3

Mahogunin RING Finger-1 (MGRN1) Suppresses Chaperone Associated Misfolded Protein Aggregation and Toxicity

MGRN1 is a RING containing E3 Ubiquitin ligase for which, so far, very few substrates have been found. Tumor susceptibility gene 101 (TSG101) is the best characterized substrate of MGRN1 E3 Ubiquitin ligase. MGRN1 has been reported to monoubiquitinate TSG101 at multiple sites. Interestingly, such monoubiquitination modifies the functionality of TSG101 rather than leading to degradation. Being a component of ESCRT1 complex, it modulates endosomal lysosomal trafficking [Kim *et al.,* 2007; Jiao *et al.,* 2009]. MGRN1 has been found involved in inhibition of melanocortin receptors (MCRs) [Perez-Oliva *et al.,* 2009]. MGRN1 regulates pigment type switching in mice [Gunn *et al.,* 2013]. Recently, MGRN1 has been shown to regulate microtubule stability through mediating alpha tubulin ubiquitination [Srivastava and Chakrabarti, 2014]. The E3 ligase has complex functionality in cellular physiology which might be because of its modulatory functions besides the normal E3 ligase functionality. Overall, various isoforms of MGRN1 are localized in cytoplasm, nucleus, endosomes and on membranes. The E3 ligase has been found with various post-translational modifications such as phosphorylation, Ubiquitin-conjugation, myristoylation and lipoylation. A distinguishing character of MGRN1 protein is that many of the protein functions are ubiquitination independent. It may also be suggested that probably, ubiquitination dependent roles of MGRN1 are less explored and yet have more promising scope of investigation.

3.1 RESULTS

Considering the common pathophysiological changes in cells, it could be assumed that like various stress responsive proteins, E3 Ubiquitin ligases may also be responding to stresses. Studies were carried out with the experiments starting from ones where MGRN1's behavior was checked in stresses; later other experiments were performed in order to explore its role in the protein quality control of cells. Following are the results of the experiments performed.

3.1.1 MGRN1 is Induced under Various Cellular Stress Conditions and Interacts with Hsp70.

It has been shown that MGRN1 interacts with the aggregates of cytosolic prion proteins (PrPs) that are associated with neurodegeneration. However, the mechanisms through which the loss of MGRN1 function impairs cellular protein quality control mechanisms and causes spongiform neurodegeneration is not known. Mitochondrial dysfunction and high levels of oxidative stress have also been observed in MGRN1 mutant mice. Therefore, to explore the possible role of MGRN1 in cellular protein quality control mechanisms, A549 cells were exposed to oxidative (H_2O_2), ER (Tunicamycin and β-mercaptoethanol), and HS (heat stress), as well as to autophagy dysfunction (Chloroquine) stress, and then examined MGRN1 mRNA and protein levels. It was noticed that MGRN1 mRNA levels were significantly increased under various stress conditions (Figure 3.1A).

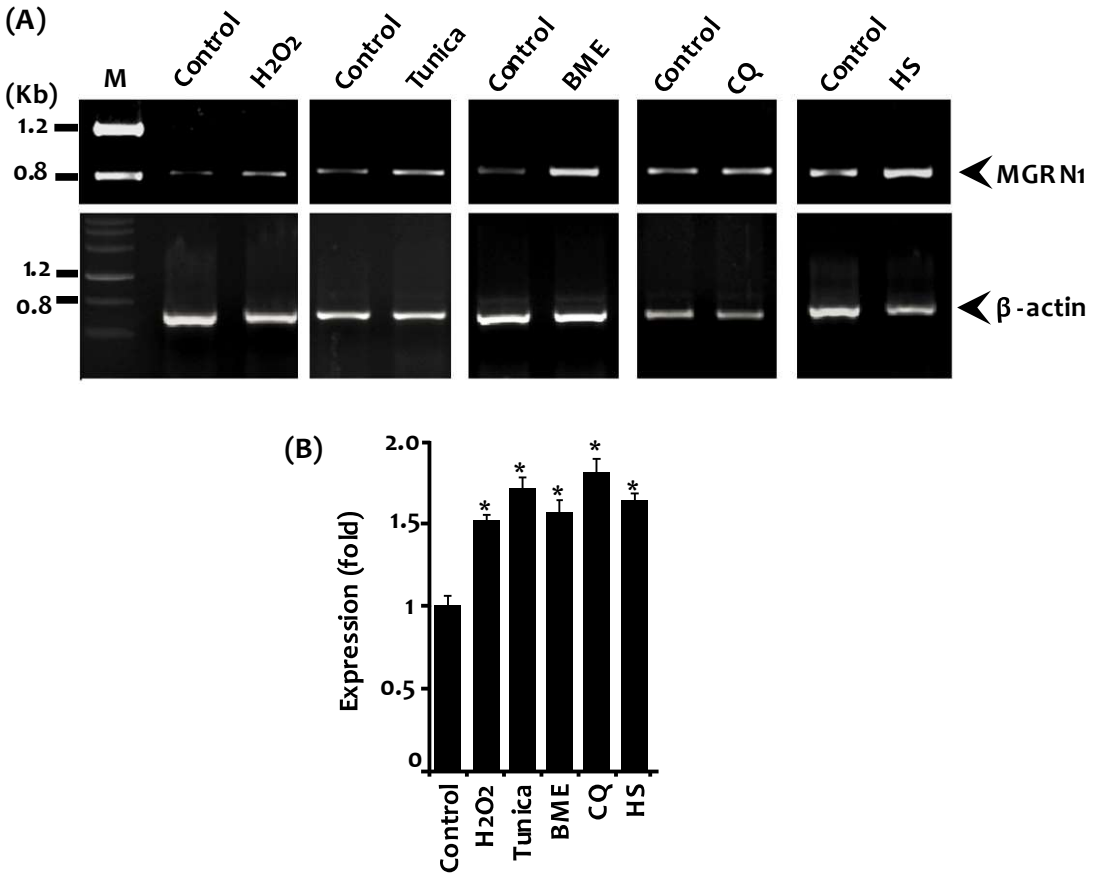

Figure 3.1 : *MGRN1 mRNA levels are elevated under various stress conditions:* (A) A549 cells were plated into six-well tissue culture plates and treated with 0.2 mM H_2O_2 for 2h, 5 µg/ml Tunicamycin for 5h, 5 mM β-mercaptoethanol for 2h, or 20 µM Chloroquine (CQ) for 8h. Alternatively, for heat stress (HS) exposure, cells were exposed to 43°C for 30 minutes and then returned to 37°C for 1h. After treatment, cells were collected, and total cellular RNA was isolated and subjected to reverse transcription polymerase chain reaction (RT-PCR) using primers for MGRN1 and β-actin. (B) Quantitation of MGRN1 mRNA levels using quantitative real time RT-PCR in the experiment as described in A.

It was also noticed that approximately 0.5 (Oxidative stress), 0.70 (ER), 0.80 (autophagy dysfunction) and 0.65 (Heat stress) fold increase in MGRN1 mRNA levels under various stress conditions as compared with control cells (Figure 3.1B). Therefore, it also became important to check MGRN1 protein levels after treatment with different types of stress-inducing agents. MGRN1 protein levels were also elevated after exposure to the various cellular insults generated by the stressor agents (Figure 3.2A and 3.2B). Surprisingly, there was less increase in levels of MGRN1 proteins compared to mRNA levels after exposure with various stress inducing agents.

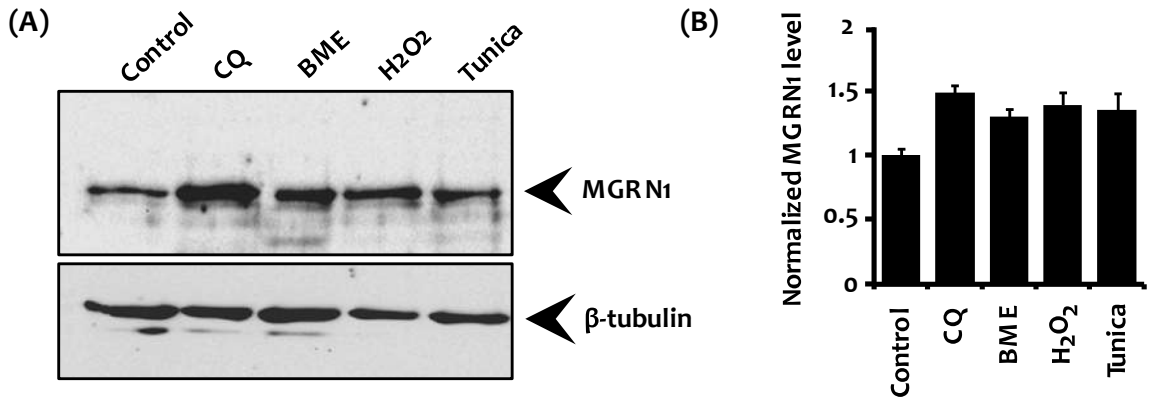

Figure 3.2 : *MGRN1 protein levels are increased after cellular stresses:* (A) Representative immunoblot showing MGRN1 protein levels in cells treated with different stress-inducing agents, as described in Figure 3.1. Blots were probed with MGRN1 and β-tubulin antibodies. (B) Quantification of the intensities of MGRN1 band shown in A, which were collected from three independent experiments. Data are expressed as a ratio of MGRN1 to β-tubulin. Values are the mean ± SD of three independent experiments.

To our understanding, the less increase in the protein levels of MGRN1 in the stressed cells could be due to its strong association with misfolded protein inclusions. To confirm this hypothesis, MGRN1-GFP construct overexpression was performed in cells and treated with various stress inducing agents. It was observed that under various stress conditions, MGRN1 protein was nicely accumulated in periphery nuclear region and make clear big inclusions like structures (Figure 3.3A). Most likely these inclusions are non-SDS-soluble in nature and therefore it is hard to observe their soluble forms.

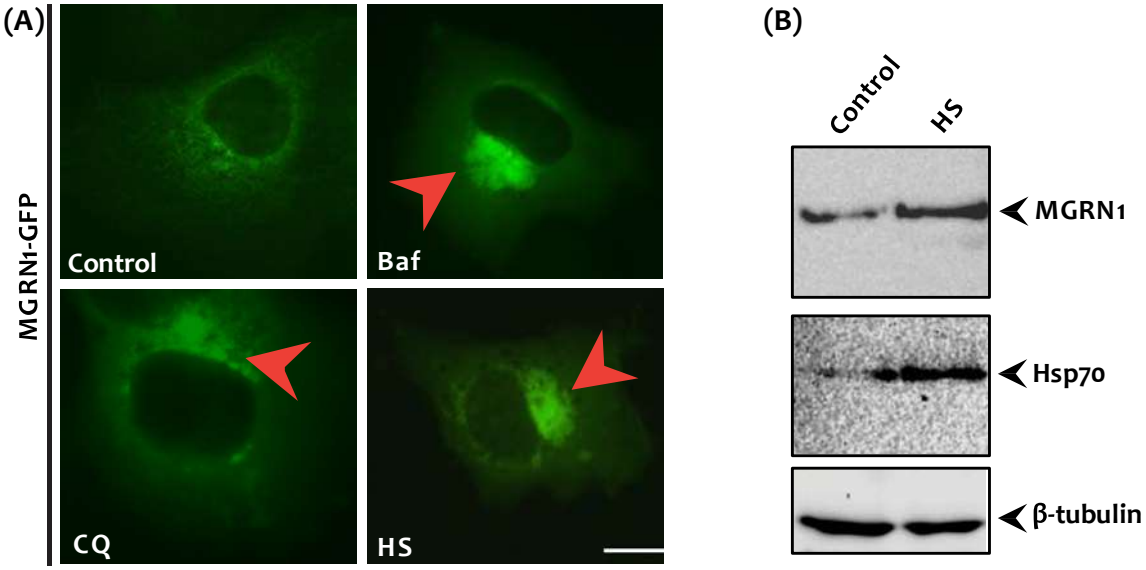

Figure 3.3 : *MGRN1 redistribution in cells with stress:* (A) Cells were transiently transfected with MGRN1-GFP construct and after 48h of transfection, treated with 50 nM Bafilomycin (Baf), 30 µM Chloroquine (CQ) or HS exposure 43°C for 30 minutes. Treated cells were directly subjected to fluorescence microscopy analysis. (B) Cells were exposed to HS at 43°C for 30 minutes and then processed for immunoblotting. Blots were probed with MGRN1, Hsp70, and β-tubulin antibodies. Scale bar, 20 µm.

It is known that heat shock proteins' levels are elevated after HS and Hsp70 is involved in the protection of cells against various types of stress. In the current study, it was found that the response of MGRN1 to HS was very similar to that of Hsp70 (Figure 3.3B). Because the expression profile of MGRN1 induced by the variety of cellular insults was similar to that of Hsp70, a hypothesis was postulated that MGRN1 may interact with the Hsp70 chaperone and this cooperative function may enhance the clearance of misfolded proteins.

Unexpectedly, it was found that MGRN1 contains one biochemically related KFERQ motif, which is important for CMA (Figure 3.4B). To strengthen this finding MGRN1 gene sequence was analyzed and it led to another finding that the 5'-untranslated region of the MGRN1 gene contains a consensus heat shock factor 1 (HSF1) binding sequence that is 90% homologous with the yeast HSF-1 binding sequence. This preliminary result indicated that this gene can respond to heat shock (HS) (Figure 3.4A).

(A)

(B)

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CLUSTAL 2.1 multiple sequence alignment

QIVDR           ------------------------------------------------------------
MGRN1           MGSILSRRIAGVEDIDIQANSAYRYPPKSGNYFASHFFMGGEKFDTPHPEGYLFGENMDL 60

QIVDR           ------------------------------------------------------------
MGRN1           NFLGSRFVQFPYVTPAPHEPVKTLRSLVNIRKDSLRLVRYKDDADSPTEDGDKPRVLYSL 120

QIVDR           ------------------------------------------------------------
MGRN1           EFTFDADARVAITIYCQASEEFLNGRAVYSPKSPSLQSETVHYKRGVSQQFSLPSFKIDF 180

QIVDR           ------------------------------------------------------------
MGRN1           SEWKDDELNFDLDRGVFPVVIQAVVDEGDVVEVTGHAHVLLAAFEKHMDGSFSVKPLKQK 240

QIVDR           QIVDR------------------------------------------------------- 5
MGRN1           QIVDRVSYLLQEIYGIENKRNQETKPSDDENSDNSNECVVCLSDLRDTLILPCRHLCLCT 300
                *****

QIVDR           ------------------------------------------------------------
MGRN1           SCADTLRYQANNCPICRLPFRALLQIRAVRKKPGALSPVSFSPVLAQSLEHDEHSCPFKK 360

QIVDR           ------------------------------------------------------------
MGRN1           SKPHPASLASKKPKRETNSDSVPPGYEPISLLEALNGLRAVSPAIPSAPLYEEITYSGIS 420

QIVDR           ------------------------------------------------------------
MGRN1           DGLSQASCPLAAIDHILDSSRQKGRPQSKAPDSTLRSPSSPIHEEDEEKLSEDVDAPPPL 480

QIVDR           ------------------------------------------------------------
MGRN1           GGAELALRESSSPESFITEEVDESSSPQQGTRAASIENVLQDSSPEHCGRGPPADIYLPG 540

QIVDR           ----------------------------------
MGRN1           RPTSMETAHGLATTSPTWPPLGGPSPDPSAAELTPL 576
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Figure 3.4 : *MGRN1 gene contains an HSF1 binding site and CMA targeting motif:* (A) Schematic diagram of the MGRN1 gene: Dark region represents the consensus yeast HSF1 binding site identified in the 5' UTR of MGRN1 gene. (B) In MGRN1 protein sequence chaperone mediated autophagy motif including the KFERQ-like motif was identified through sequence alignment using ClustalW2 tool.

Co-immunoprecipitation was used to explore the possibility of an interaction between MGRN1 and Hsp70. An immunoprecipitation experiment using the lysates of Cos-7 cells that overexpressed MGRN1-Myc and Hsp70-EGFP revealed that Hsp70 interacts with MGRN1 (Figure 3.5A). To further confirm the observed interaction, a more detailed immunoprecipitation study was performed; the same co-transfected cell lysates pulled down with MGRN1 antibody were subjected to immunoblot analysis using anti-Myc (Figure 3.5B) and anti-GFP (Figure 3.5C). Now, it was important to check reverse interaction between Hsp70 and MGRN1; therefore, in the next immunoprecipitation experiment, co-transfected cell lysates were pulled down using an Hsp70 antibody and blots were developed using MGRN1 antibody (Figure 3.6A). Figure 3.6B represents the pulled down Hsp70 proteins detected through immunoblotting using a GFP antibody.

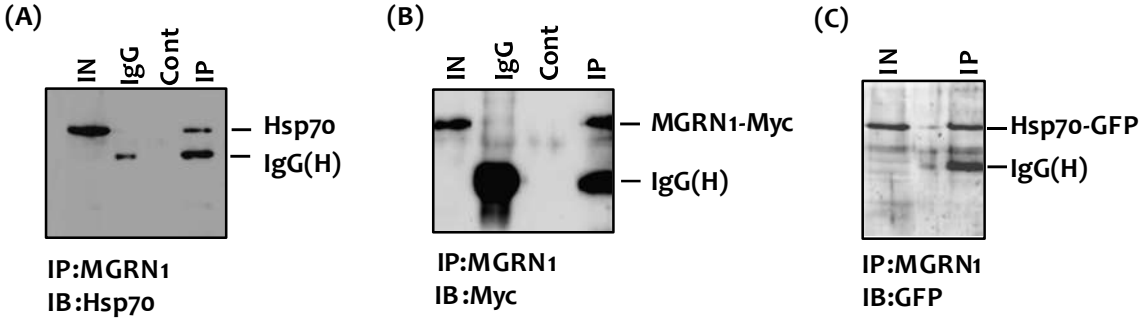

Figure 3.5 : *MGRN1 interacts with Hsp70 protein:* Transfection was used to overexpress the MGRN1-Myc and Hsp70-EGFP in Cos-7 cells. Cells were collected, lysed, and processed for immunoprecipitation (IP) with an MGRN1 antibody 24h post-transfection. Blots were probed with an Hsp70 (A) antibody and Myc (B) antibody. (C) Hsp70-EGFP transfected cell lysate were pulled down by MGRN1 antibody and blot was developed with GFP antibody.

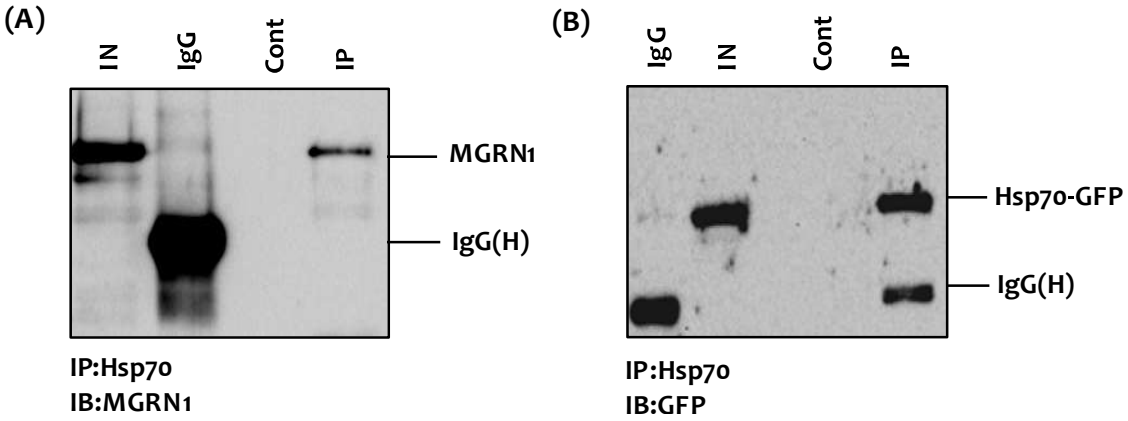

Figure 3.6 : *Hsp70 interacts with MGRN1 protein:* As described in Figure 3.5, the samples were processed for IP using an Hsp70 antibody and the blots were probed with anti-MGRN1 (A) and anti-GFP (B).

Experiments were performed using different controls; the same cell lysate was pulled down with beads only, and analyzed the blots using anti-MGRN1, anti-Hsp70 and anti-GFP antibodies (Figure 3.7). This confirms the interaction of MGRN1 protein with Hsp70 chaperone.

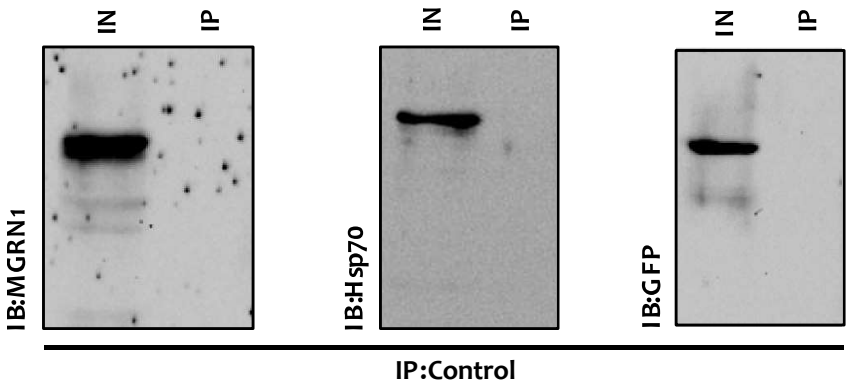

Figure 3.7 : *Negative control for MGRN1 and Hsp70 interaction analysis:* As described in section Figure 3.5; same samples were pulled down by beads only (control) and blots were obtained with anti-MGRN1, anti-Hsp70 and anti-GFP antibodies.

3.1.2 Recruitment of MGRN1 to Components of Inclusion Bodies Following Inhibition of Autophagy

Inclusion bodies (IBs) are generally positive for p62 and ubiquitin. The key observation of the interaction between MGRN1 and Hsp70 raised the critical question that whether there are cumulative effects of MGRN1 and Hsp70 in the selective autophagy pathway. To assess whether MGRN1 is recruited to the general components of IBs, immunofluorescence staining of p62 was performed in MGRN1-GFP overexpressing cells after treatment with Bafilomycin (Baf). Treatment with Baf resulted in the formation of distinct IBs that were co-localized with p62. MGRN1 was also clearly recruited towards these IBs (Figure 3.8). In Baf-treated cells, these IBs were also positive for both Hsp70 and p62 (Figure 3.9).

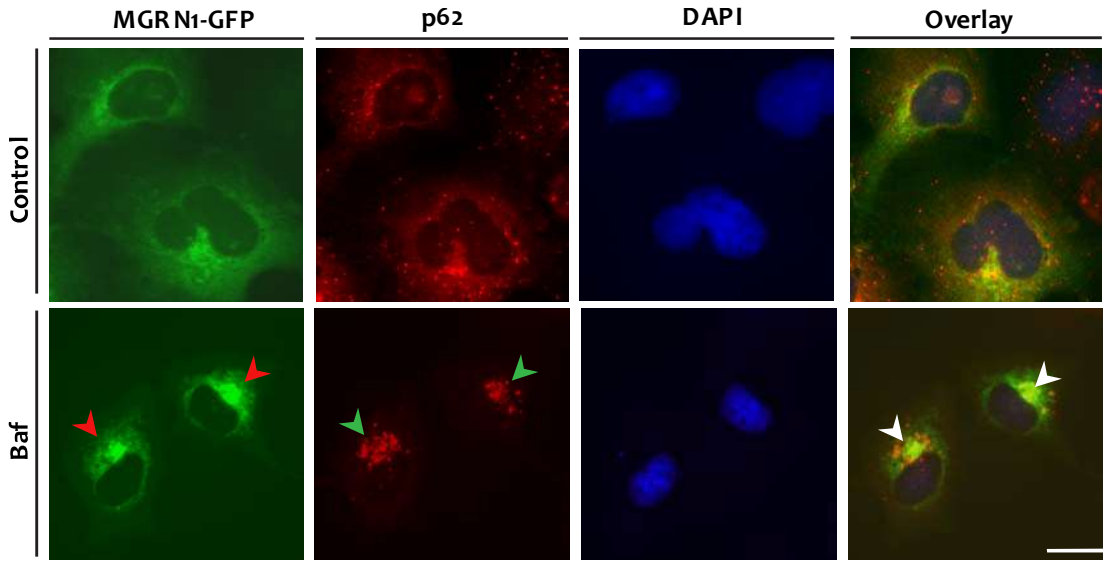

Figure 3.8 : *MGRN1 is recruited to accumulated p62 after Bafilomycin treatment:* Cos-7 cells were plated on two-chamber slides. On the following day, the cells were transiently transfected with the MGRN1-GFP construct. After 36h of transfection, the cells were treated with 50 nM Bafilomycin (Baf) for 12h. Post-treatment, cells were fixed and subjected to immunofluorescence staining using p62 antibody and then subsequently with a rhodamine-conjugated secondary antibody antibody. 4',6-diamidino-2-phenylindole (DAPI) was used to stain nuclei. Arrows indicate the recruitment of MGRN1 to p62 aggregates.

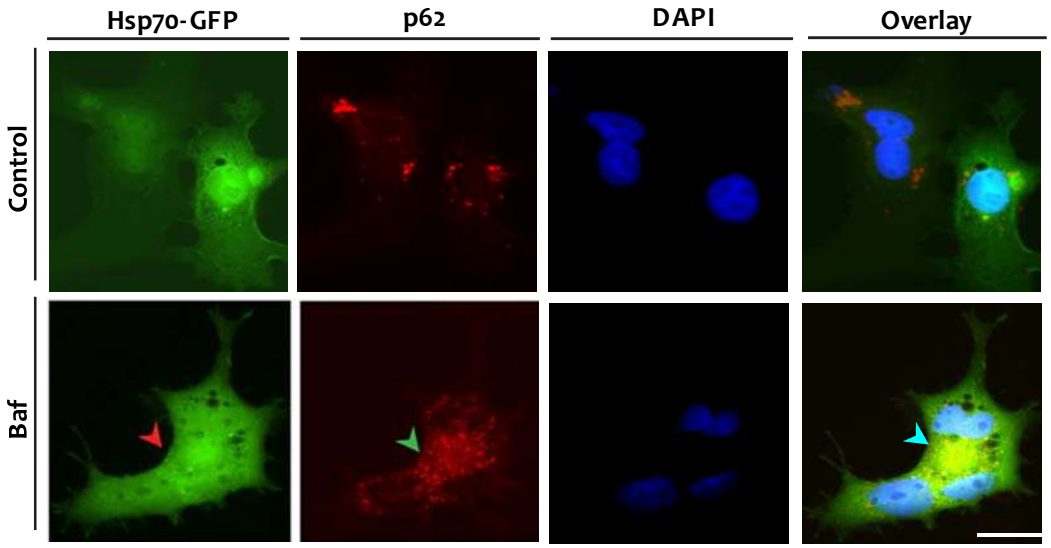

Figure 3.9 : *Hsp70 colocalizes with p62 aggregates followed by Bafilomycin treatment:* Cells were transfected with Hsp70-EGFP and treated with Bafilomycin as described in Figure 3.8. Cells were observed using a fluorescence microscope and their nuclei were stained using DAPI. Arrows indicate the co-localization of Hsp70 with p62 aggregates.

Next, it was observed that Baf treatment caused the recruitment of MGRN1 primarily towards perinuclear Ubiquitin-positive IB structures (Figure 3.10). Overall, these results clearly indicate that MGRN1 is predominantly redistributed in perinuclear regions and recruited to p62-, Hsp70-, and Ubiquitin-positive IBs.

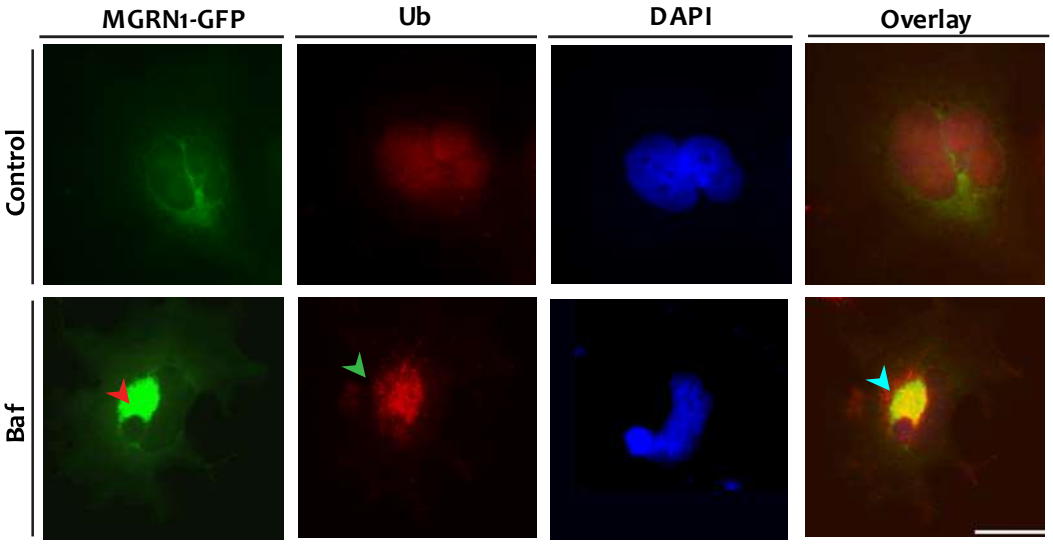

Figure 3.10 : *MGRN1 gets colocalized with Ubiquitin positive aggregates after Bafilomycin treatment:* Cells were transiently transfected with the MGRN1-GFP construct and treated with Baf as described in Figure 3.9; after treatment, cells were processed for immunofluorescence staining using Ubiquitin (Ub) antibody. Rhodamine-conjugated secondary antibody was used to label ubiquitin. Nuclei were stained with DAPI. Arrows indicate the redistribution of MGRN1 with Ub-positive aggregates. Scale bar, 20 μm.

3.1.3 MGRN1 is Colocalized with Hsp70-anchored Misfolded Luciferase Inclusion Formations

The interaction, recruitment, and co-localization of MGRN1 with the pre-formed general components of IBs known to be induced in response to impaired autophagy, were analyzed. It was found that MGRN1 colacalizes with heat-denatured Luciferase protein; MGRN1 levels were increased and MGRN1 was recruited towards perinuclear regions with denatured Luciferase (Figure 3.11) and Hsp70 chaperone (Figure 3.12), after heat stress.

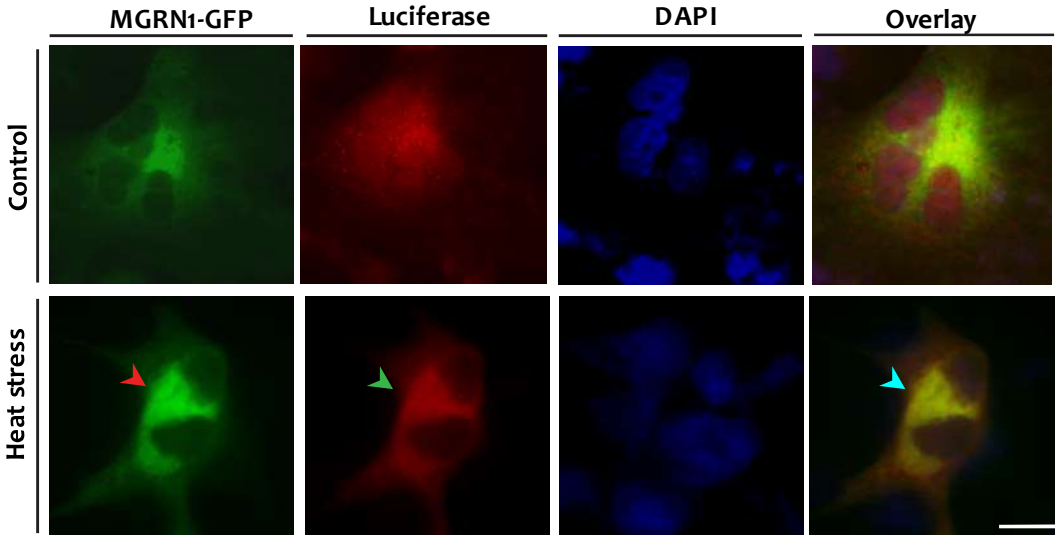

Figure 3.11 : *MGRN1 is recruited to accumulated Luciferase protein after heat stress:* Cos-7 cells were plated on two-chamber slides and co-transfected with MGRN1-GFP and Luciferase constructs. After 36h, cells were exposed to heat stress (43°C for 1h) and then processed for immunofluorescence staining using Luciferase antibody. A rhodamine-conjugated secondary antibody was used to label Luciferase. Arrows indicate the co-localization of MGRN1 with heat denatured Luciferase protein.

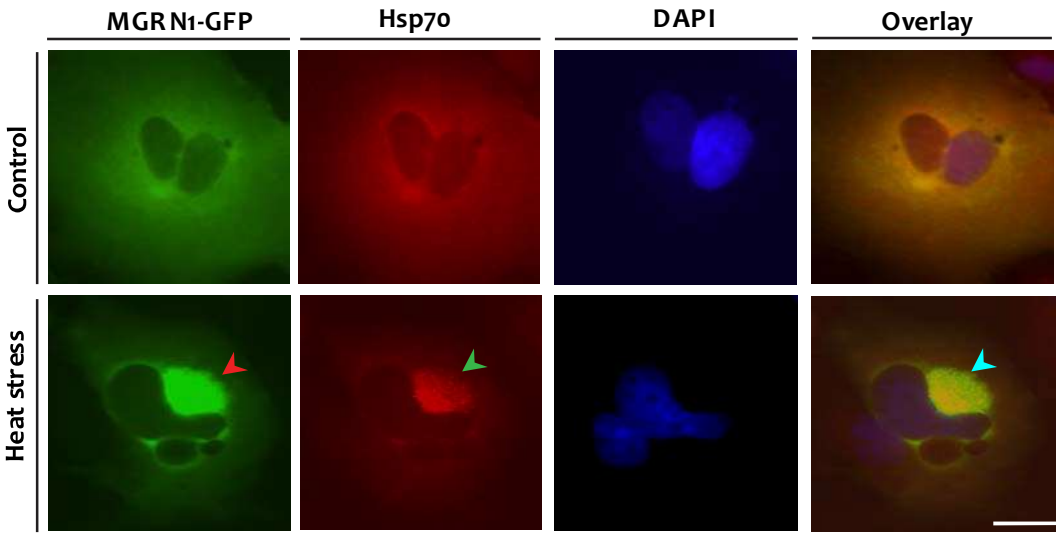

Figure 3.12 : *MGRN1 is colocalized with accumulated Hsp70 protein after heat stress:* The cells were transfected with MGRN1-GFP construct. The cells were exposed to HS and processed for immunofluorescence staining using an Hsp70 antibody. Arrows indicate the redistribution of MGRN1 with Hsp70 following HS exposure.

To more clearly confirm that this perinuclear distribution was specifically associated with the selective autophagy pathway, Baf-pretreated cells were used for heat shock. In this experiment, denatured Luciferase aggregates were observed to be dispersed throughout the cytoplasm and strongly co-localized with both MGRN1, while Hsp70 was also found to be colocalized with MGRN1 upon heat stress followed by Bafilomycin treatment (Figure 3.13). These results suggest that MGRN1 may target misfolded proteins through the Hsp70 chaperone and promote their clearance through autophagy.

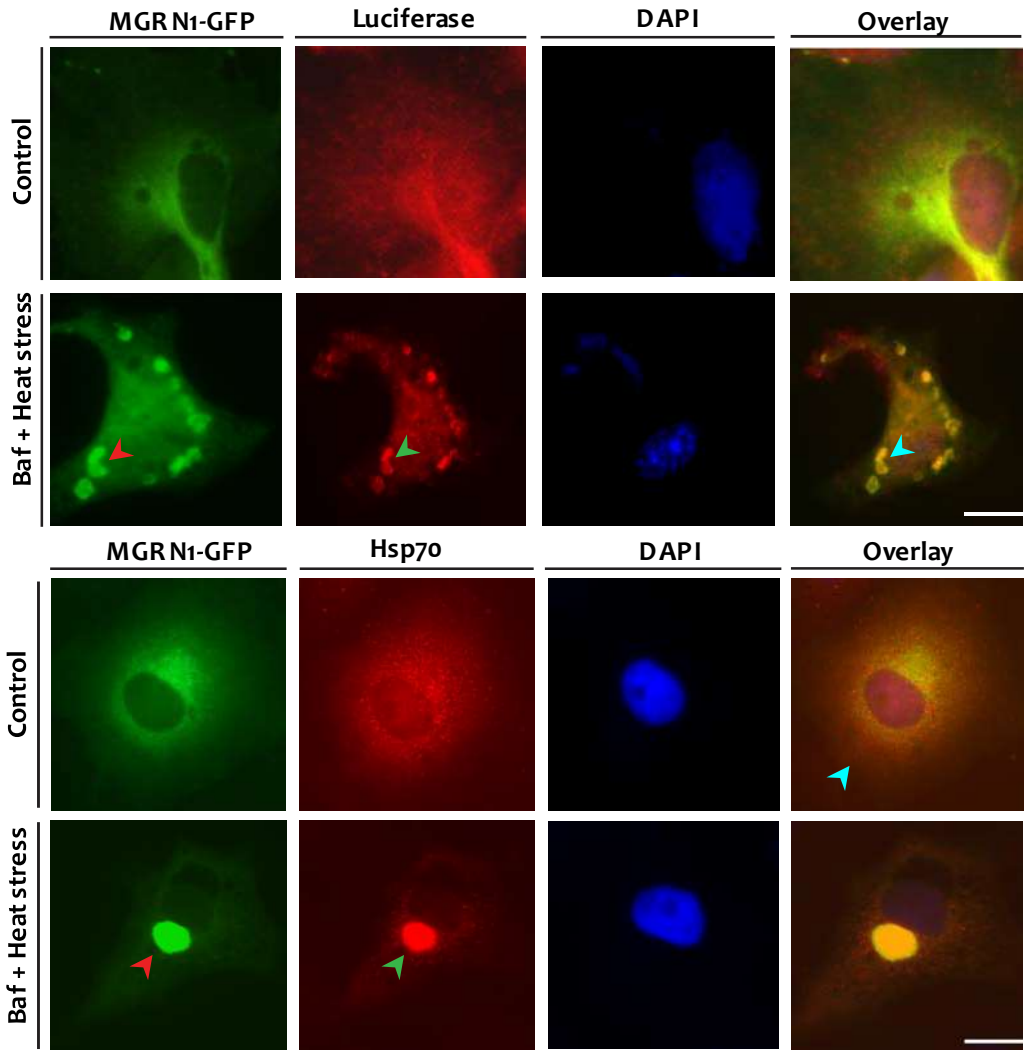

Figure 3.13 : *MGRN1 is recruited to heat-denatured Luciferase protein aggregates and Hsp70 protein followed by heat stress and Bafilomycin treatment:* Cos-7 cells were grown on two-chamber slides and co-transfected with Luciferase and MGRN1-GFP expression constructs. Thirty-six hours later, a portion of the samples were treated with 50 nM Bafilomycin (Baf) for 12h. Cells were heat stressed via exposure to 43°C for 1 hour and then subjected to immunofluorescence staining with anti-Luciferase antibody for a set of cells while the another set of cells were processed for immunocytochemistry using the Hsp70 antibody. A rhodamine-conjugated secondary antibody was used to label Luciferase. Overlay images include DAPI nuclear staining (blue color). Arrows indicate cytosolic aggregation and co-localization of MGRN1 with Luciferase following treatment with Baf + HS.

3.1.4 MGRN1 Alleviates Cellular Insults Generated by Various Stress-inducing Agents.

Thus far, the results indicated that MGRN1 may play a significant role in preventing the accumulation of misfolded proteins. To explore the underlying mechanism, it was further hypothesized that MGRN1 targets the misfolded proteinaceous species associated with the Hsp70 chaperone through autophagy. To confirm this hypothesis and simultaneously investigate the role of MGRN1 in protecting against various stresses, control and MGRN1 overexpressing cells were treated with 10 µg/ml Tunicamycin for 12h or 5 mM β mercaptoethanol for 3 h to induce ER stress, 0.5 mM H_2O_2 for 5 h to induce oxidative stress, and 50 µM CQ for 12 h to induce autophagy dysfunction. After treatment with the stress inducers, cells were visualized using bright field microscopy (Figure 3.14A). Overexpression of MGRN1 was confirmed using both reverse transcription polymerase chain reaction (RT-PCR) (Figure 3.14B) and immunoblot analysis with an MGRN1 antibody (Figure 3.14C).

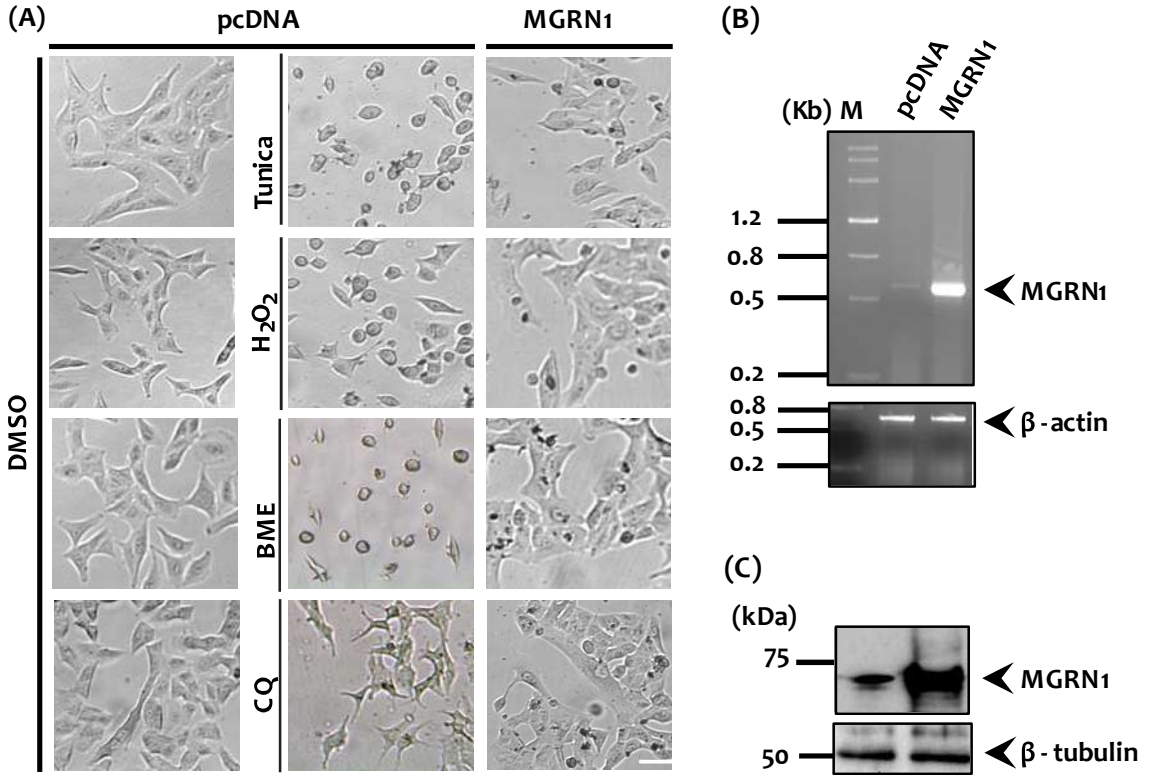

Figure 3.14 : *MGRN1 overexpression confers cytoprotection under various stress conditions*: A549 cells were transiently transfected with pcDNA (control) and MGRN1-Myc constructs (transfected with a 24-well tissue culture plate). After 48 hours of transfection, cells were treated with 10 µg/ml Tunicamycin (Tunica) for 12h, 5 mM β-mercaptoethanol (BME) for 3h, 0.5 mM H_2O_2 for 5h and 50 µM CQ for 12h. Cells treated with the various stressors were visualized using a bright field microscope as indicated in A. After treatment, cells were collected, and total cellular RNA was isolated and subjected to RT-PCR using primers for MGRN1 and β-actin (B). Some of the transfected cells were processed for immunoblot analysis using MGRN1 and β-tubulin antibodies (C).

To further confirm the protective effect of MGRN1 against various cellular insults, knockdown experiments were performed using siRNA oligonucleotides against MGRN1 that partially knockdown endogenous MGRN1. Scrambled siRNA was used as a control.

Approximately, upto 80% knockdown of endogenous MGRN1 levels were achieved. After transfection, cells were treated with various stress-inducing agents and processed for bright field microscopy image analysis as shown in Figure 3.15A. MGRN1 knockdown was confirmed by both reverse transcription polymerase chain reaction (RT-PCR) (Figure 3.15B) and immunoblot analysis using an MGRN1 antibody (Figure 3.15C). These results further substantiate that MGRN1 participates in cellular defense mechanisms in response to the proteotoxic effects caused by various stress-inducing agents.

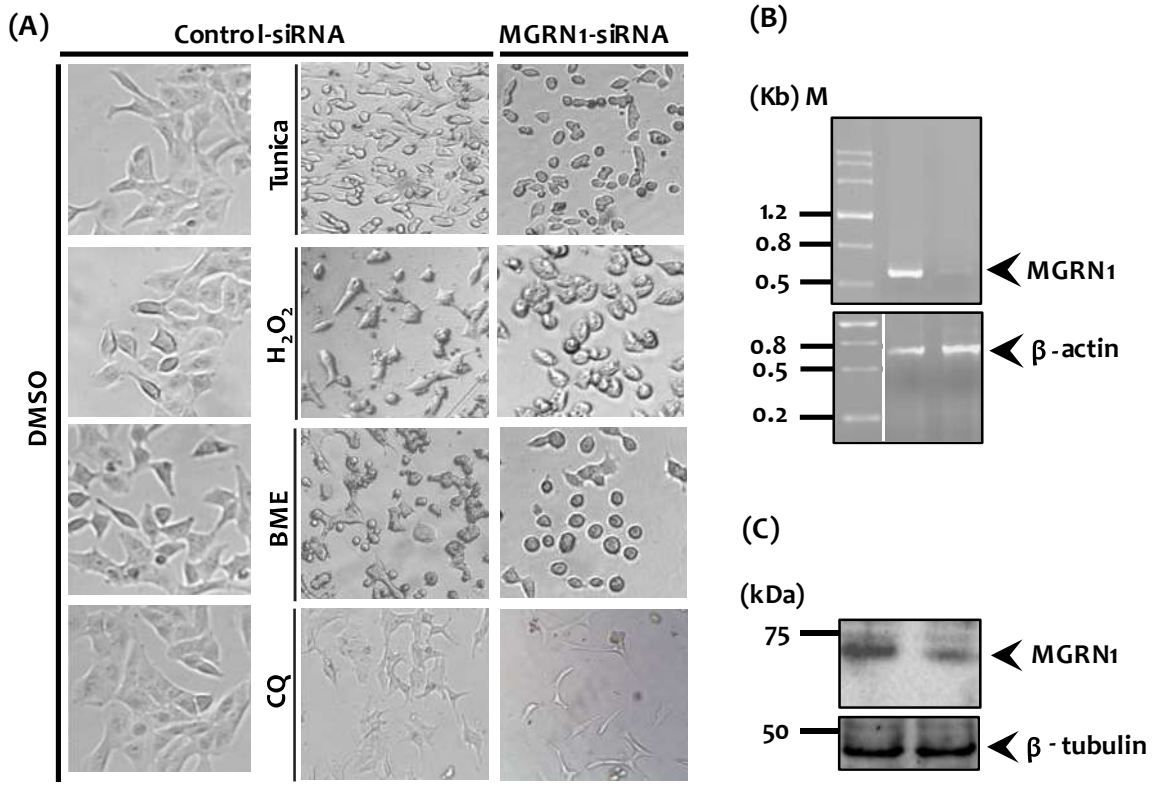

Figure 3.15 : *MGRN1 knockdown makes cells more vulnerable under stress conditions:* The A549 cells were transiently transfected with control (scrambled siRNA) and MGRN1 siRNA oligonucleotides and treated with various stress-inducing agents such as 05 µg/ml Tunicamycin (Tunica) for 8h, 2.5 mM β-mercaptoethanol (BME) for 1.5h, 0.1 mM H_2O_2 for 4h and 25 µM CQ for 10h. The cells treated with the various stressors were visualized using bright field microscopy as indicated in A. Post-treatment, the transfected cells were used to isolate total cellular RNA and processed for RT-PCR using primers for MGRN1 and β-actin (B). Some cells were used to make cell lysates, which were subjected to immunoblot analysis with anti-MGRN1 and β-tubulin antibodies (C).

3.1.5 MGRN1 Overexpression Induces the Degradation of Misfolded Luciferase Protein and Knockdown of MGRN1 Leads to Mitochondrial Membrane Depolarization and Cytochrome c Release

The MGRN1 E3 Ubiquitin ligase regulates endosomal trafficking through the proteasomal-independent ubiquitylation pathway. In the current study, it was unexpectedly found that MGRN1 interacts with the Hsp70 chaperone. MGRN1 also co-localizes with both perinuclear IBs and misfolded proteins following the inhibition of autophagy. These results prompted us to further investigate the functional role of MGRN1 in the degradation of

misfolded proteins. To directly demonstrate the role of MGRN1 in the degradation of denatured Luciferase protein, overexpression of MGRN1 along with a Luciferase construct was performed in Cos-7 cells. Transfected cells were exposed to 43°C for 30 minutes and then returned to 37°C for 2 h of recovery. In some experiments, cells were treated with 100 μM CQ before the HS exposure. Cells were then processed for immunofluorescence staining (Figure 3.16A) and a Luciferase activity assay (Figure 3.16B). As shown in Figure 3.16A and 3.16B, MGRN1 overexpression led to the efficient degradation of heat-denatured Luciferase protein, which was prevented by the CQ-mediated inhibition of autophagy (Figure 3.16B). Overexpression of MGRN1 was confirmed by performing an immunoblot using MGRN1 antibody (Figure 3.16C).

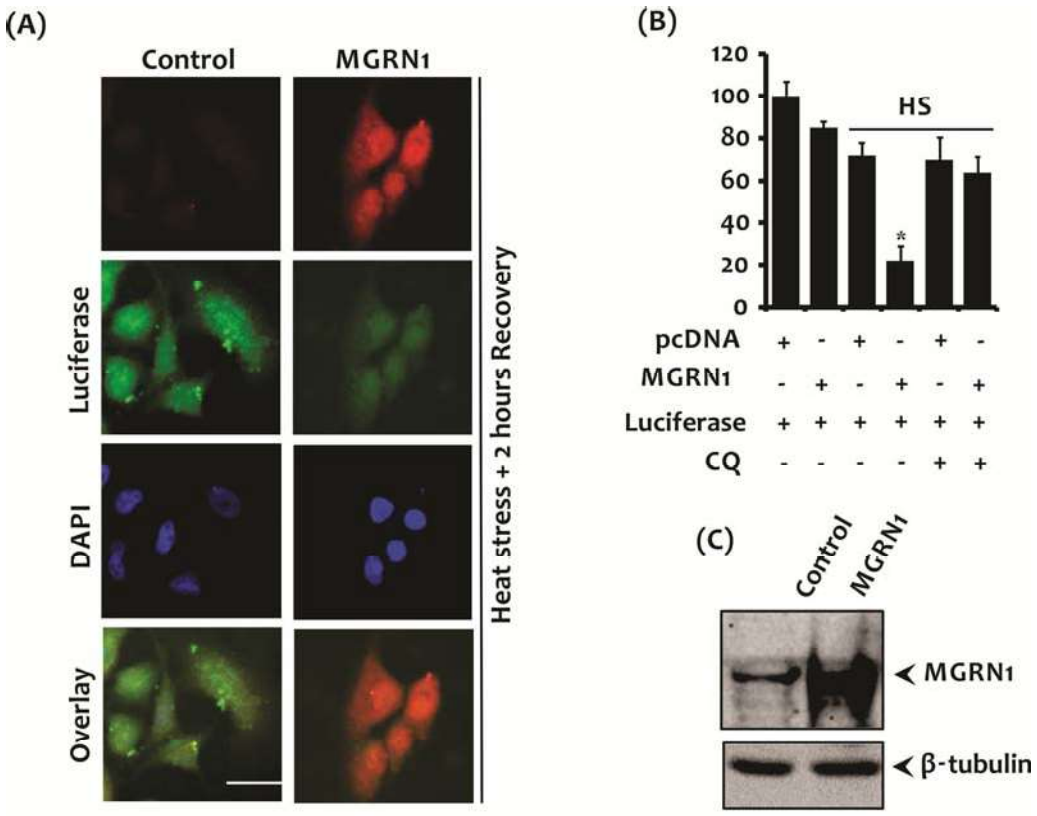

Figure 3.16 : *MGRN1 promotes heat-denatured Luciferase protein degradation:* (A) Cos-7 cells were co-transfected with an MGRN1-Myc plasmid along with a firefly Luciferase expression construct. Cells were processed for immunofluorescence staining using anti-Luciferase and MGRN1 antibodies. A Luciferase activity assay was performed (B). Similar set of cells was then subjected to immunoblot analysis using MGRN1 and β-tubulin antibodies (C). Values are presented as the mean ± SD of three independent experiments. *, $p < 0.05$ compared with the control group that was heat-stressed but transfected with an empty pcDNA construct.

To further confirm this result, cotransfection of catalytically inactive form of MGRN1 (MGRN1^{AVVA}-GFP) along with Luciferase constructs were performed. Transfected cells were exposed to heat stress treatment with CQ. Cells were then subjected to immunofluorescence staining (Figure 3.17A) and a Luciferase activity assay (Figure 3.17 B). It was noticed that overexpression of catalytically inactive form of MGRN1 did not degrade heat denatured Luciferase. Taken together, the results suggest that MGRN1 overexpression significantly reduces the levels of heat-denatured, misfolded Luciferase protein through autophagy.

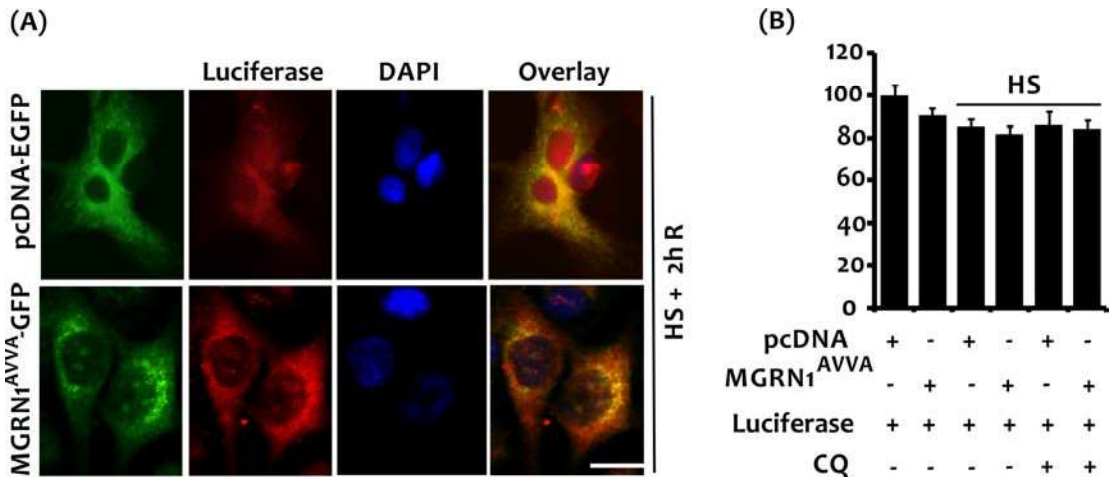

Figure 3.17 : *Catalytically inactive MGRN1 does not promote Luciferase degradation:* Cells were cotransfected with pcDNA-EGFP, MGRN1-AVVA-GFP mutant and Luciferase plasmids and after heat treatment and recovery, cells were processed for immunofluorescence staining (A) using anti-Luciferase antibody and Luciferase assay (B) as described in materials and methods. Values are presented as the mean ± SD of three independent experiments. *, $p < 0.05$ compared with the control group that was heat-stressed but transfected with an empty pcDNA construct. Scale bar, 20 μm.

It was noticed that MGRN1 defends against oxidative stress in cells and generates cytoprotective effects. In order to explore this finding further, the effect of MGRN1 was investigated on mitochondrial membrane depolarization and cytochrome c release. Cells were transiently transfected with control (scrambled siRNA) or MGRN1-specific siRNA oligonucleotides in a concentration-dependent manner. Following 48h of transfection, the same cells were subjected to mild oxidative stress (0.05 mM H_2O_2 for 10 minutes) and then processed for JC-1 staining to examine changes in mitochondrial membrane depolarization. In addition, some cells were used for immunofluorescence staining of cytochrome c. The voltage sensitive fluorescence dye JC-1 stains polarized mitochondria red. Conversely, green fluorescence indicates a low or depolarized mitochondrial membrane potential. As compared to treatment with control siRNA, partial knockdown of MGRN1 in a concentration-dependent manner made cells highly sensitive to mild oxidative insults, as determined by the signal detected with fluorescence microscopy (Figure 3.18A-D). Partial knockdown of MGRN1 released cytochrome c from the mitochondria (Figure 3.18E-H). Mitochondrial dysfunction in MGRN1 mutant mice has also been reported. A significant increase in TUNEL-positive cells was also observed in the current study in the MGRN1 siRNA-transfected cells, treated with oxidative stress inducing agent (Figure 3.18I). It was also noticed that knockdown of MGRN1 made cells highly sensitive to oxidative insults. Taken together, these results suggest that a loss of MGRN1 function plays a critical role in mitochondrial dysfunction and the associated oxidative stress.

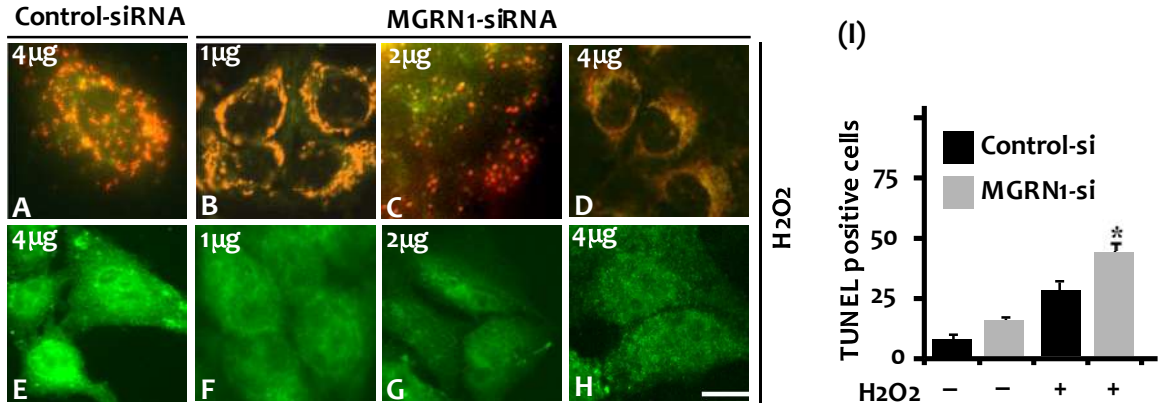

Figure 3.18 : *MGRN1 knockdown leads to mitochondrial dysfunction:* (A-H) A549 cells were transiently transfected with control (scrambled) siRNA (A, E) or with different concentrations of MGRN1 siRNA oligonucleotides; post transfection cells were treated with 0.05 mM H_2O_2 for 10 minutes and subjected to either 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) staining to detect the changes in mitochondrial membrane potential (functional coupled mitochondrial activity as red and the depolarized membrane becomes green) (A-D) or immunostaining using anti-cytochrome c (E-H). (I) Percentage of TUNEL positive control (scrambled) siRNA and MGRN1-siRNA transfected cells treated with H_2O_2. Values are presented as the mean ± SD of three independent experiments. *, $p < 0.05$ compared with the control-si cells treated with H_2O_2. Scale bar, 20 µm.

3.1.6 MGRN1 Protects against Cell Death mediated by ER and Oxidative Stress.

The current report shows that the endogenous levels of MGRN1 are increased after exposure to different stressors and promote the degradation of misfolded proteins. All these results suggest that MGRN1 may contribute to a critical protective survival response under stressed conditions. To evaluate this possibility, both overexpression as well as knockdown of MGRN1 was performed and confirmed the same with the help of immunocytochemistry (Figure 3.19A and 3.20A respectively) and reverse transcriptase PCR (Figure 3.19B and 3.20B respectively). Overexpression and knockdown of MGRN1 were also confirmed through immunoblotting using an MGRN1 antibody (Figure 3.19C and 3.20C respectively).

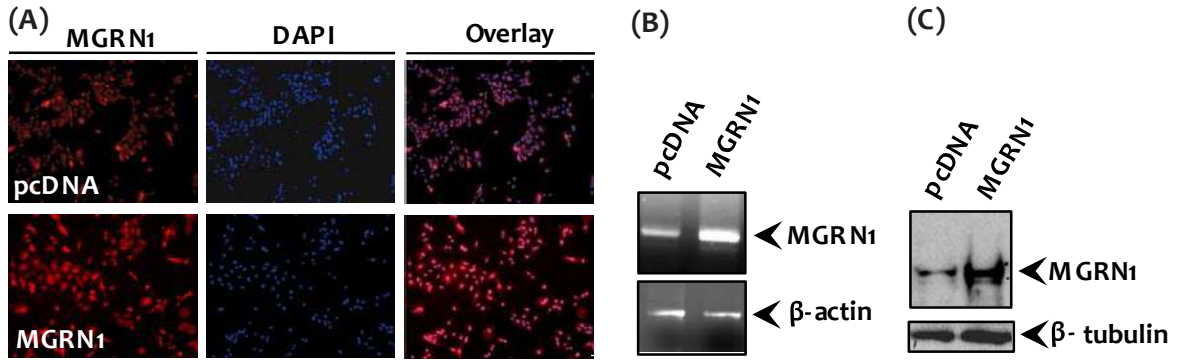

Figure 3.19 : *MGRN1 overexpression representation*: Some MGRN1 transfected cells along with pcDNA overexpression as control were processed for and for immunofluorescence staining (A) and reverse transcriptase-PCR (B) and immunoblotting (C). Scale bar, 20 µm.

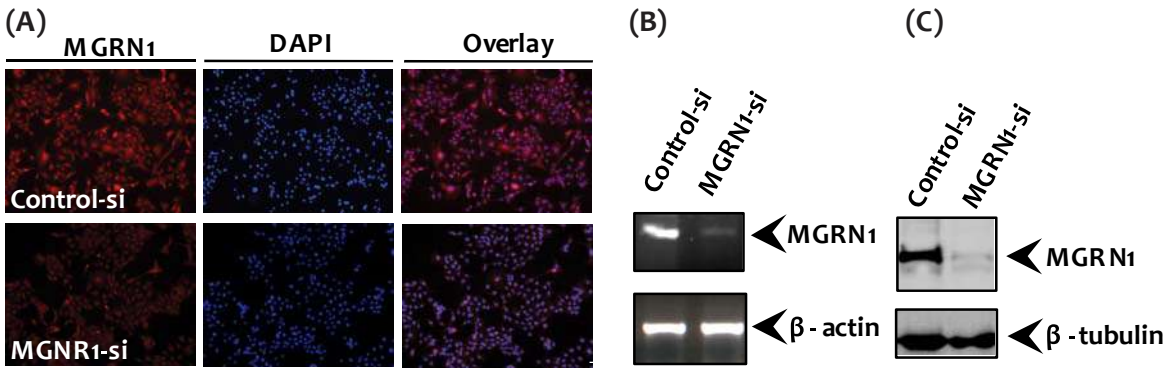

Figure 3.20 : *Immunocytochemistry and represntative blot and RT-PCR analysis for MGRN1 knockdown in cultured A549 cells:* (A-C) A549 cells were transfected with control (scrambled) and MGRN1 siRNA; few cells were collected and processed for RT-PCR (B) and some were used to make protein sample to perform immunoblotting (C) while similar a set of cells was processed for immunofluorescence staining (A) with an MGRN1 antibody. For nuclear staining, fixed cells were incubated with DAPI for 10 minutes. Scale bar, 20 μm.

The overexpression of MGRN1 alleviated cytotoxicity and protected against the cell death induced by ER (Figure 3.21A and 3.21B) and oxidative stress (Figure 3.21C). Overexpression of Hsp70 along with MGRN1 generated an additional protective effect against cytotoxic insult. Overexpression of GFP-tagged Hsp70 was confirmed by immunoblotting using a GFP antibody (Figure 3.21D). To further confirm the cytoprotective nature of MGRN1, knockdown experiments of MGRN1 with specific siRNA oligonucleotides were also performed where the cells were later exposed to ER and oxidative stress as described earlier.

Similar sets of cells were used for cell viability assays. One set with MGRN1 overexpression was used with or without co-transfection of Hsp70. After transfection, cells were treated with different stress-inducing agents. ER (Thapsigargin and Tunicamycin) and oxidative stress (H_2O_2) cause a decline in cell viability.

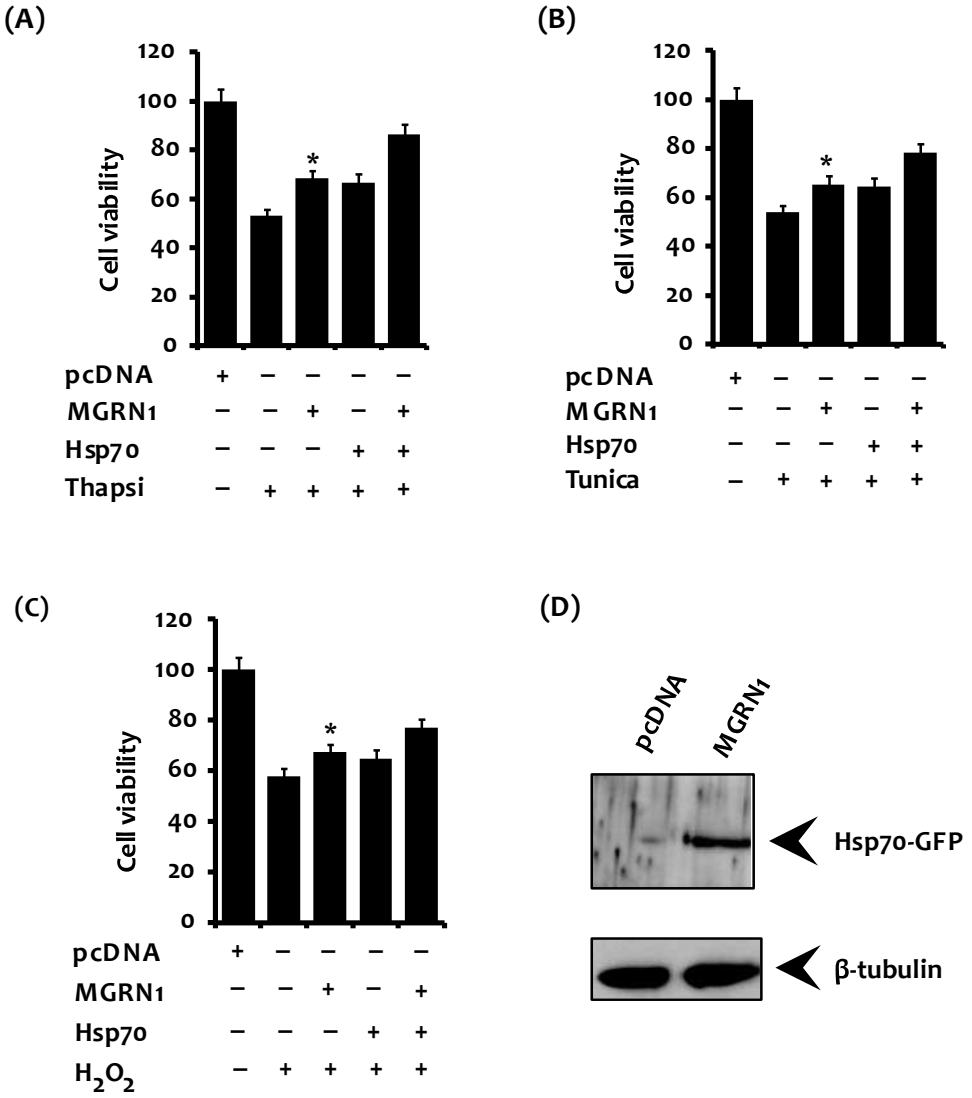

Figure 3.21 : *MGRN1 leads to relatively increased cell viability under endoplasmic and oxidative stress conditions:* (A–C) A549 cells were transiently transfected with the MGRN1-Myc plasmid and some of the cells co-transfected with the Hsp70-EGFP construct. After 48h of transfection, cells were treated with 5 µg/ml Thapsigargin for 8h (A), 10 µg/ml Tunicamycin for 10h (B), or 0.5 mM H_2O_2 for 5h (C) and cell viability was measured using an MTT assay. (D) Representative blot for overexpression of Hsp70. Values are presented as the mean ± S.D. of three independent experiments, each performed in duplicate. *, $p < 0.05$ compared with the empty pcDNA-transfected (stress inducing treated) experiment.

It was observed that cells were highly sensitive to the stress; cell viability was significantly reduced after the partial knockdown of MGRN1 in cells under ER stress (Figure 3.22A and 3.22B) and oxidative stress (Figure 3.22C). Overall, all these results show that MGRN1 plays an important role in the cytoprotection of cells under ER and oxidative stresses.

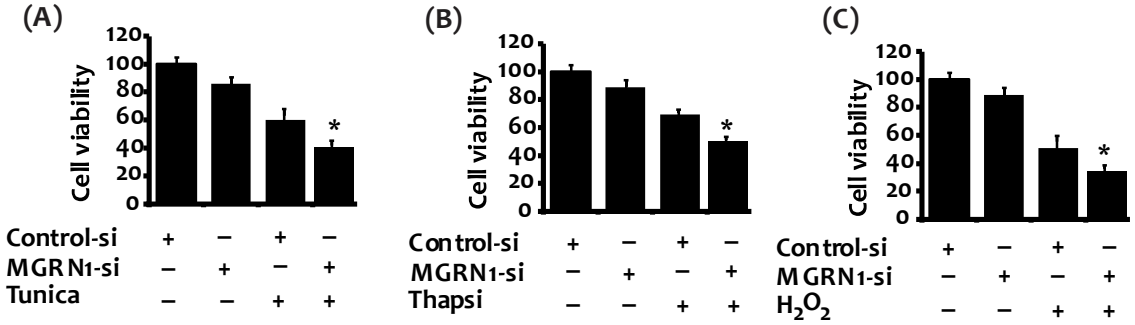

Figure 3.22 : *MGRN1 knockdown decreases cell viability under stress conditions:* (A-C) A549 cells were transiently transfected with control (scrambled siRNA) or MGRN1 siRNA oligonucleotides and treated with Thapsigargin (A), Tunicamycin (B) and H_2O_2 (C) for MTT cell viability assay as described in Figure 3.20.

3.2 DISCUSSION

The study presents the first report that mahogunin ring finger-1 (MGRN1), a putative E3 Ubiquitin ligase, interacts with Hsp70-anchored misfolded protein inclusions and promotes their degradation via autophagy. The end product of the MGRN1 gene is a member of the "really interesting new gene" (RING) domain family protein which contains the E3 Ubiquitin ligase activity [He *et al.,* 2003]. Under proteotoxic insult, cells generate defense mechanisms that maintain a delicate balance between protein folding and degradation with the help of molecular chaperones and E3 Ubiquitin ligases. A recent study showed that some newly synthesized and pre-existing proteins sequester with amyloidogenic aggregates. Furthermore, the lack of function of those critical proteins may generate toxicity in cells [Olzscha *et al.,* 2011]. Nearly 30% of newly synthesized proteins are not folded properly [Schubert *et al.,* 2000]. Under cellular stress conditions; this imbalance in proteostasis may be aggravated by an overabundance of misfolded protein aggregates or an insufficient chaperone capacity.

It has been shown that the massive aggregation of misfolded proteins impairs the Ubiquitin proteasome system [Bennett *et al.,* 2005]. How cells survive after proteasomal inhibition and which other pathways are adopted for the clearance of misfolded proteins remains a valid and open question. Numerous reports suggest that the existence of CMA also reduces the overabundance of misfolded proteins [Bejarano and Cuervo, 2010]. However, how CMA recognizes and specifically targets misfolded proteins for degradation through autophagy is not fully understood. The possibility of an interaction between lysosomes, chaperones, and E3 Ubiquitin ligases remains unresolved. The pentapeptide consensus motif retaining cytosolic proteins are recognized by chaperones for autophagy; this degradation pathway does not require vesicle trafficking [Massey *et al.,* 2006]. Neural precursor cells that express both developmentally down-regulated protein 4 (NEDD4) and atrophin-interacting Protein 4 (AIP4)/Itch-E3 Ubiquitin ligases and have been implicated in the degradation of melanocytic transmembrane proteins (*i.e.*, Melan-A/MART-1) by lysosomes in pigmented cells. This study suggests that E3 Ubiquitin ligases mediate the ubiquitylation process that controls lysosomal sorting [Levy *et al.,* 2005]. In mouse mutation in MGRN1 Ubiquitin ligase cause embryonic lethality and generates spongiform neurodegeneration [He *et al.,* 2003]. MGRN1 monoubiquitinates tumor susceptibility gene 101 (TSG101) under *in vitro* and *in vivo* conditions [Kim *et al.,* 2007]. MGRN1 physically interacts with melanocortin receptors (MCRs), and the overexpression of Gαs eliminates the inhibitory effect of MGRN1 on MC1R signaling [Perez-Oliva *et al.,* 2009]. Previous reports support the current findings which suggest that MGRN1-E3 Ubiquitin ligases may have a potential role in the degradation of misfolded proteins via autophagy. Here, for the first time, it is shown that MGRN1 mRNA and protein levels were

substantially increased after treatment with various stress-inducing agents. Other E3 ligases, including E6-AP, CHIP, and Parkin, are also increased after exposure to various stressors and play a cytoprotective role against stress-mediated cell death [Imai *et al.,* 2002; Jana *et al.,* 2005; Dikshit and Jana, 2007; Mishra *et al.,* 2009].

Recently, it has been reported that the molecular chaperone Hsp70 protein interacts with bis (monoacylglycero) phosphate (BMP) protein, and this interaction stabilizes the lysosomal membrane and thus reduces lysosomal pathology in Niemann-Pick disease in fibroblasts. Because it was noticed that MGRN1 follows the same induced expression profile as Hsp70 following heat shock stress which leads to the hypothesis that MGRN1 might be able to recognize Hsp70-associated misfolded proteins and promote their degradation through autophagy. It was also noticed that MGRN1 interacts with the Hsp70 chaperone. Notably, when the work was continued, another finding was made that the 5′-untranslated region of MGRN1 retained a heat shock factor 1 consensus binding site and one CMA motif; this further supports the current findings. It has been reported previously that Hsp70 interacts with E3 Ubiquitin ligases and regulates a delicate balance between protein folding and the degradation of misfolded proteins [McClellan *et al.,* 2005]. Because of our previous results, it was important to demonstrate the functional significance of MGRN1 and Hsp70 under conditions of cellular stress. To answer this question, a series of immunofluorescence experiments were performed. It was observed that following the inhibition of autophagy, MGRN1 was nicely co-localized with p62, Hsp70, and Ubiquitin-positive cytoplasmic IBs. It has been shown previously that p62 binds to ubiquitinated proteins and co-localizes with Ubiquitin-positive aggregates after Baf treatment [Korolchuk *et al.,* 2009]. It was also found that MGRN1 colocalizes with both Hsp70 protein and denatured Luciferase protein after HS treatment. The immunofluorescence staining analysis demonstrated that MGRN1 is recruited with Hsp70-associated heat-denatured Luciferase protein after the inhibition of autophagy. The recruitment of MGRN1 with heat-denatured Luciferase inclusion-like structures generated possibilities to further investigate the functional role of MGRN1 in the degradation of misfolded cytoplasmic proteins. The study shows that MGRN1 overexpression promotes the degradation of heat-denatured Luciferase protein through autophagy. Autophagy inhibits the aggregation of non-native proteins and cell organelles and subsequently supports cell survival under stressed conditions [Levine and Kroemer 2008; Mizushima *et al.,* 2008]. All these studies support the current findings that Hsp70-associated misfolded proteins are preferentially targeted by MGRN1 for selective autophagy.

Exposure to various cellular stressors causes protein misfolding and aggregation and may be a causal factor for known neurodegenerative diseases. After stress exposure, cells can respond in different manners; our current results demonstrate that MGRN1 actively participates in cell survival under various proteotoxic insults. Further confirmation was done through studying the cytoprotective role of MGRN1 during aberrant cellular stress responses; it was noticed that MGRN1 overexpression counteracts the effects of various cellular stressors and promotes cell survival. Partial knockdown of MGRN1 induced cytotoxicity and consequently suppressed the cell growth mediated by various stress-inducing agents. Mitochondrial malfunction and oxidative stress have been observed in Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD) [Beal, 2005]. It has been established that mitochondrial dysfunction and cellular stress are causally associated with neurodegeneration. MGRN1 null mutant mice have lower expression levels of mitochondrial proteins, which lead to mitochondrial dysfunction at an early age [Sun *et al.,* 2007]. The current study demonstrates that down-regulation of MGRN1 dramatically altered mitochondrial membrane depolarization and cytochrome c release. Previous studies and our current results clearly indicate that MGRN1 generates an oxidative stress response, and loss of its function most likely leads to mitochondrial defects in cells. Growing evidence suggests that the overexpression of molecular

chaperones and E3 Ubiquitin ligases alleviates the toxic effects of misfolded proteins in cells and plays a significant role in the biology of protein misfolding and neurodegenerative diseases [Cummings *et al.,* 2001; Wyttenbach *et al.,* 2002; Tsai *et al.,* 2003; Mishra *et al*., 2009].

3.3 CONCLUDING REMARKS

Finally, the study shows that knockdown of MGRN1 under conditions of stress induces cell death and overexpression of MGRN1 protects against cell death induced by various stressors; this cytoprotective effect is more prominent with congruent Hsp70 overexpression. Altogether, our study provides evidences that MGRN1 is recruited to Hsp70-associated misfolded protein inclusions and promotes their degradation via autophagy. In future, a detailed understanding of the molecular pathomechanism underlying the role of MGRN1 in protein-folding diseases will provide greater knowledge of how misfolded proteins lead to cellular damage and death.

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