Chapter 4:

Materials and Methods

4.1 Materials

4.1.1 Cell lines:

HEK293, MCF-7, and HeLa cell lines were obtained from ATCC (Manassas, USA). HEK293 and HeLa were grown in Dulbecco's modified Eagle's media (DMEM) (Gibco, Thermo Fisher Scientific, USA). MCF-7 cells were grown in Minimal Essential Media (MEM, HyClone, GE, USA). Both media were supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, USA), 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Gibco, Thermo Fisher Scientific, USA). Cells were incubated at 37 °C, 5% CO2 in specific media.

4.1.2 Chemicals and reagents:

Etoposide, Puromycin, Cycloheximide, Sodium Azide, Digitonin, Cytochrome c, NADH, Sodium Succinate, Sodium Dithionite, H2O2, Oligomycin, EBSS, Sodium Pyruvate, Rapamycin, Ammonium Chloride, Rotenone, Wortmannin, MG132, EZviewTM Red Anti-Flag M2 Affinity Gel, EZviewTM Red Anti-HA Affinity Gel and Protease inhibitor cocktail were purchased from Sigma-Aldrich, USA. Recombinant Human TNF-α was purchased from Milteny Biotech, Germany and PeproTech, USA. G418, Opti-MEM, Lipofectamine 2000, Lipofectamine 3000, MicroAmp Fast Optical 96-Well Reaction Plate and MicroAmp Optical Adhesive Film were purchased from Thermo Fisher Scientific Inc, USA. RNAiso Plus (total RNA extraction reagent), TB Green Premix Ex Taq II (Tli RNase H Plus) (SYBR Green) and PrimeScript cDNA synthesis kits were purchased from Takara Bio Inc, Japan. T4 Polynucleotide Kinase, T4 DNA Ligase, and BbsI-HF® were purchased from New England Biolabs, USA. Caspase-Glo® 3/7 Assay System and Dual Glow Luciferase Reporter Kits were purchased from Promega, USA. 3-(4,5-dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT reagent) was purchased from MP

Biomedicals, USA. DMSO and diaminobenzidine (DAB) were obtained from Sisco Research Laboratories (SRL), India. Immun-Blot® PVDF Membrane, Protein Assay Dye Reagent Concentrate and Clarity Western ECL Substrate were purchased from Bio-Rad Laboratories, USA. TaqMan probes specific for TRIM genes were procured from Applied Biosystems, Inc., USA and all other oligos were synthesized from Integrated DNA Technologies (IDT), USA. siRNA targeting TRIMs and Control-siRNA were purchased from Qiagen, Germany.

4.1.3 Generation of stable cell lines:

HEK293-mCherry-LAMP1 and HEK293-GFP-LC3 stable cell lines were generated as previously described (Tomar et al., 2012a). Initially, cells were seeded at a density of $4.5X10^5$ in a 6-well plate and transfected with mentioned constructs using standard $CaPO_4$ transfection method. After 24 hours of transfection, the media was substituted with G418 (500 µg/ml) containing DMEM. Cells were incubated in G418-containing media and media was changed every 24 hours until stable clones were visible. Later, using serial dilution method single clones were obtained from harvested stable colonies in a 96-well plate. The single clones were then transferred and 24-well plates and expanded. After expansion for 7 days, the cells were transferred to a 25 cm^2 culture flask and maintained in DMEM supplemented with 250 µg/ml of G418.

4.1.4 Constructs:

CRISPR/Cas9 knockout constructs of TRIM1, TRIM8 and RelA gene were generated by following protocol described by Ran et al., 2013. Detailed cloning protocol is described in the Methods section. Oligonucleotide sequences and primers for confirmation of clones are mentioned in **Table 4.3**. The details of all other constructs used in the current study and their sources are mentioned in **Table 4.1**.

Table 4.1: Details of constructs used in the study.

Sr. No.	Construct name	Source
1	HA-TRIM8 and HA-TRIM8ΔR	Gift from Dr. S Hatakeyama (Department of Biochemistry, Hokkaido University Graduate School of Medicine)
2	TRIM8-shRNA 1 & 2	Gift from Dr. Edurne Berra Ramírez (Gene Silencing Platform, CICbioGUNE, Derio, Spain)
3	HA-TRAF2	Gift from Dr. S M Srinivasula (IISER, Thiruvananthapuram, Kerala, India)
4	TRIM1, TRIM1-GFP, TRIM2, TRIM8, TRIM15, TRIM15-YFP, TRIM16	Gift from Dr. Walther H. Mothes (Section of microbial Pathogenesis, Yale School of Medicine, USA)
5	YFP tagged TRIM15 deletion domains and mutant constructs	Gift from Dr. Pradeep Uchil (Yale School of Medicine, USA)
6	Flag-TAK1	Dr. Yan-Yi Wang (Wuhan Institute Of Virology, Chinese Academy Of Sciences)
7	Flag tagged TRIM1 and deletion domain constructs	Gift from Dr. Paul Young (University College Cork, Ireland)
8	pCMV-tag4A	purchased from Stratagene, USA
9	pDsRed2-mito Vector (mtRFP), pAcGFP-N1	purchased from Clontech, Takara-Bio, Japan
10	pSpCas9(BB)-2A-Puro (PX459) V2.0	Gift from Dr. Feng Zhang (Addgene plasmid # 62988)
11	TRIM8-sgRNA1 & 2, TRIM1-sgRNA, RelA-sgRNA	In-house, guide RNA cloned into pSpCas9(BB)-2A-Puro (PX459) V2.0 vector
12	GFP-LC3	Gift from Dr. T. Yoshimori (National Institute of Genetics, Shizuoka, Japan)

13	mCherry-GFP-LC3 and mCherryGFP-p62	Gift from Dr. Terje Johansen (Dept. of Biochemistry, Institute of Medical Biology, University of Tromsø)
14	mCherry-LAMP1	Gift from Dr. J. Lippincott-Schwartz, HHMI, Virginia, USA
15	HA tagged Ub and lysine mutant constructs	Gift from Dr. Kunitada Shimotohno, Chiba Institute of Technology, Chiba, Japan

4.1.5 Antibodies:

The details of the antibodies used in the study and its source are given in **Table 4.2**.

Table 4.2: Details of antibodies used in the study.

Sr. No.	Antibody	Source	Dilutions
1	anti-TRIM15	St John's Laboratory, UK	1:2500
2	anti-β-Actin	Santa Cruz Biotechnology, Inc. USA	1:5000
3	anti-UQCRC2	Santa Cruz Biotechnology, Inc. USA	1:5000
4	anti-PARP	Cell Signaling Technology, USA	1:2500
5	anti-Caspase-3	Cell Signaling Technology, USA	1:1000
6	anti-XIAP	Cell Signaling Technology, USA	1:1000
7	anti-Flag M2 peroxidase (HRP)	Sigma-Aldrich, USA	1:1000
8	anti-HA-peroxidase (HRP)	Sigma-Aldrich, USA	1:1000
9	Secondary antibodies HRP conjugated anti-	Jackson ImmunoResearch Lab, Inc, USA	1:5000-1:20000

	rabbit and anti-mouse		
10	anti-LAMP1	Cell Signaling Technology, USA	1:2500
11	anti-p62	Cell Signaling Technology, USA	1:2500
12	anti-NDP52	Cell Signaling Technology, USA	1:2500
13	anti-LC3	Sigma-Aldrich, USA	1:10000
14	anti-Lamin A/C	Abcam, USA	1:5000

4.2 Methodology

4.2.1 Guide RNA cloning and validation of clones:

Guide RNA cloning was performed with minor modifications using protocol described earlier (Ran et al., 2013). PX459 V2.0 vector was digested overnight using BbsI-HF enzyme and dephosophorylated using Alkaline Phosphatase. Guide RNA upper strand (US) and lower strand (LS) were phosphorylated and annealed in T4 Polynucleotide Kinase enzyme and buffer using manufacturer's protocol. Phosphorylate guide RNA oligos and digested vectors were ligated using T4 DNA ligase (NEB) by following manufacturer's protocol. Ligated construct were transformed using Stbl3 competent cells and selected on amplicillin plates. List of guide RNA US and LS are provided in **Table 4.3**.

Table 4.3: Guide RNA sequence used for cloning and clone verification.

Sr. No	Name	Description	Sequence 5'' to 3'
1	TRIM8-sgRNA1-US	For TRIM8-sgRNA1 clone	CACCGTATCTGCCTGCACGTTTTCG
2	TRIM8-sgRNA1-LS		AAACCGAAAACGTGCAGGCAGATAC
3	TRIM8-sgRNA2-US	For TRIM8-sgRNA2 clone	CACCGCTGGCTCCACGAAAACGTGC
4	TRIM8-sgRNA2-LS		AAACGCACGTTTTCGTGGAGCCAGC
5	TRIM1-sgRNA –US	For TRIM1-sgRNA clone	CACCGGTTATCTCGCTGAACCACCG
6	TRIM1-sgRNA –LS		AAACCGGTGGTTCAGCGAGATAACC

7	RelA-sgRNA-US	For RelA-sgRNA clone	CACCGTCAATGGCTACACAGGACCA
8	RelA-sgRNA-LS		AAACTGGTCCTGTGTAGCCATTGAC

4.2.2 Screening of positive guide RNA clones:

Obtained colonies were grown in 1 ml LB media and colony PCR was performed using U6 forward primer and LS strand of respective guide RNA for detection of positive clones. Positive clones were amplified and further validated by sanger sequencing.

4.2.3 Transfection of cell lines:

HEK293 cells were transfected using the standard calcium phosphate transfection method (Kingston et al., 2003). MCF-7 and HeLa cells were transfected using Lipofectamine 3000 reagent following the manufacturer's protocol (Thermo Fisher Scientific Inc, USA). MCF-7 and HeLa cells were grown in reduced serum medium for transfection and complete media was supplemented to cells after 24 hours of transfection. The co-transfection of siRNA and plasmid DNA constructs were done by Lipofectamine 2000 reagent using the manufacturer's protocol.

4.2.4 Cell survival and cell death assays:

4.2.4.1 Trypan blue dye exclusion assay

To monitor cell death trypan blue dye exclusion assay were performed. HEK293 cells were plated at a density of 1.5 x10^5 cells/well in 24-well plate and transfected with indicated constructs. After 24 hours of transfection, cells were treated with indicated reagents for mentioned time. After treatment cells were collected and stained with trypan blue dye. Minimum of 100 cells per view were counted and the percentage of trypan blue negative cells was plotted as percentage cell survival.

4.2.4.2 Cellular proliferation assay using MTT

MTT assay was performed to check cell proliferation. HEK293 cells were plated at the density of 10000 cells/per well in 96-well plate and transfected with indicated constructs. After 24 hours of transfection, cells were treated with indicated reagents. After treatment, MTT (0.1 mg/ml) reagent was added in each well and incubated for 1.5 hours at 37 °C. Media was replaced by DMSO (MTT solubilization agent) (SRL, India) to dissolve the precipitate of purple-colored formazan crystals. Absorbance was measured using a colorimetric microplate reader (BioTek Instruments, Inc. USA) at 595 nm wavelength.

4.2.5 Caspase-3/7 luciferase assay:

The activation of Caspase-3/7 was monitored using Caspase-Glo® 3/7 Assay Systems (Promega, USA) according to manufacturer instructions as described previously (Tomar et al., 2013). Indicated constructs were transfected in white bottom 96 well plate using Lipofectamine® 2000 transfection reagent by forward transfection method. 20,000 cells per well were plated into 96 well plate and DNA: transfection reagent mixture was dispensed. After 24 h of transfection, cells were treated with etoposide (100 μM) for the indicated time. The caspase-3/7 substrate was added and incubated for 1 hour at room temperature. Luminescence was measured post incubation using a luminometer (Berthold Technologies, Germany).

4.2.6 NF-κB luciferase reporter assay:

Dual-Glo luciferase assay system was used for detecting NF-κB activity (Promega, USA) as described previously (Tomar et al., 2012b). HEK293 cells were plated at a density of 2.5X105 cells per well in 12 well plates. siRNA targeting specific TRIMs or indicated vectors were co-transfected with NF-κB firefly and Renilla luciferase reporter constructs (ratio 9:1). siRNA and vector co-transfection was done using Lipofectamine 2000 following manufacturer's

protocol. Transfected cells were treated with TNF-α or indicated reagent for 10 hours or indicated time points and Firefly/Renilla activity was measured by following the manufacturer's protocol using BioTek Synergy HTX multimode plate reader. The Firefly/Renilla ratio was plotted to show the activation of NF-κB pathway.

4.2.7 Fluorescence microscopy:

4.2.7.1 Detection and quantitation of TRIM15

HEK293 cells were seeded at a density of $1.5X10^5$ cells on coverslips in 24 well plates. Cells were transfected with TRIM15-YFP or TRIM15-RFP. After 24 hours of transfection, cells were treated with indicated chemicals and fixed with 4% Paraformaldehyde (PFA). Coverslips were removed from 24 well plates and slides were prepared using SlowFade™ Gold Antifade Mountant (Thermo Scientific, USA). Images were acquired using Nikon Eclipse Ti2 Inverted Microscope and processes using NIS-Elements-AR (Advanced Research) software (Nikon, Japan). Automated image analysis was also performed using NIS-Elements-AR software. For measurement of mean fluorescent intensity (MFI) and binary area "Field measurement" feature of the "Automated image analysis" tool was used. Acquisition and measurement parameters for YFP and RFP images were kept the same for all the images.

4.2.7.2 Autophagy and autophagic flux measurement using GFP-LC3, mCherry-GFP-LC3 and mCherry-GFP-p62constructs

Autophagy and flux measurement was done using IX83 fluorescent microscope (Olympus, Japan) and analyzed by cellSens Imaging Software (Version 1.12, Olympus, Japan). Autophagy was checked by monitoring GFP-LC3 puncta formation, whereas autophagy flux was checked using tandem mCherry-GFP constructs of LC3 or p62 as previously described (Tomar et al., 2012a). HEK293-GFP-LC3 cells were plated at density of $1.5X10^5$ cells per well

and transfected with indicated constructs. After 24 hours of transfection GFP fluorescence was monitored using a fluorescence microscope for autophagic puncta formation. Numbers of puncta per cell were counted in a minimum of 100 cells and the graph was plotted for the average number of LC3 puncta per cell.

Similarly, HEK293 cells were co-transfected with tandem mCherry-GFP constructs of LC3 or p62 and indicated construct to determine autophagy flux. After 24 h of transfection GFP and RFP puncta were monitored using a fluorescence microscope. The numbers of GFP/RFP (orange/yellow) and only RFP puncta representing autophagosome and autophagolysosomes respectively were counted in a minimum of 100 cells and graph plotted for the average number of LC3 and p62 puncta per cell.

4.2.8 Confocal microscopy

4.2.8.1 Cellular localization and dynamics of TRIM15

HEK293 cells were seeded at a density of $1.5X10^5$ cells on coverslips in 24 well plates, and transfected with TRIM15-YFP. Cells were treated with indicated reagents and fixed with 4% Paraformaldehyde (PFA). Coverslips were removed for slide preparation and SlowFade™ Gold Antifade Mountant (Thermo Scientific, USA) was used for staining the nucleus. Images were acquired using Nikon Confocal Microscope A1R HD25 (Nikon, Japan).

The "object count" feature of the "Automated image analysis" tool was used for counting the YFP puncta of TRIM15. For each condition at least 6 frames of images (> 100 cells per frame) were analyzed. The output of object counts from the automated image analysis was further processed using Microsoft Excel. TRIM15-YFP puncta were sorted based on their size (<2 µ, 2-10 µ and >10 µ) and % populations for each size range puncta were plotted.

4.2.8.2 Subcellular localization of MID2/TRIM1

The cellular localization of MID2/TRIM1 was monitored using confocal microscopy. Briefly, HEK293 were seeded at a density of $1.5X10^5$ cells on coverslips in 24 well plates. TRIM1-GFP was transfected alone or co-transfected with mCherryp62, mCherryLAMP1 or mtRFP. After 24 hours of transfection, cells were treated with indicated reagent and fixed with 4% Paraformaldehyde (PFA). Coverslips were removed from 24 well plates and slides were prepared using glycerol mounting medium. GFP images were acquired with an inverted Leica TCS SP8 confocal microscope system (Leica Microsystems GmbH, Germany).

4.2.9 Quantitative analysis of gene expression:

4.2.9.1 Gene expression analysis of TRIM family proteins using TaqMan probes

HEK293 cells were treated with TNF-α (10 ng/ml) for 10 hours and collected in RNA ISO plus reagent (Takara, Japan). Total RNA was isolated using RNAiso Plus Reagent and was reverse transcribed to synthesize cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. The expression of TRIM proteins was analyzed by quantitative Real-Time PCR using TaqMan probes specific for the indicated TRIM gene (Applied Biosystems, Inc., USA). Data were processed using DataAssist v3.01 (Applied Biosystems, Inc., USA). 18S rRNA was used as endogenous control and fold change values ($2^{-\Delta\Delta ct}$) were plotted.

4.2.9.2 Gene expression using SYBR Green chemistry

To analyze quantitative gene expression RNA isolation and cDNA synthesis were performed as described above. mRNA expression of indicated genes was confirmed using real-time PCR by SYBR Premix Ex Taq II (Tli RNase H Plus)

(Takara, Japan) as per the manufacturer's instruction. β-Actin and GAPDH genes were used as multiple endogenous control and expression of indicated genes were calculated using QuantStudio 3 and 5 system's Design and Analysis Software v1.5.1. Fold change values ($2^{-\Delta\Delta ct}$) of a minimum of three independent biological replicates were plotted.

The details of the primers used in the study are listed in **Table 4.4**.

Table 4.4: List of primers used in the study.

Gene	Forward Primer (5′ to 3′)	Reverse Primer (5′ to 3′)
TRAF2	GCTCATGCTGACCGAATGTC	GCCGTCACAAGTTAAGGGGAA
RELA	ATGTGGAGATCATTGAGCAGC	CCTGGTCCTGTGTAGCCATT
TRIM8	GAATGAAATCCGGAAGATGC	CTTCAGTTGGTTCACTTTCTC
p62/SQSTM1	AATCAGCTTCTGGTCCATCG	TTCTTTTCCCTCCGTGCTC
LAMP1	GCTCTTCCAGTTCGGGAT	TAGGAATTGCCGACTGTG
LAPTM4A	GCCTGTGTTCTTTTTGCCGT	GGCAACTGAGGACGAAGTCA
ATP6V0D1	TCTGGTGATGACTTGAAACTGC	GTCTAGGAAGCTGGCGAGTG
MID2/TRIM1	GAGGCCTGTATAATTCAGTAG	GTTTGTTTTTAGTCGGGTAGG
TRIM2	AAGCAGAAAGCTGTGAAAAG	CCCACTCGAAAGATCAAATC
TRIM15	AGACTTTTGTGAGTCCTGAG	CCCTGAATCTATTTCCAGATG
TRIM16	CAAAGTTATCACGGAATCCAC	TCCTCTTCCTTGGAAAACTC
ACTB	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATG
GAPDH	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG
U6	GAGGGCCTATTTCCCATGATTC	

4.2.10 Nuclear-cytoplasmic fractionation:

The nuclear and cytosolic fractions were prepared as described previously with minor modifications (Tomar et al., 2012b). HEK293 cells were plated at a density of $1X10^6$ in a 60-mm2 dish and transfected with indicated vectors. After 24 hours of transfection, the cells were treated with indicated chemicals. Cells were washed with DPBS (Hyclone, GE, USA) and resuspended in 300 µl of

buffer-A (10 mM HEPES buffer, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 0.4% (v/v) NP40, 0.5 mM dithiothreitol (DTT), and 1X protease inhibitor cocktail (Roche, Germany) and incubated on ice for 30 min and lysed. Lysates were centrifuged at 15,000g for 15 min at 4 °C and supernatant was collected as the cytosolic fraction. Pellets were washed thrice with buffer-A and resuspended in 70 µl of ice-cold buffer-B (20 mM HEPES buffer, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT and 1X protease inhibitor cocktail). Resuspended pellet was subjected to high-speed vortex twice at an interval of 30 min. The nuclear lysate was centrifuged at 15000g for 15 min at 4 °C and supernatant was collected as the nuclear fraction. Protein concentration was measured by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, USA) and an equal amount of proteins (for both cytosol and nuclear fraction) were resolved on 10.5% SDS-PAGE. Protein expressions in nuclear and cytosolic fractions were analyzed by western blotting using specified antibodies.

4.2.11 SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting:

Western blotting was performed to detect protein levels in total cells, cytosolic and nuclear fractions. Total cells pellets were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl, 5 mM EDTA, 0.4% NP40 or 1% Triton-X100, 1% Glycerol and 1 x protease inhibitor cocktail), whereas nuclear fractions were lysed in buffer-B. Protein concentration was determined by Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad, USA) and an equal amount of proteins were resolved on 10.5% SDS-PAGE. In case of LC3 western blotting, the proteins were run on 12.5% gel. Proteins were electro-blotted on PVDF membrane (Immun-Blot® PVDF Membrane, Bio-Rad, USA) at 110 V for 1 h at 4 °C. The membrane was blocked then with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 h at room temperature and incubated with

primary antibody overnight at 4 °C. After incubation membrane was washed thrice with TBS-T (TBS containing 0.1% Tween-20) and incubated with a secondary antibody for 1 hour at room temperature. The proteins were detected by using Clarity Western ECL Substrates (Bio-Rad, USA) and exposing to X-ray film or using ChemiDoc MP Imaging System (Bio-Rad, USA).

4.2.12 Co-immunoprecipitation and western blotting:

To detect protein-protein interaction, immunoprecipitation experiments were performed as reported earlier (Tomar et al., 2013b). HEK293 cells were seeded at a density of 3 X 10^6 cells per 10 cm^2 dish and co-transfected with indicated constructs. After 24 h of transfection, cells were treated with indicated reagents and collected in ice-cold DPBS. The pellet was resuspended in IP buffer (100 mM NaCl, 50 mM Tris–HCl, 1% Triton-X 100 and 1X protease inhibitor cocktail) and incubated on ice for 30 minutes. Later Cells were disrupted by passing through a 24G sterile syringe needle (50 strokes) and incubated over ice for 30 minutes. Disrupted cells were centrifuged at 12000 rpm for 15 minutes and supernatant were collected and incubated with HA or FLAG beads overnight on a roller shaker at 4˚C. The gel beads were washed four times with IP buffer, resuspended in 5× SDS-PAGE sample buffers and separated on 10.5% SDS-PAGE. Western blotting was performed and proteins were detected using specific antibodies.

4.2.13 Analysis of public cancer databases:

4.2.13.1 TIMER Database Analysis for expression correlation

Tumor IMmune Estimation Resource (TIMER) is a web server for Comprehensive Analysis of Tumor-Infiltrating Immune Cells (Li et al., 2017). The “Correlation” module of the web server was used to get the expression scatterplots, Spearman’s correlation and estimated statistical significance between REL, RelA, NFKB1, and MID2/TRIM1 expression. It was also for

correlation between MID2/TRIM1 and TRAF2 expression in indicated cancers. The partial correlation conditioned was adjusted to none.

4.2.13.2 Gene expression analysis in GEPIA and Oncomine

Gene Expression Profiling Interactive Analysis (GEPIA) and GEPIA2 (enhanced version of GEPIA) webserver were used for analyzing expression in cancer and normal tissues (Tang et al., 2017). "Expression DIY" module was used to check the expression of MID2/TRIM1 in Tumor vs Paired Normal analysis. Similarly, the "Multiple gene comparison" of "Expression DIY" module was used to check the Tumor vs Paired Normal expression of MID2/TRIM1 and TRAF2 expression. The Cancer Genome Atlas (TCGA) normal and Genotype-Tissue Expression (GTEx) data was also included in the analysis.

The Oncomine cancer microarray database and data-mining platform was also used to check the expression of MID2/TRIM1 in cancers vs normal tissues (Rhodes et al., 2004).

4.2.13.3 Gene expression analysis from GEO datasets

The Gene Expression Omnibus dataset (GEO) GDS3809 was explored for TRIM15 expression (probe set: 1451916_s_at). The microarray expression values of TRIM15 were plotted as mRNA expression for indicated time points (Lotzer et al., 2010). Similarly, we explored the GDS4062 dataset for expression of TRIM15 (probe set: 36742_at, 210885_at, and 210177_at) in normal, psoriasis lesional and non-lesional dataset. Microarray transformed counts were plotted for mRNA expression for each probe set separately.

4.2.14 Statistical analysis:

All the data in this study are shown as mean ± SEM or mean ± SD for n observations. Comparisons of groups were performed using one-way ANOVA (Newman-Keuls post test) for each group or by unpaired two-tailed Student's

t-test to determine the levels of significance for each data set using GraphPad Prism®5. The experiments were repeated minimum three times independently and probability values of $p < 0.05$ were considered as statistically significant. In case of normalized graphs, the data was normalized by considering maximum value as 100% and 0 (minimum value) as 0% for all data set. Obtained normalized values were plotted in form of graph. All figure images representing results were prepared with Adobe Photoshop© 8 CS.