reduced in TRIM8 transfected etoposide treated cells as compared to control however its level increased in TRIM8 transfected wortmannin-etoposide co-treated cells (Figure 8.6 B) suggesting that cleaved subunit is available for downstream action during autophagy inhibition. Hence it was hypothesized that TRIM8 mediated autophagy may regulate levels of cleaved subunit of caspase-3 through autophagy. This hypothesis was further confirmed by monitoring Caspase-3 levels in presence of autophagy induced rapamycin. The western blotting showed increased cleaved caspase-3 subunits in vector transfected rapamycin-etoposide co-treated cells compared to only etoposide treated cells (Figure 8.6 C). Whereas, TRIM8 expression reduced active caspase-3 levels in rapamycin-etoposide co-treated cells compared to vector transfected cells (Figure 8.6 C).

Further the effect of TRIM8 on active caspase-3 levels was reconfirmed by TRIM8 knockdown in HeLa cells. Western blotting showed increased active caspase-3 levels in etoposide treated TRIM8-sgRNA transfected cells (Figure 8.6 D). More increase in the bands representing active caspase-3 was observed in TRIM8-sgRNA transfected cells co-treated with etoposide and wortmannin. These results suggested that the active caspases-3 can be a substrate of autophagy. To further confirm the hypothesis that cleaved caspase-3 is degraded by TRIM8 mediated autophagy, western blotting was performed. Cells were transfected with control vector or TRIM8 and treated

with etoposide in presence of autophagy inhibitors (NH_4Cl and Bafilomycin A1) and UPS inhibitor (MG132).

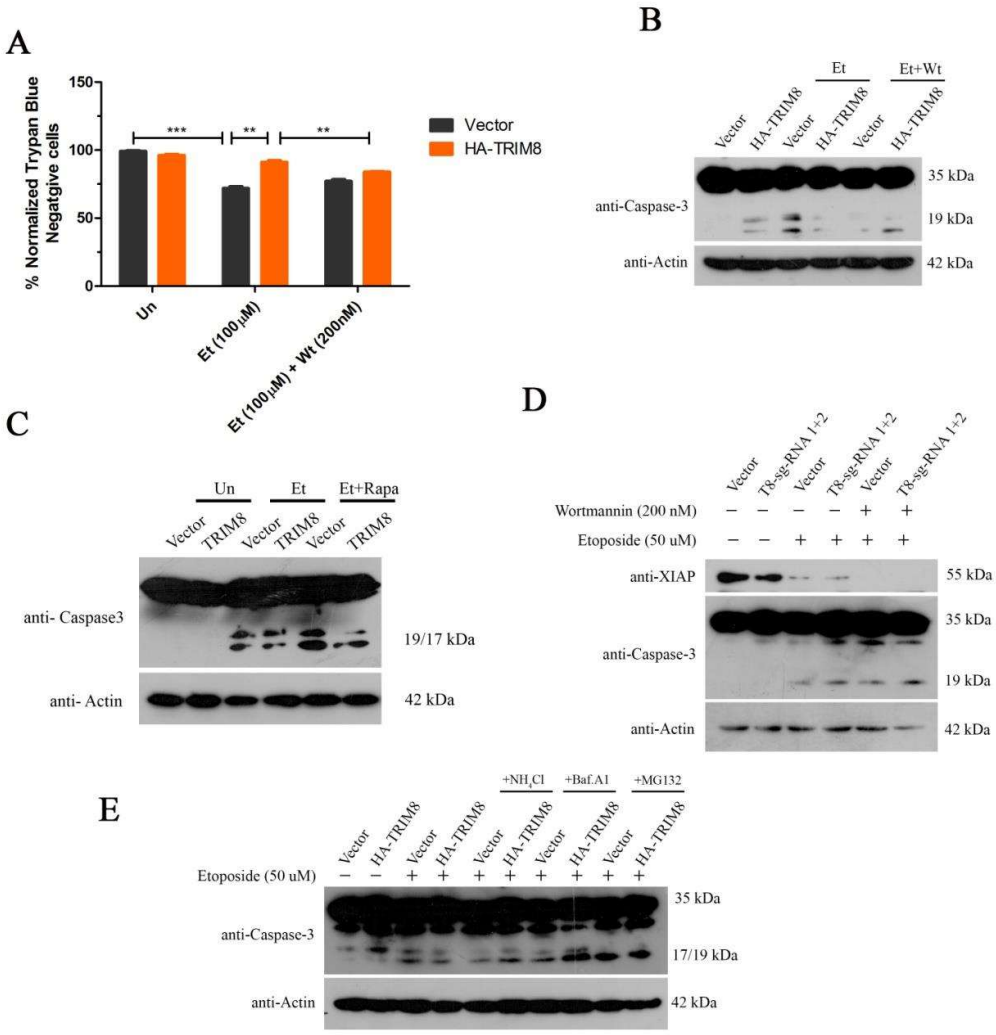

Figure 8.6: TRIM8 mediated autophagy is required for cell survival during genotoxic stress. *(A) HEK293 cells were transfected with Vector or TRIM8. Cells were treated with etoposide and wortmannin as indicated for 24 hours. Trypan blue dye exclusion assay was performed to check cell viability. Asterisk (**), (***) indicates normalized % trypan blue negative cells statistically significant from vector; p value <0.01 and <0.001 (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 hours 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 hours. 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 hours in presence of NH4Cl (20 mM, 4 hours), Bafilomycin A1 (25 nM, 8 hours) or MG132 (10 µM, 10 hours). Cleaved caspase-3 levels were monitored by western blotting using caspase-3 specific antibody.

Interestingly, the level of 17/19 kDa band corresponding to cleaved/active caspase-3 subunits were increased in TRIM8 transfected cells co-treated with autophagy inhibitors NH_4Cl and Bafilomycin A1 whereas MG132 co-treatment did not affect the cleaved caspase-3 levels (Figure 8.6 E). These results confirmed that TRIM8 mediated autophagy promotes degradation of cleaved subunit of caspase-3 and results in cytoprotection.

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

E3 ligases Inhibitor of apoptosis proteins (IAPs) are known to regulate apoptotic pathways during genotoxic stress, and XIAP known to directly bind and inhibit caspase-3 activation (Deveraux et al., 1997; Silke and Meier, 2013).

X-Linked Inhibitor of Apoptosis (XIAP) has also been identified as an autophagy regulator. XIAP activated NF-κB signalling enhances Beclin-1 expression and in turn activate autophagy (Lin et al., 2015; Lu et al., 2007). Therefore, the effect of TRIM8 on XIAP mediated regulation of genotoxic stress induced cell death was investigated. HEK293 cells were transfected with TRIM8 and XIAP levels were monitored treated after etoposide treatment. It was observed that the level of 53 kDa band representing XIAP decreased in etoposide treated cells compared to untreated, suggesting destabilization of XIAP; corresponding increase in cleaved caspase-3 subunits was also observed (Figure 8.7 A). The blot also showed increased XIAP levels in TRIM8 transfected cells as compared to vector transfected cells treated with etoposide. Suggesting TRIM8 mediated XIAP stabilization during genotoxic stress conditions (Figure 8.7 A). Moreover, corresponding reduction in cleaved caspase-3 subunits was observed in TRIM8 transfected cells treated with etoposide as compared to control (Figure 8.7 A). Also, the knockdown of TRIM8 in HeLa cells showed reduced XIAP levels in untreated cells (Figure 8.6 D). Furthermore, XIAP the stabilization and caspase-3 cleavage was monitored temporally in presence of etoposide. Surprisingly, there was no difference in the level of 17/19 kDa band representing cleaved caspase-3 subunits at 8 and 16 hours etoposide treated cells, however its levels reduced at 24 hours (Figure 8.7 B) in TRIM8 transfected cells compared to control transfected cells. On the other hand endogenous XIAP levels were more in TRIM8 transfected cells at 16 hours etoposide treated cells compared to control treated cells (Figure 8.7 B). These observations suggested that XIAP stabilization precedes reduced caspase-3 activation in TRIM8 transfected cells during genotoxic stress.

XIAP directly interact with caspase-3 via linker/BIR2 domain to inhibit its activity (Bratton et al., 2001; Riedl et al., 2001; Takahashi et al., 1998). Since

TRIM8 affected both XIAP and caspase-3 it was checked whether TRIM8 forms a part of XIAP and Caspase-3 complex. HEK293 cells were transfected with vector or TRIM8 and treated with or without etoposide in presence of MG132 and NH_4Cl. Pull down of TRIM8 using HA affinity beads followed by western blotting showed a 53 kDa band corresponding to XIAP in HA-TRIM8 pull down (Figure 8.7 C).

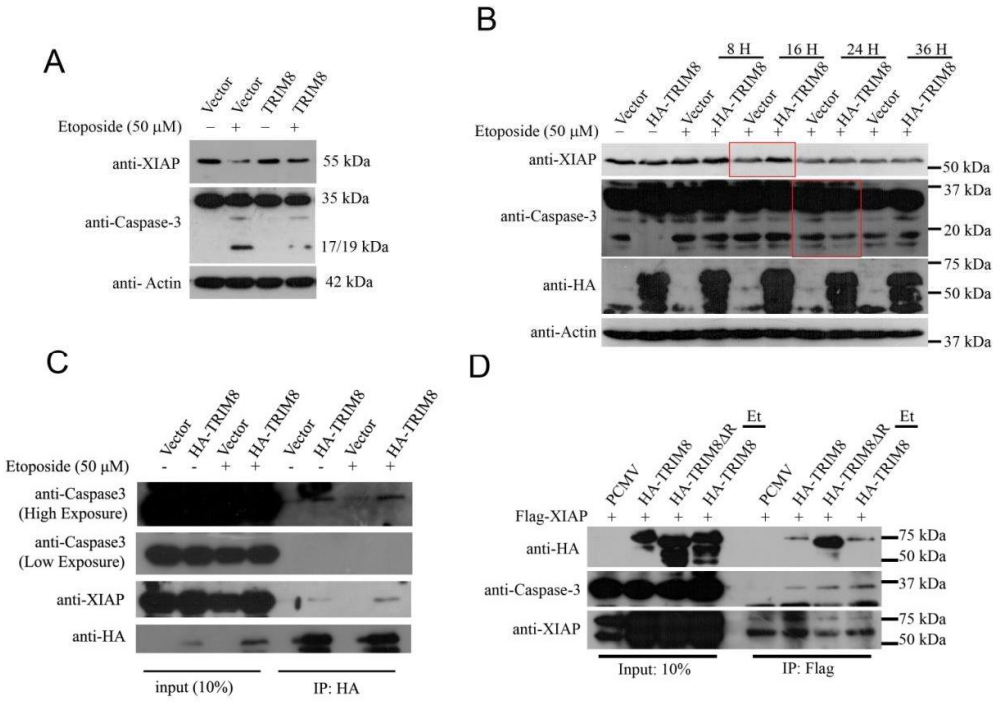

Figure 8.7: TRIM8 interacts with XIAP and caspase-3 and stabilizes XIAP. *(A) HEK293 cells were transfected with Vector or TRIM8 and treated with etoposide for 24 hours. Western blotting was performed to check the levels of XIAP and cleaved caspase-3 by using specific antibodies. (B) HEK293 was transfected 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 hours in presence of MG132 (10 μM, 10 hours) and*

wortmannin (20 mM, 4 hours). 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 hours) in presence and absence of etoposide (50μM, 10 hours). FLAG immunoprecipitation and western blotting was performed. Indicated antibodies were used to detect interaction.

Further increase level of XIAP in pulldown was observed in etoposide treated cells as compared to untreated cells. Since XIAP interacts with caspase-3 and inhibits its activation, therefore the blot was checked for caspase-3. Interestingly, the 35 kDa band corresponding to pro-caspase-3 was observed in HA-TRIM8 pull down. This result indicates that TRIM8, XIAP and pro-caspase-3 may form a complex during genotoxic stress (Figure 8.7 C). Further, the interaction was reconfirmed by reverse-IP using Flag-XIAP pull down. FLAG-XIAP was co-transfected with either vector, HA-TRIM8 or HA-TRIM8ΔR, cells were treated with etoposide and FLAG-IP was performed. Both the full length and RING domain deleted TRIM8 bands were observed in the pull down of FLAG-XIAP (Figure 8.7 D). This reconfirmed the interaction of TRIM8-XIAP and also showed that RING domain of TRIM8 is dispensable for interaction with XIAP (Figure 8.7 D). Moreover, pro-caspase-3 also appeared in was observed in FLAG-XIAP pull down lanes containing TRIM8 or TRIM8ΔR. In Similar increase in pro-caspase-3 band was also observed in etoposide treated cells compared to its control pull down lanes (Figure 8.7 D). These observations confirm that TRIM8 is a part of XIAP-caspase-3 complex during genotoxic stress and thus may prevent caspase-3 activation in presence of etoposide.

8.8 Discussion:

Autophagy is emerging as a regulator of genotoxic stress and determinant of cell fate (Alexander et al., 2010; Eliopoulos et al., 2016; Wang et al., 2016). Regulators of cell death-autophagy crosstalk and their role in cell fate determination is poorly understood. The present study identifies TRIM8 as a novel regulator of DNA damage response which plays a cytoprotective role by promoting autophagy flux through lysosomal biogenesis.

TRIM proteins are sub class of RING family of E3 Ligases. They can form unique subcellular structures and dynamically localize to different subcellular compartments during stress conditions and infections (Mandell et al., 2014; Reymond et al., 2001; Tomar et al., 2015; Tomar et al., 2013a; Tomar and Singh, 2014; Tomar et al., 2012a). TRIMs homo- and heterodimerization is attributed to its coiled coil domain, whereas their E3 Ligase activity lies predominantly within the RING domain. They can regulate turnover of their substrate through conventional UPS or by autophagy pathway (Kozakova et al., 2015; Mandell et al., 2014; Marin, 2012; Napolitano and Meroni, 2012). Previous reports have confirmed that TRIMs may form higher molecular weight complexes and self ubiquitinate to regulate their own turnover. The evidences in this study suggest that TRIM8 has very high turnover which is regulated both by UPS and autophagy pathway. More interestingly it is stabilized during genotoxic stress, indicative of its role regulation of DDR. Previously, it has been observed that TRIM8 expression is upregulated by UV radiation in p53 dependent manners which in turn stabilizes p53 and inhibit cell proliferation (Caratozzolo et al., 2012; Caratozzolo et al., 2014). The present study shows argues that its expression is increased in cells under genotoxic stress independent of cellular p53 status. Moreover, stress induced stabilization further suggest an additional layer of control over cellular

functions of TRIM8 which requires elaborate exploration. Also its implication in different pathophysiological conditions needs to be further studied.

Activation of NF-κB during DNA damage promotes TNF-α production which acts as a feedforward loop for caspase-8 induced cell death induction under severe or persistent DNA damage (Biton and Ashkenazi, 2011). More evidences are emerging that suggest that NF-κB activation regulates autophagy which plays essential role in DDR (Copetti et al., 2009; Eliopoulos et al., 2016; Ye et al., 2011). The present study identifies that genotoxic stress stabilized TRIM8 potentiates autophagy as TRIM8 expressing cells show increased number of autophagy puncta, LC3-II levels. Moreover autophagy is induced by TRIM8 was observed in cells having functional p53 (MCF-7 and MX1) and non-functional p53 (HEK-293 and HeLa). Confirming TRIM8 mediated autophagy is independent of p53 regulated mechanisms. Further, TRIM8 activates transcription of lysosomal genes to promote lysosomal biogenesis and consequently enhances autophagy flux. This could 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 supported by observation confirming autophagy mediated clearance\degradation of nuclear DNA during DNA damage conditions (Lan et al., 2014). Moreover, accumulation of damaged DNA has been observed in autophagy deficient cells, whereas autophagy competent cells can repair DNA damage and helps in survival (Lan et al., 2014; Pankiv et al., 2007; Qiang et al., 2013; Wang et al., 2016). These observations require further investigation during genotoxic stress conditions to better understand the autophagy-DDR nexus. Interestingly, TRIM8 transcriptionally promotes expression of p62 which has pleiotropic functions including selection of autophagic cargo, inflammation restriction by promoting mitophagy (Zhong et al., 2016) and

DNA damage associates inflammation and senescence (Kang et al., 2015), hence oncogenesis. These evidences highlight the complex relationship of TRIM8 mediated regulation of p62 and possible role beyond autophagy and implication in tumorigenesis.

This also provides evidences of autophagy-DDR that regulates cell death during genotoxic stress condition. TRIM8 expression enhanced cellular viability, whereas its knock down sensitized the cells to death during genotoxic stress induced by etoposide. Autophagy has been shown to regulate Caspase-8 turnover in normal conditions (Hou et al., 2010; Tomar et al., 2013a). During persistent genotoxic stress, Caspase-8 is activated leading to cell death. In this study it was consistently observed that expression of TRIM8 does not affect Casapse-8 activation or turnover during genotoxic stress however, regulates the activation of critical executioner caspase-3. TRIM8 transfected cells show reduced level of cleaved caspase-3 subunit suggesting that TRIM8 mediated autophagy may promote degradation of active caspase-3. Additionally, the rescue o cleaved subunit of caspase-3 levels in presence of inhibitors of autophagy (NH_4Cl and Bafilomycin A1) and its decrease in autophagy inducer rapamycin further supported this hypothesis.

XIAP is a major regulator of cell death and autophagy (Lin et al., 2015). It inhibits the proteolytic activity of caspase-3, -7 and -9 by directly binding to these proteins (Riedl et al., 2001; Scott et al., 2005). Hence, under stress conditions cellular XIAP levels may determine cell fate. The current study showed that TRIM8 knock down reduced the XIAP levels, whereas its enhanced expression stabilizes XIAP during genotoxic stress conditions. XIAP is also and activator of NF-κB. It acts via BIR (Baculovirus Inhibitor of apoptosis protein Repeat) domain mediated dimerization and binding to TGF-beta activated kinase 1 (MAP3K7) binding protein 1 (TAB1) (Lu et al., 2007). XIAP mediated NF-κB activation also induced expression of genes involved in

autophagy like Beclin-1(Lin et al., 2015). 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 XIAP stabilization leading to formation of multiprotein complexes sequestering caspase-3 and preventing its cleavage and activation. Therefore TRIM8 expression under genotoxic stress and radiation may provide cancer cells an additional survival advantage and possibly resistance. Further validation of this hypothesis in various in-vivo cancer models is required.

In summary the current study describes TRIM8, as a novel regulator of DDR-autophagy cross-talk with a cytoprotective role during genotoxic stress. The evidences here further support previous report from our lab suggesting TRIM8 as potential oncogene (Tomar et al., 2012b). 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 (Caratozzolo et al., 2012). The loss of heterozygosity is observed in glioblastoma (Micale et al., 2015; Vincent et al., 2000) however TRIM8 level is maintained through STAT3 suggesting its oncogenic functions (Zhang et al., 2017a). Similarly in another report TRIM8 had been shown to enhance STAT3 signaling and promote src mediated anchorage independent growth, characteristic of tumor cells (Okumura et al., 2010). 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.