4
Mahogunin RING Finger-1 Suppresses Misfolded Polyglutamine Aggregation and Cytotoxicity

A striking clinical hallmark of many neurodegenerative diseases is the presence of Ubiquitin-positive intracellular inclusions formed by the aggregation of non-native neurotoxic proteins [Kopito, 2000; Ross and Poirier, 2004; Ravikumar and Rubinsztein, 2006]. Polyglutamine diseases are caused by a (CAG)*n* expansion within the coding region of the responsible gene. Polyglutamine diseases such as Huntington's disease (HD), spinocerebellar ataxias (types 1, 2, 3, 6, 7 and 17) and X-linked spinal bulbar muscular atrophy (SBMA) are most likely caused by the neuronal dysfunction or neuronal cell death that results as the lengths of the glutamine stretches/repeats increase; early disease onset and the accumulation of intracellular aggregates are strongly linked with increased polyglutamine tract length [Zoghbi and Orr, 2000; Margolis and Ross, 2001; Jana *et al.*, 2005]. The question of whether the molecular pathology that underlies polyglutamine disease is caused by a gain or a loss of function is controversial. However, the formation of intraneuronal aggregates and the deposition of inclusion bodies represent a failure of the cellular protein quality control mechanism (QC). Currently, there are no known treatments that effectively prevent or eliminate aggregate formation in polyglutamine diseases.

4.1 RESULTS

In the present work, study was performed to understand the detailed mechanism of the ubiquitination and degradation of expanded polyglutamine proteins by mahogunin ring finger-1 (MGRN1) E3 Ubiquitin ligase through the autophagy pathway. This report provides evidence that MGRN1 interacts with expanded polyglutamine proteins and recruits with Ubiquitin and p62-positive expanded polyglutamine protein aggregates in both cellular and R6/2 transgenic mouse models. Moreover, MGRN1 is involved in the ubiquitination and degradation of expanded polyglutamine proteins and thereby could be a potential protein of the disease.

4.1.1 Misfolded and Ubiquitinated Expanded Polyglutamine Proteins Dysregulate MGRN1 Expression

To examine the effect of polyglutamine on endogenous MGRN1 levels, cells were transfected with normal (Ataxin-3(28Q) and EGFP-HDQ23, EGFP tagged Huntingtin exon 1 protein with 23 polyQ repeats) and expanded polyglutamine (Ataxin-3(84Q) and EGFP-HDQ74-EGFP-tagged Huntingtin exon 1 protein with 74 polyQ repeats). These polyglutamine proteins with expanded repeat lengths form aggregates when overexpressed. Total RNA were isolated from cells with GFP-positive aggregate and RT-PCR analysis was performed for MGRN1. Significant decrease in MGRN1 mRNA levels in the expanded polyglutamine Ataxin-3(84Q) (Figure 4.1A and 4.1C) and EGFP-HDQ74 (Figure 4.1B and 4.1D) expressing cells was observed. Approximately, a 0.5-fold decrease in MGRN1 mRNA levels has been observed in the expanded polyglutamine expressing cells compared with control cells (Figure 4.1E).

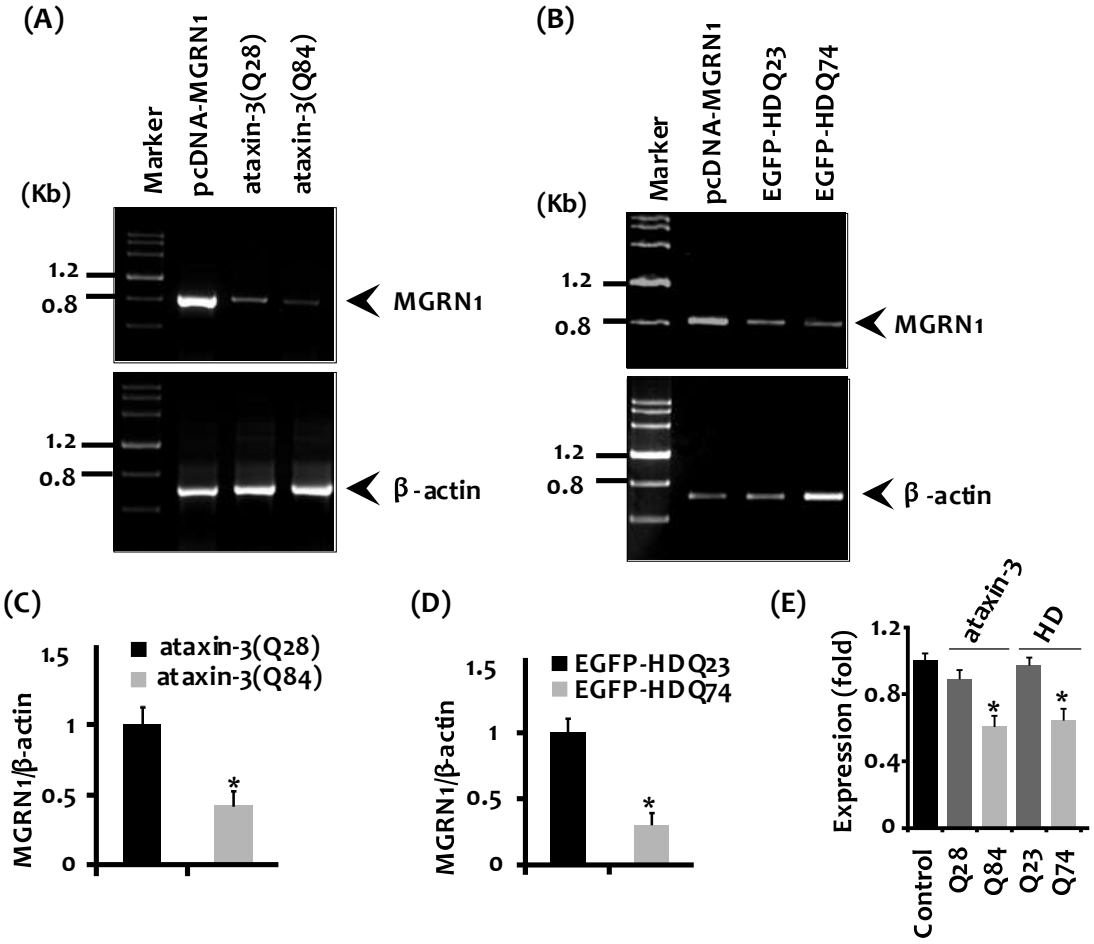

Figure 4.1 : *MGRN1 dysregulation in expanded polyglutamine-expressing cells*: (A-B) A549 cells were transfected with Ataxin-3 (A) or EGFP-HD (B) normal and polyglutamine-expanded expression plasmids as indicated. After 48h, total RNA was isolated from the cells and processed for RT-PCR analysis using MGRN1 and β-actin primers. (C-D) The bar diagram represents the quantitation of the MGRN1 band intensities shown in A and B. Data were collected from three independent experiments using NIH image analysis software and normalized to the levels of β-actin. Values are the mean ± S.D. *, $p < 0.05$ compared with Ataxin-3(Q28)- (C) and EGFP-HDQ23- (D) expressing cells. (E) Cells were transiently transfected with Ataxin-3 or EGFP-HD normal and polyglutamine-expanded expression plasmids including control (pcDNA) plasmid. After 48h, quantitation of MGRN1 mRNA levels using quantitative reverse transcriptase-PCR in the experiment.

Overexpression of the polyglutamine constructs was done in Cos-7 cells where 50-60% cells were found to be positive for expanded polyglutamine Ataxin-3 (Figure 4.2A) as well as for Huntingtin expanded polyglutamine aggregates (Figure 4.2B) after 48h. The expression of polyglutamine proteins tagged with GFP was also confirmed through immunoblotting analysis using anti-GFP and anti-Actin antibodies (Figure 4.2C).

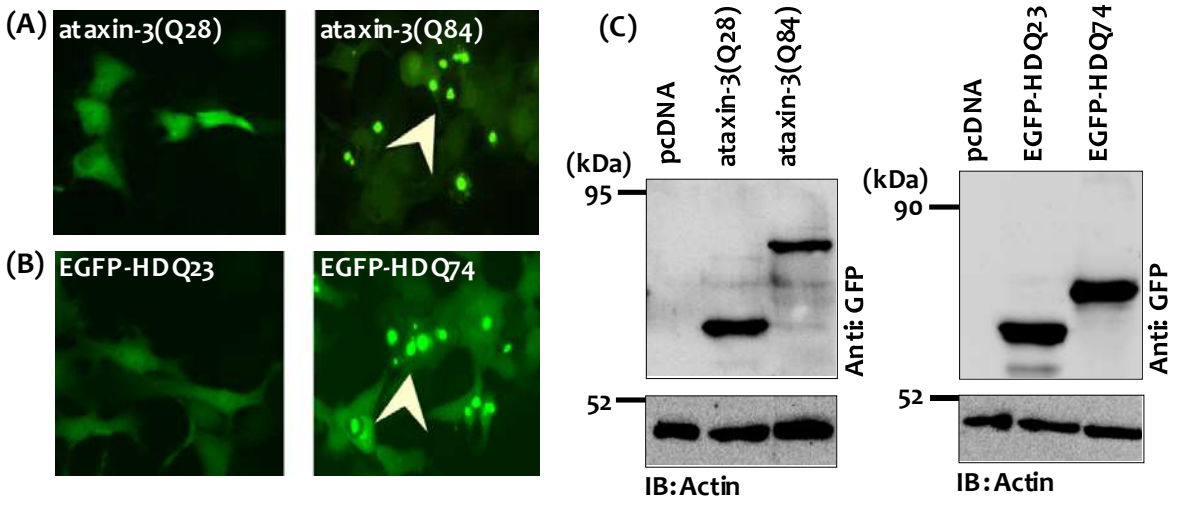

Figure 4.2 : *Expression and immunoblotting of polyglutamine proteins:* Aggregate formation in Ataxin-3(Q84)- (A) and EGFP-HDQ74- (B) expressing cells. (C) The same cells were subjected to immunoblotting analysis using GFP and actin antibodies.

Next was to test the endogenous levels of MGRN1 in normal and expanded polyglutamine expressing cells (Figure 4.3A). Dysregulation in MGRN1 protein levels were speculated to be most likely due to sequestration of endogenous MGRN1 with expanded polyglutamine proteins. To examine, a filter trap assay was performed with cell lysates obtained from expanded polyglutamine expressing cells, where MGRN1 was majorly observed in association with insoluble aggregates of expanded polyglutamine proteins (Figure 4.3B).

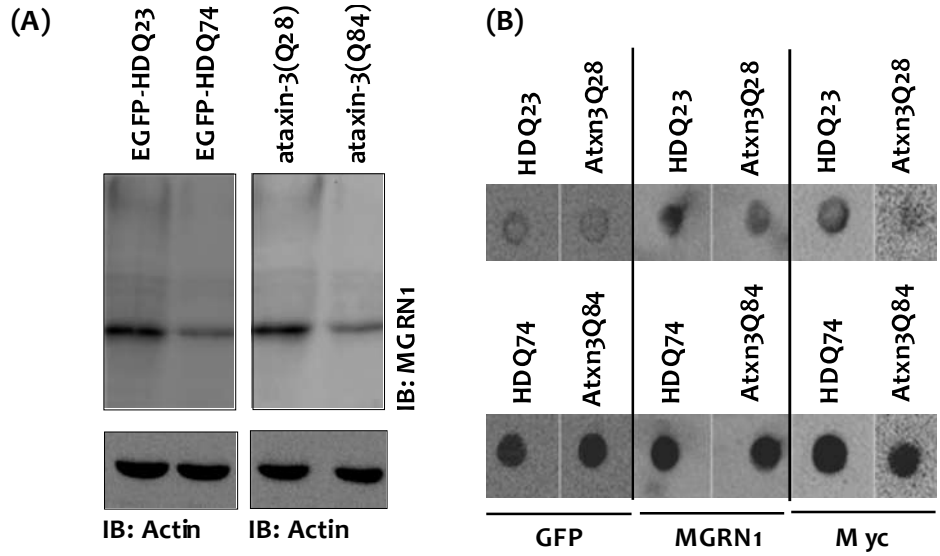

Figure 4.3 : *MGRN1 protein levels are decreased after overexpressing polyglutamine proteins*: (A) Cells were co-transfected as described above with Ataxin-3, normal (Ataxin-3(Q28)) and expanded-polyglutamine (Ataxin-3(Q84)) and Huntingtin, normal (EGFPHDQ23) and polyglutamine-expanded (EGFP-HDQ74) constructs. Forty-eight hours after transfection, cells were collected and processed for immunoblotting with MGRN1 and actin antibodies (A). Some cells were co-transfected with Myc-MGRN1 plasmid. Same cell lysates were processed for filter-trap analysis by probing with antibodies against GFP, MGRN1 and Myc (B).

It was hypothesized that aggregation of expanded polyglutamine promotes their sequestration and these events might compromise MGRN1 protein function in the expanded polyglutamine expressing cells. To test the hypothesis, normal and expanded polyglutamine proteins were overexpressed in cells and processed for immunoblotting using a Ubiquitin antibody. As shown in Figure 4.2, approximately more than 50% of the EGFP-HDQ74 and Ataxin-3(84Q)-expressing cells formed GFP-positive aggregates. The Huntingtin proteins with 74Q repeats and the Ataxin-3 proteins with 84Q repeats were ubiquitinated, whereas the proteins with normal glutamine repeats did not exhibit ubiquitination. To confirm the hypothesis, same samples were processed for an immunoblotting analysis of MGRN1. Further analysis showed the dysregulation in the endogenous levels of MGRN1 protein in cells that expressed ubiquitinated expanded polyglutamine proteins compared with the cells expressing non-ubiquitinated proteins with normal numbers of glutamine repeats (Figure 4.4A, 4.4B and 4.4C).

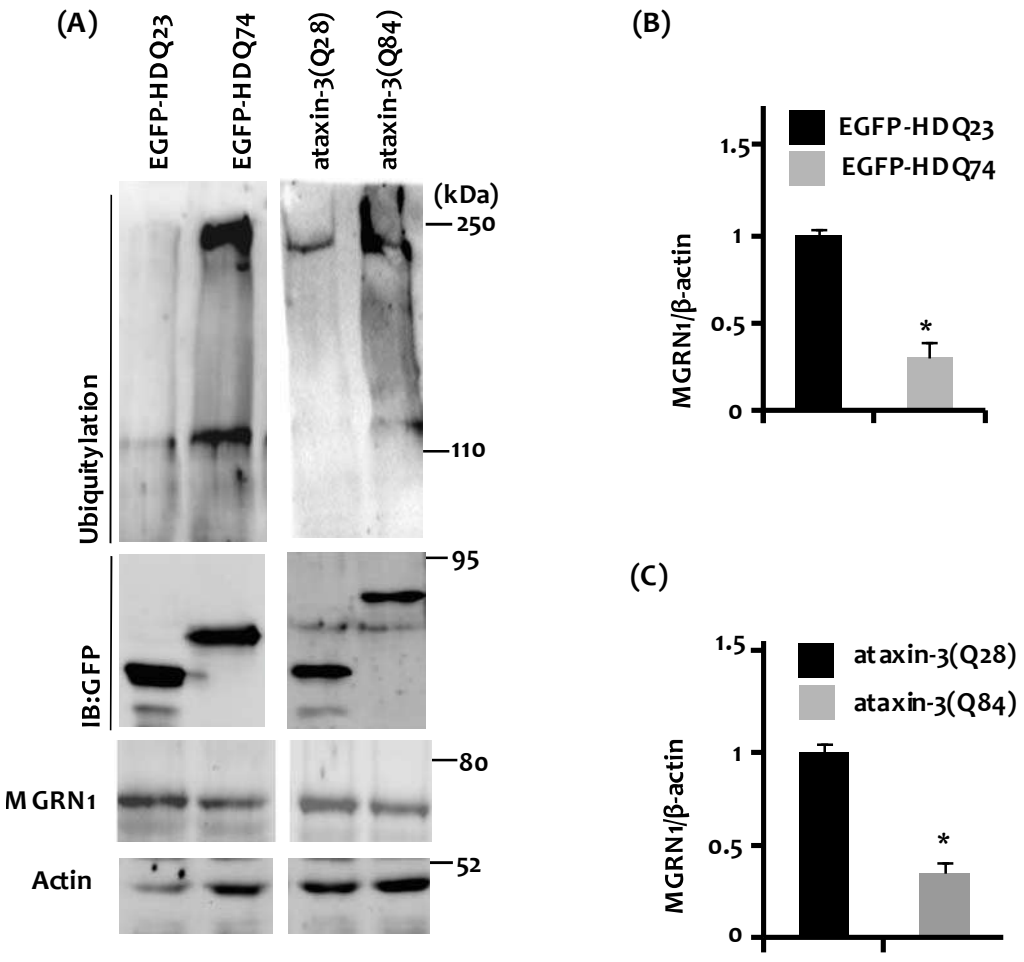

Figure 4.4 : *Ubiquitination of expanded polyglutamine proteins depletes the endogenous level of MGRN1 protein and interaction of MGRN1 with soluble misfolded normal and expanded polyglutamine proteins:* (A) Ataxin-3 normal (Ataxin-3(Q28)) and expanded (Ataxin-3(Q84) polyglutamine, and EGFP-HD normal (EGFP-HDQ23) and expanded (EGFP-HDQ74) polyglutamine plasmids were transiently transfected in 293T cells; 48h after transfection, the cells were subjected to immunoblotting using ubiquitin, GFP, MGRN1 and actin antibodies (B-C). Quantification of endogenous MGRN1 levels collected from three independent experiments using NIH Image analysis software. Data were normalized using actin. Values are the mean ± S.D. *P<0.05 with respect to cells expressing proteins with normal numbers of glutamine repeats.

4.1.2 MGRN1 Interacts with the N-terminal of Truncated Misfolded Huntingtin and Ataxin-3 with Expanded Polyglutamine Tracts

The preliminary results suggested that MGRN1 interacts with mutant expanded polyglutamine proteins which may be one of the factors involved in the dysregulation of endogenous MGRN1. A stochastic interaction between MGRN1 and expanded polyglutamine proteins was likely to be there and could lead to a huge depletion of MGRN1 at the site of localization or function. To validate this assumption, interaction of MGRN1 with expanded polyglutamine proteins were checked. First, the normal (Ataxin-3(28Q) and EGFP-HDQ23) or expanded polyglutamine (Ataxin-3(84Q) and EGFP-HDQ74) constructs were overexpressed in cells. Same set of cells were co-transfected with MGRN1 construct as shown in Figure 4.2 MGRN1 was strongly and specifically immunoprecipitated with the Huntingtin (EGFP-HDQ74) and Ataxin-3(84Q) compared with the Huntingtin (EGFP-HDQ23) and Ataxin-3(28Q). Figure 4.5B shows the same samples as in Figure 4.5A respectively, probed with anti-GFP. A detailed immunoprecipitation study was performed using different control experiments to confirm the strong interaction of MGRN1 with the N-terminals of expanded polyglutamine Huntingtin and Ataxin-3 proteins.

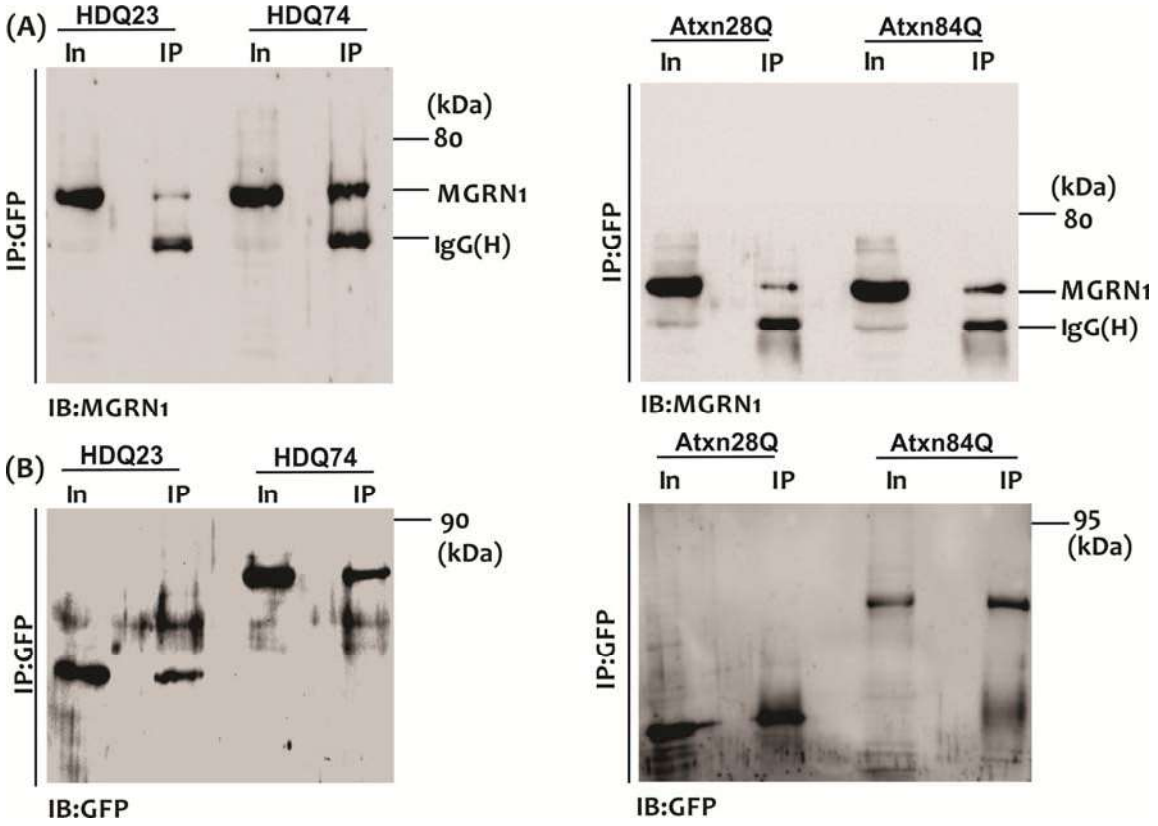

Figure 4.5 : *MGRN1 interacts with polyglutamine proteins:* (A-B) Transfection was used to overexpress Myc-MGRN1 and normal (EGFP-HDQ23) and polyglutamine-expanded (EGFP-HDQ74) and Ataxin-3 normal (Ataxin-3(Q28)) and expanded polyglutamine (Ataxin-3(Q84)) constructs in Cos-7 cells. After 48h of transfection, the cell lysates were processed for immunoprecipitation (IP) with a GFP antibody. Blots were sequentially probed with anti-MGRN1 (A) and anti-GFP (B).

Further transfections of normal and expanded polyglutamine constructs of the N terminals of Huntingtin or Ataxin-3 proteins were done and the cell lysates were immunoprecipitated with beads only (control) (Figure 4.6) and normal IgG (Figure 4.7A and 4.7B), and the blots were probed with anti-MGRN1 and anti-GFP antibodies as indicated in the figures. To further confirm the interaction of MGRN1 and expanded polyglutamine proteins, cell lysates were also pulled down with MGRN1 antibody and blots were processed with anti-GFP (Figure 4.7C) and for control experiment, only GFP expressing cell lysates were used for immunoprecipitation with MGRN1 antibody and blot was detected with GFP antibody (Figure 4.7D). These results confirmed the finding that MGRN1 strongly interacts with the N-terminals of Huntingtin and Ataxin-3 proteins with expanded polyglutamine tracts.

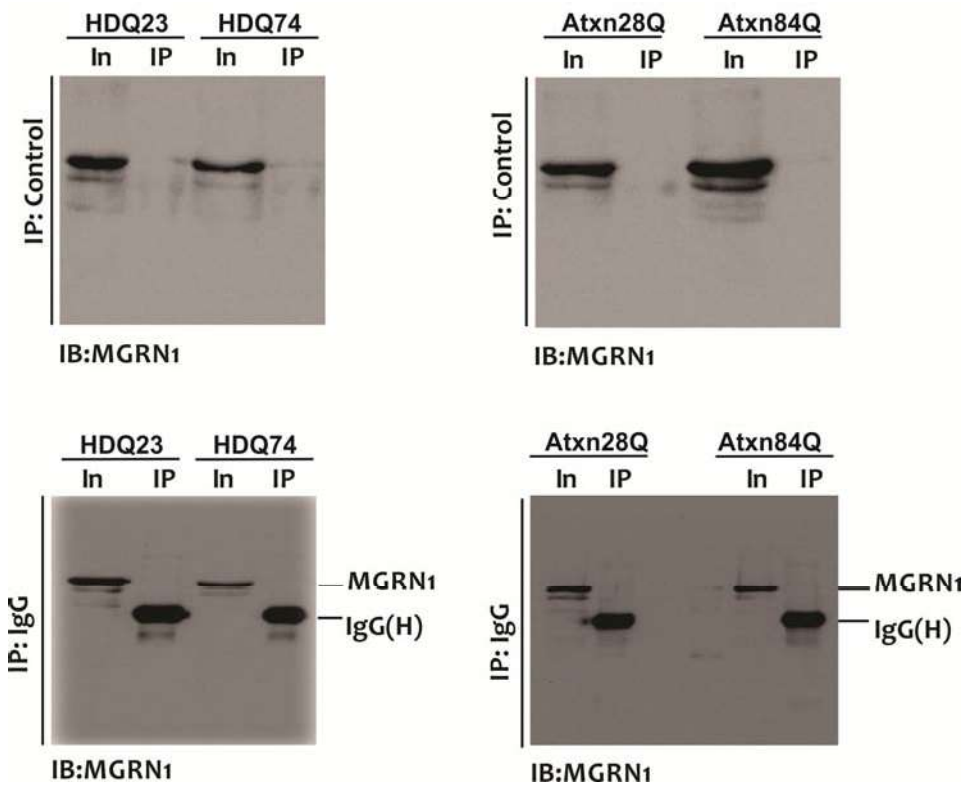

Figure 4.6 : *Negative controls for interactions of MGRN1 and polyglutamine proteins:* Cell lysates were collected from Ataxin-3 and EGFP-HD normal and expanded polyglutamine-expressing cells and processed for IP by beads alone (control-IP) and blots were developed by anti-MGRN1.

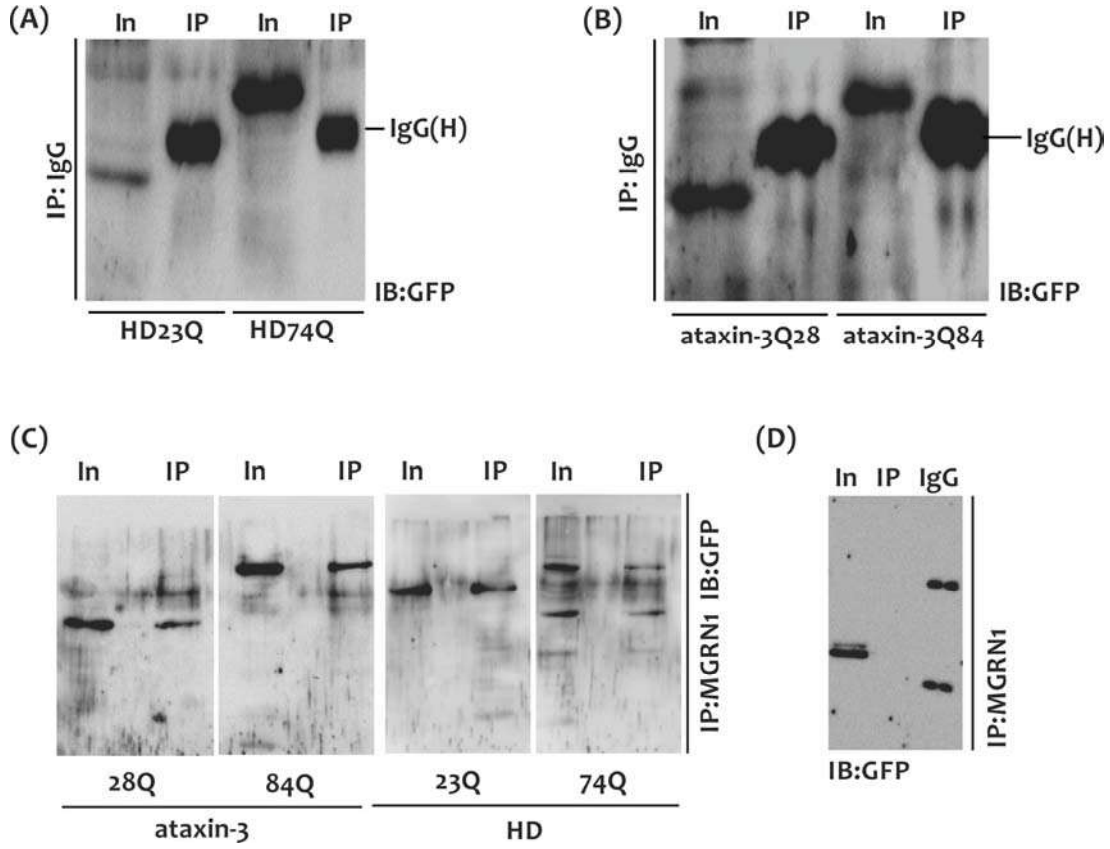

Figure 4.7 : *Polyglutamine proteins interact with endogenous MGRN1 protein*: (A-D) As described above some cell lysates were used immunoprecipitation (IP) with normal IgG and blots were probed with anti-GFP antibodies (A-B). (A) Cells were transfected with normal (EGFP-HDQ23) and polyglutamine-expanded (EGFP-HDQ74) constructs while another set of cells were transfected with normal (Ataxin-3(Q28))and expanded polyglutamine (Ataxin-3(Q84)) and lysates were processed for IP by anti MGRN1 and blots were developed with anti-GFP. (C) Cells were transfected with only GFP expressing construct and lysates were processed for IP with MGRN1antibody and blot were developed with GFP antibody. Immunoprecipitants were subject to immunoblotting analysis with the indicated antibodies.(D) Cells were transfected with only GFP and immunoprecipitated by MGRN1 antibody and then blot was developed by GFP antibody.

4.1.3 Association and Recruitment of MGRN1 with Polyglutamine Aggregates

To examine whether the subcellular localization of endogenous MGRN1 is affected by polyglutamine aggregates, transfected normal and expanded polyglutamine protein Huntingtin (EGFP-HDQ23 (Figure 4.8A) and EGFP-HDQ74) (Figure 4.8B) and Ataxin-3 (Ataxin-3(28Q) (Figure 4.8C) and Ataxin-3(84Q)) (Figure 4.8D) constructs in cells. After transfection, cells were subjected to immunofluorescence staining using MGRN1 antibody. Endogenous MGRN1 was recruited with the Huntingtin and Ataxin-3 expanded polyglutamine aggregates in cells as shown in Figure 4.8. Next, co-transfection of Huntingtin (EGFP-HDQ23 and EGFP-HDQ74) and Ataxin-3 (Ataxin-3(28Q) and Ataxin-3(84Q)) constructs with the MGRN1 plasmid were performed. After 48h of transfection, cells were subjected to immunofluorescence experiments using the anti-MGRN1 antibody. MGRN1 was localized to the cytoplasm with partial or diffuse staining in the nucleus of cells expressing normal numbers of glutamine repeats, shown in Figure 4.9A (EGFP-HDQ23), and Figure 4.9C (Ataxin-3(28Q)). Overexpressed MGRN1 was found to be associated and colocalized with the aggregates of proteins with expanded polyglutamine repeats in EGFP-HDQ74- and Ataxin-3(84Q)-expressing cells (Figure 4.9B, and Figure 4.9D respectively).

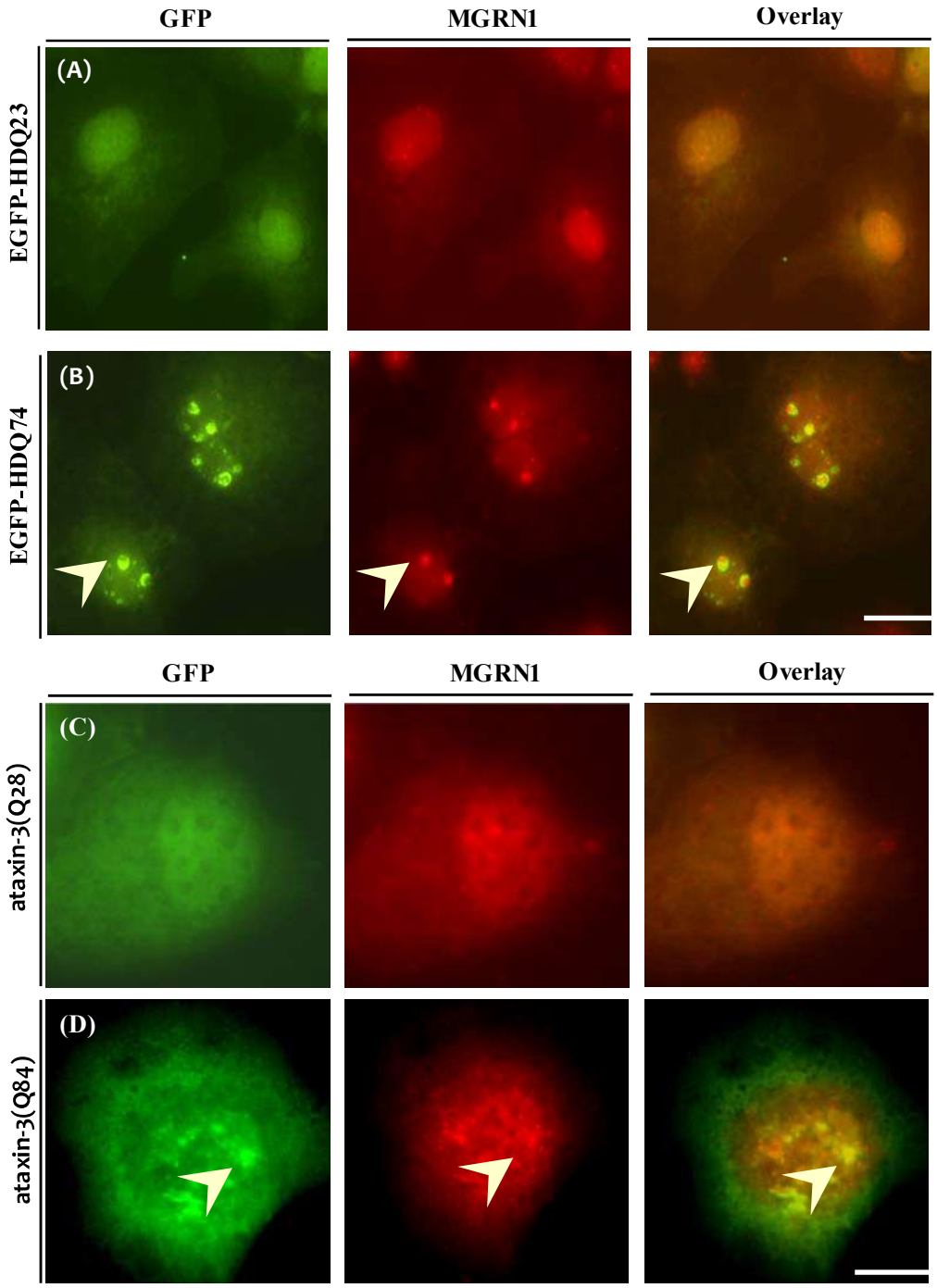

Figure 4.8 : *MGRN1 associates with Huntingtin expanded polyglutamine aggregates and recruitment of MGRN1 to Ataxin-3 aggregates:* (A-B) Cos-7 cells were transiently transfected with EGFP-HDQ23 (A) EGFP-HDQ74 (B) construct and after 48h of transfection, the cells were processed for immunofluorescence staining for MGRN1 (red) and normal and expanded polyglutamine expression (green). (C-D) Cos-7 cells were transiently transfected with Ataxin-3-GFP fusion normal (C) (Ataxin-3(Q28)) and expanded (D) polyglutamine (ataxin- 3(Q84)) constructs. Post transfected cells were subjected to immunofluorescence staining for MGRN1 (red) and normal and expanded polyglutamine expression (green).

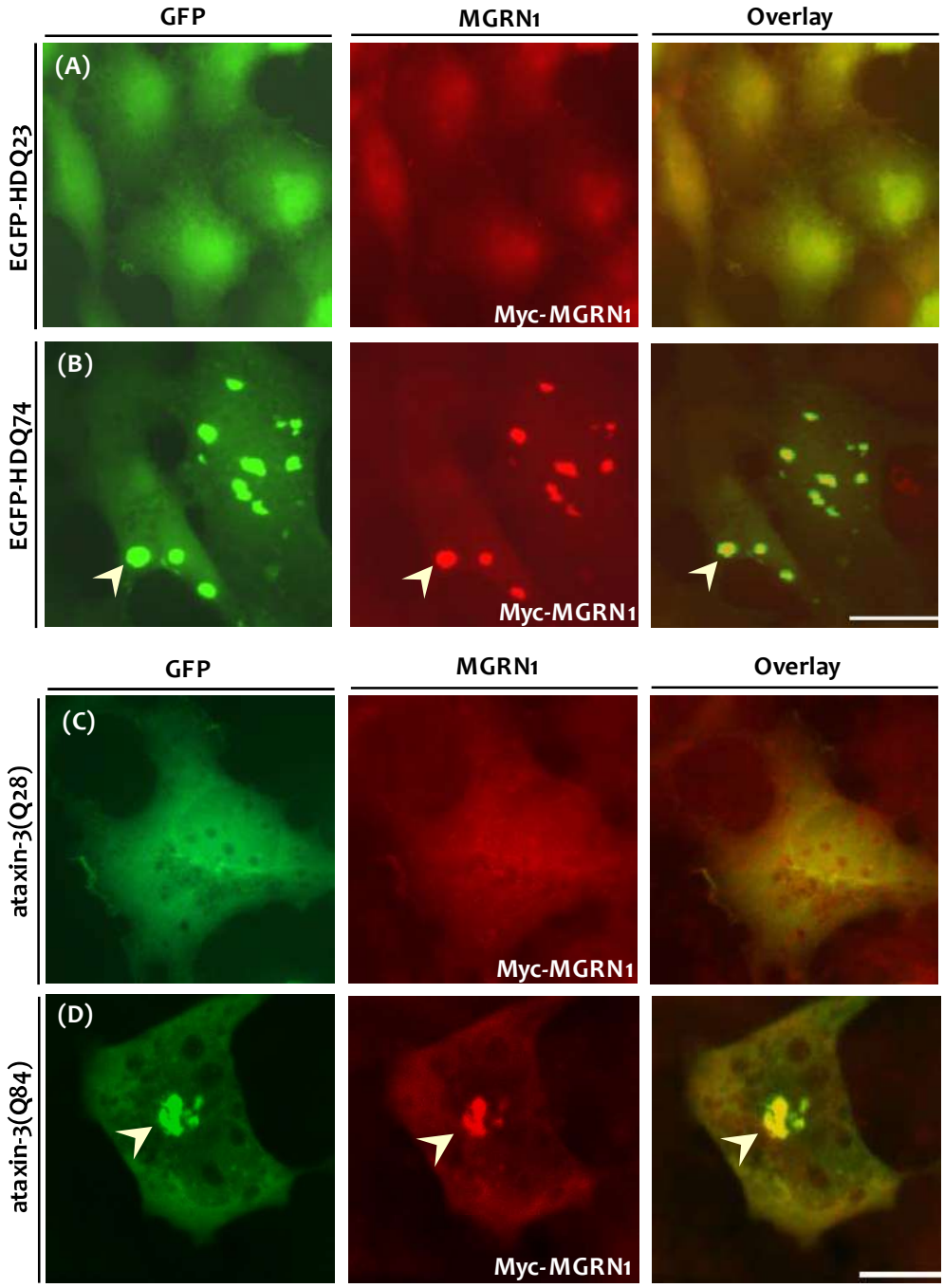

Figure 4.9 : *MGRN1 colocalizes with expanded polyglutamine protein aggregates in cells:* (A-B) EGFP-HDQ23 (A) and EGFP-HDQ74 (B) expressing Cos-7 cells were transiently transfected with Myc-MGRN1 plasmid. Thirty-six hours after transfection, cells were subjected to immunofluorescence staining with anti-MGRN1 (red). A rhodamine-conjugated secondary antibody was used to label MGRN1. Arrows indicate the recruitment of MGRN1 to the Huntingtin aggregates. (C-D) Sub-confluent Cos-7 cells were plated into two-well chamber slides and then cotransfected with Ataxin-3-GFP constructs containing (Ataxin-3(Q28)) (C) and (Ataxin-3(Q84)) (D) polyglutamine constructs along with Myc-MGRN1 plasmid as indicated. After transfection, the cells were fixed in 4% paraformaldehyde and then processed for immunofluorescence with MGRN1 antibody. MGRN1 was visualized with rhodamine-conjugated secondary antibody (red). Arrows indicate the recruitment of MGRN1 to Ataxin-3 aggregates. Scale bar, 20 μm.

To further confirm that the polyglutamine aggregates are positive for MGRN1, a triple-immunofluorescence staining was performed. As shown in Figure 4.10, MGRN1 was present in the cytoplasm and had a diffuse localization or staining pattern with p62 and Ubiquitin in EGFP-HDQ23-expressing cells with a normal number of glutamine repeats. Strikingly, MGRN1 recruits and co-localizes with p62 and Ubiquitin double-positive aggregates of expanded polyglutamine proteins (Figure 4.10A and 4.10B). These results indicate a strong and specific interaction between MGRN1 and misfolded-expanded polyglutamine proteins or their aggregates.

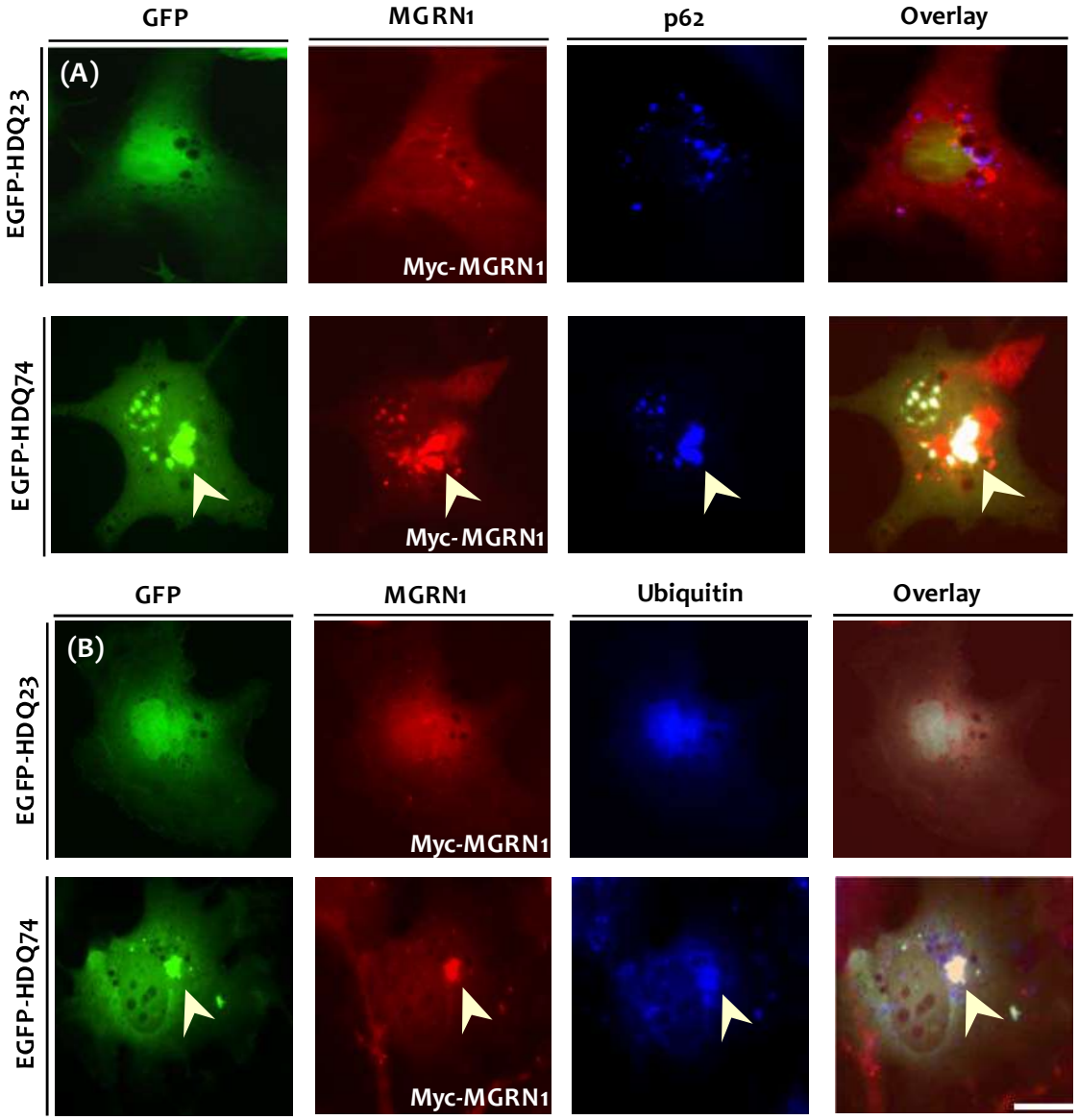

Figure 4.10 : *MGRN1 colocalization with Huntingtin expanded polyglutamine protein, p62 and Ubiquitin-positive aggregates in cells:* Cos-7 cells were transiently co-transfected with EGFP-HDQ23 or EGFP-HDQ74 constructs and Myc-MGRN1 plasmid. After 48h of transfection, the cells were subjected to triple-immunofluorescence staining for MGRN1 (red), p62 (blue) or Ubiquitin (Blue) and EGFP-HDQ74 expression (green) as described in the methods. Arrows indicate the recruitment of MGRN1 to Huntingtin expanded-polyglutamine aggregates that are positive for p62 staining. Scale bar, 20 μm.

4.1.4 Recruitment and Colocalization of MGRN1 with p62 and Ubiquitin into Neuronal Aggregates of Mutant Huntingtin in Brain of R6/2 Transgenic Mice

To confirm the current finding that MGRN1 interacts and associates with p62 and ubiquitin positive polyglutamine aggregates in animal models, the recruitment of MGRN1 in the R6/2 transgenic mouse brain, which models HD onset, was examined. The localization of MGRN1 in control and R6/2 mice was compared. Immunofluorescence results show cytoplasmic staining of MGRN1 in cerebellar Purkinje cells and in cortical neurons of control mice. However, in R6/2 HD transgenic mice, MGRN1 was strongly recruited to neuronal p62-positive aggregates in cerebellar Purkinje cells and in cortical neurons (Figure 4.11).

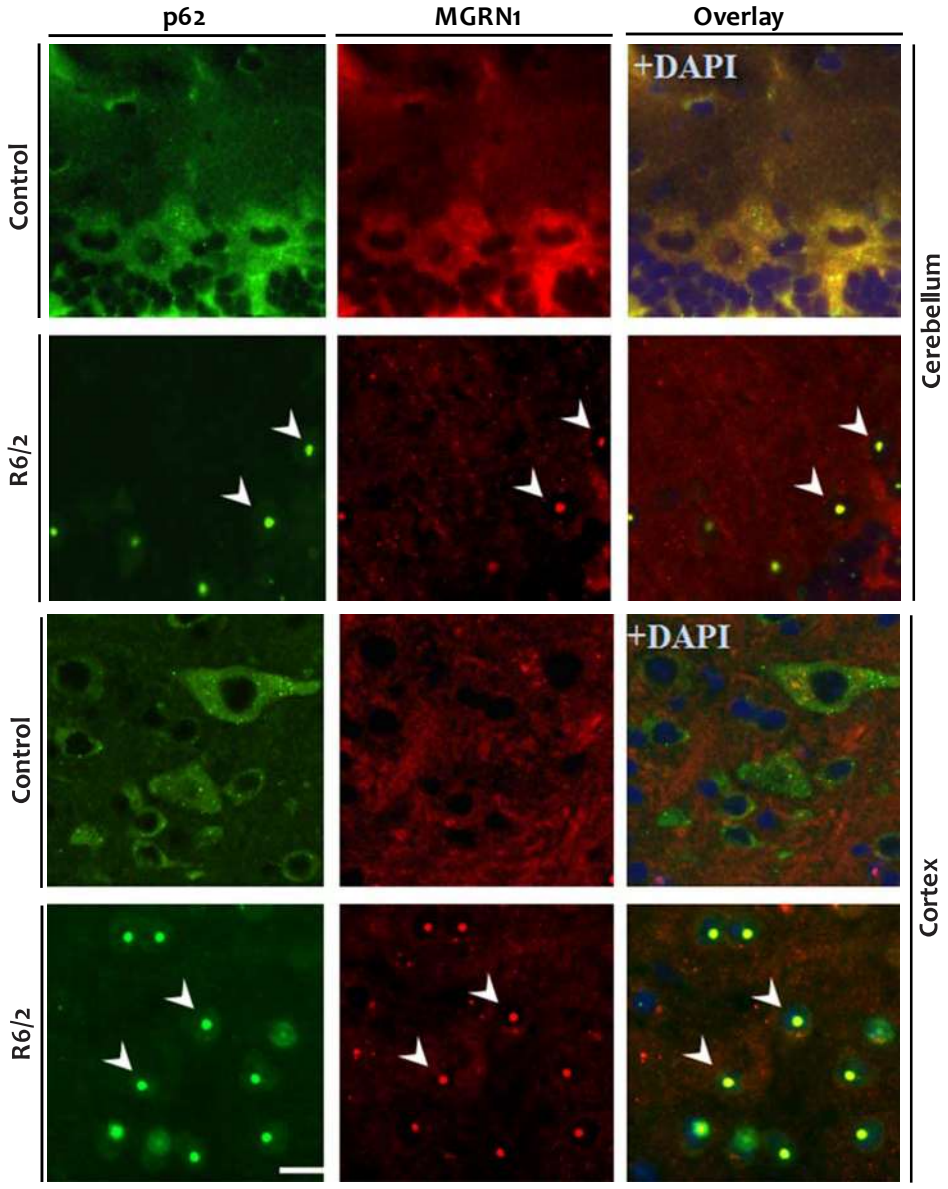

Figure 4.11 : *Redistribution and association of MGRN1 with p62-positive aggregates in R6/2 transgenic mice cerebellum and cortex:* Immunofluorescence double-labeling of MGRN1 and p62 in the brain of R6/2 mouse shows adult mouse brain sections of the cerebellum from control and R6/2 mice, incubated with p62 (green) and MGRN1 (red) antibodies. In the overlay (yellow) image, arrows indicate the aggregates of the expanded Huntingtin that co-localize with p62 and MGRN1 with DAPI (blue) staining. Scale bar, 20 μm.

To confirm the recruitment of MGRN1 with neuronal aggregates of mutant Huntingtin in the R6/2 HD mouse model, double-immunofluorescence staining studies were done using MGRN1 and Ubiquitin antibodies. Analysis of these results showed that more than 70-80% of the Ubiquitin positive aggregates were co-localized and recruited with the MGRN1 protein in the different brain regions of the HD R6/2 transgenic mouse model (Figure 4.12).

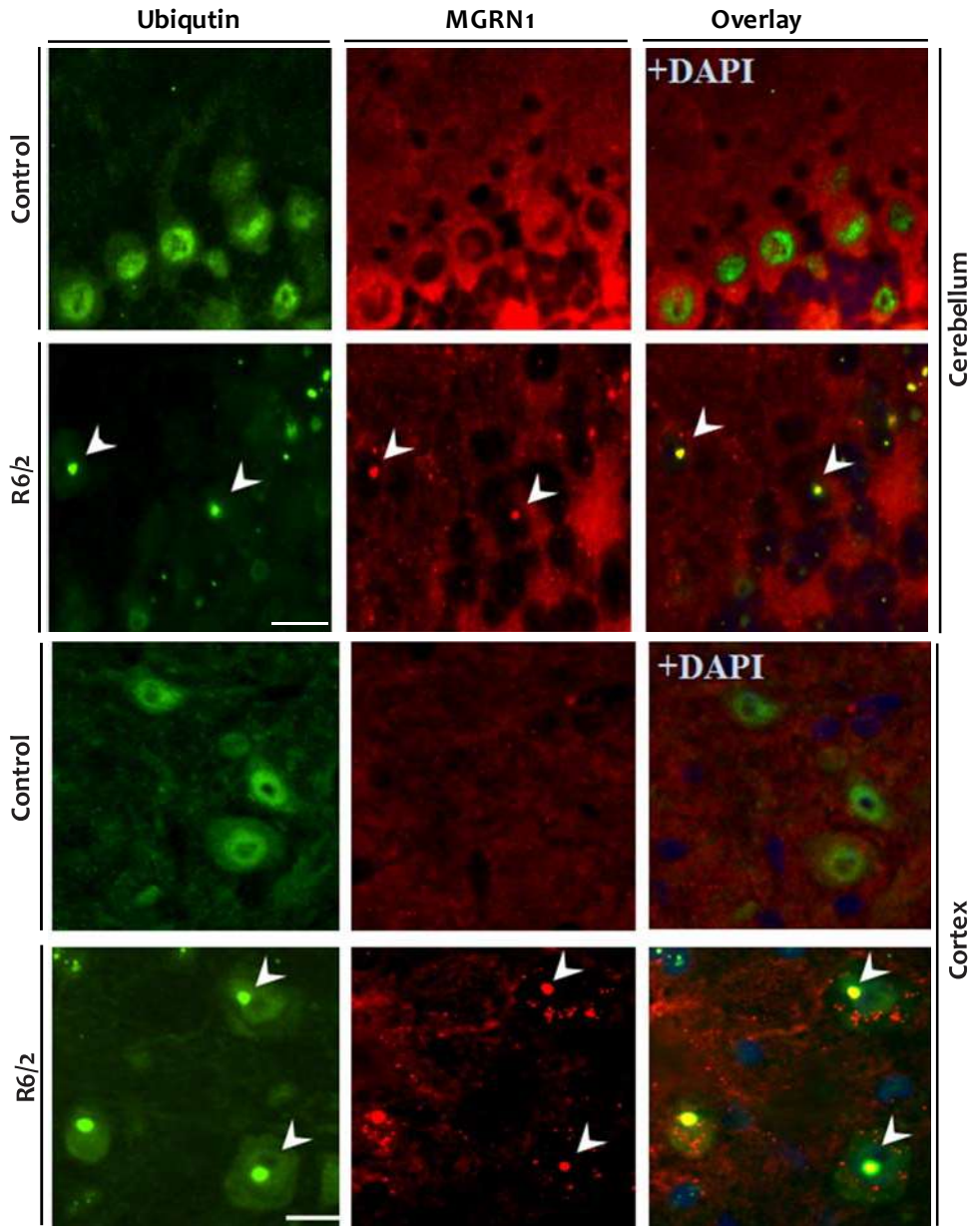

Figure 4.12 : *Colocalization of MGRN1 with Ubiquitin-positive aggregates in the neurons of the brain cerebellum and cortex of transgenic R6/2 mouse model of Huntington's Disease:* Sections from the adult mouse brain subjected to immunofluorescence staining with anti-Ubiquitin and anti-MGRN1 in the cerebellum of control and R6/2 transgenic mice. The FITC-conjugated secondary antibody was used to label the ubiquitin, and rhodamine-conjugated secondary antibody was used to stain MGRN1. Arrows indicate the aggregates of expanded Huntingtin that co-localize with MGRN1 and ubiquitin. The overlay images also show DAPI staining of the cell nuclei. Scale bar, 20 μm.

It was further confirmed that these neuronal (NeuN positive) aggregates (Figure 4.13A and 4.13B) were also positive for Huntingtin staining and were colocalized with MGRN1 and p62 inclusions in R6/2 transgenic mice brain (Figure 4.14A and 4.14B). The quantification of same samples were performed and observed that the normal MGRN1 bearing neurons were decreased in HD R6/2 transgenic mouse model (Figure 4.14C and 4.14D).

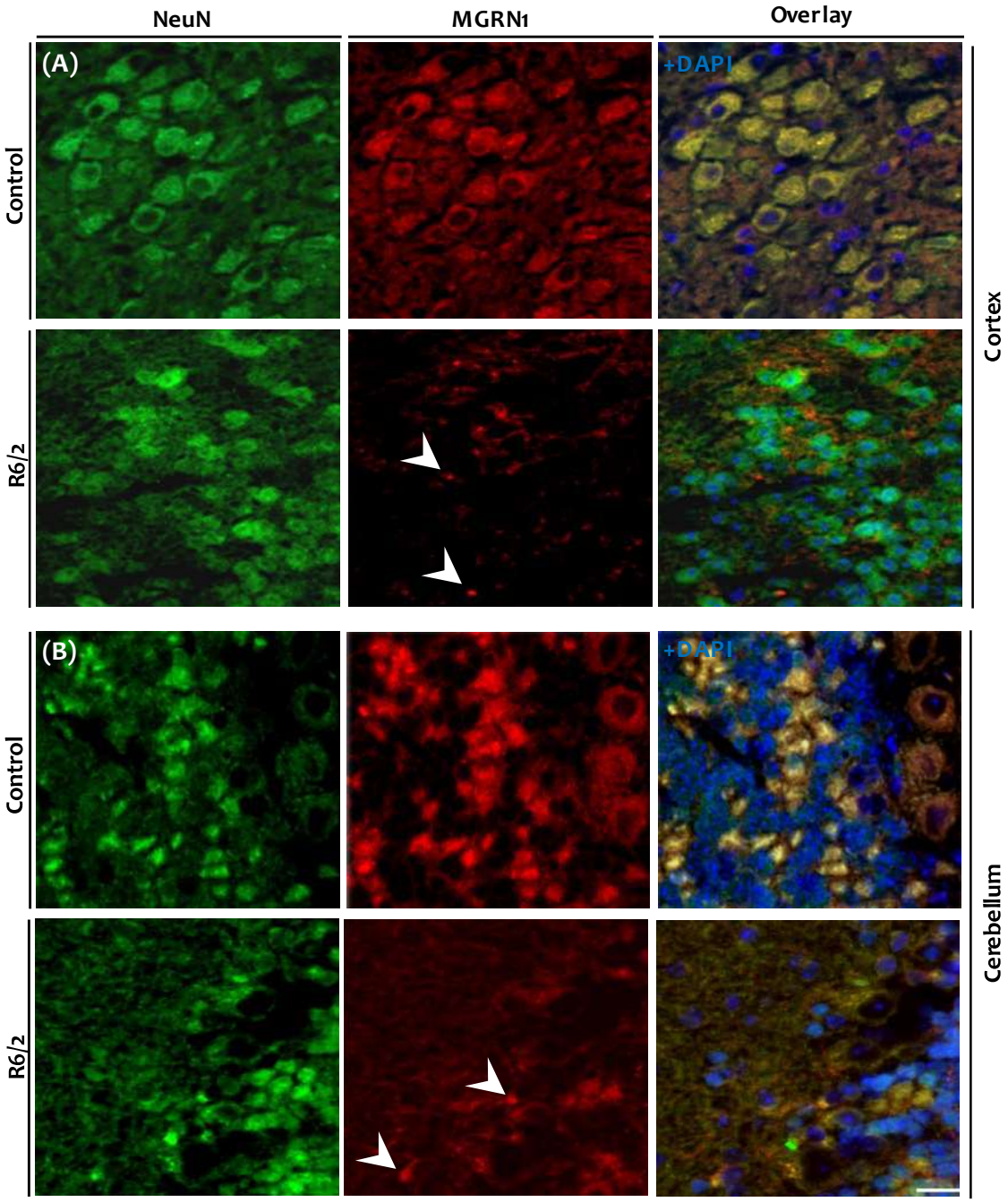

Figure 4.13 : *Recruitment of p62 and MGRN1 with Huntingtin-positive aggregates in the neurons of the transgenic R6/2 mouse model of Huntington's Disease:* (A-B) Brain sections of R6/2 mice were immunostained for NeuN (green) and MGRN1 (red) in cortex (A) and cerebellum (B) regions. The FITC-conjugated secondary antibody was used to label the NeuN, and rhodamine-conjugated secondary antibody was used to stain MGRN1. Arrows indicate MGRN1-positive aggregates in the neurons.

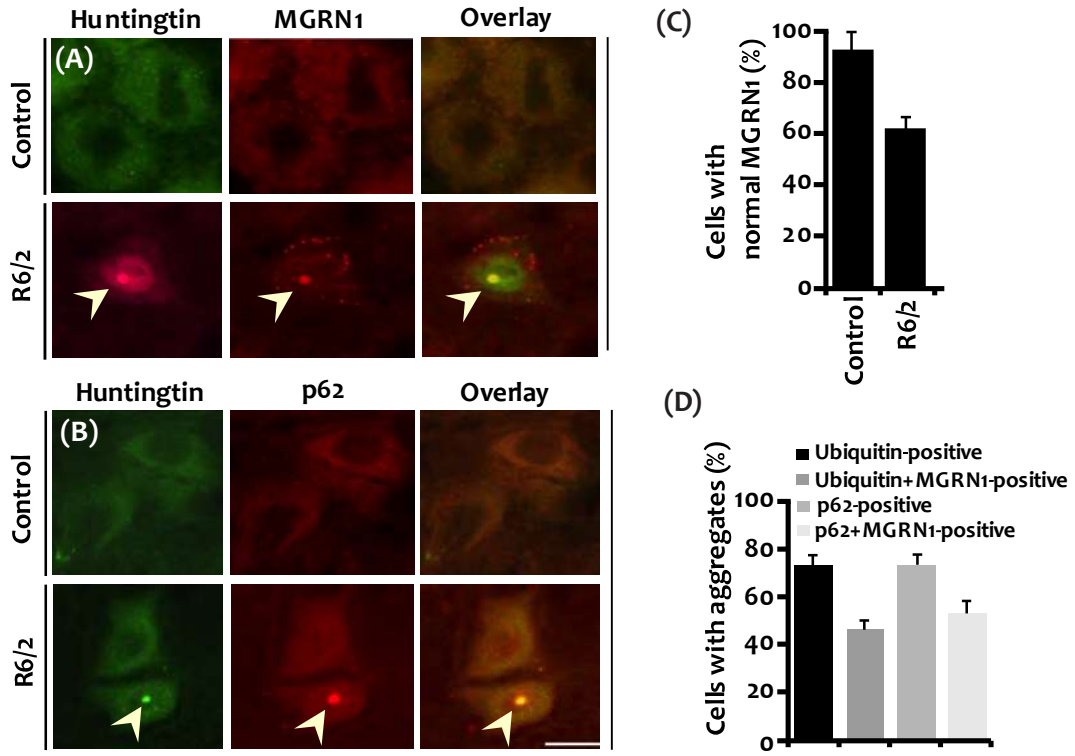

Figure 4.14 : *Colocalization of MGRN1 with Huntingtin and p62-positive aggregates in model mice:* (A) Sections from the adult mouse brain processed to immunofluorescence staining with anti-Huntingtin and MGRN1 antibody in the control and R6/2 transgenic mice. The FITC-conjugated secondary antibody was used to label the Huntingtin, and rhodamine-conjugated secondary antibody was used to stain MGRN1. Arrows indicate the aggregates of expanded Huntingtin that co-localize with MGRN1. (B) Double immunofluorescence staining of p62 and Huntingtin in the brain of an R6/2 mouse. Adult mouse brain sections from control and R6/2 mice were probed with Huntingtin (green) and p62 (red) antibodies. In the overlay (yellow) image, arrows indicate the aggregates of the expanded Huntingtin that co-localize with p62 staining. Merging (yellow) illustrates co-localization of MGRN1 and Huntingtin in the inclusions. Scale bar, 20 μm. (C) Percentage of neurons with normal MGRN1 staining per total cortical neurons from B6. (D) Quantitative analysis of p62, Ubiquitin and MGRN1 positive inclusions in the HD transgenic mice brain sections along with their age-matched control.

4.1.5 MGRN1 Promotes the Ubiquitination of Polyglutamine-expanded Proteins

The overburden or massive production of misfolded proteins can impair the Ubiquitin proteasome system. To avoid such a drastic problem and to promote the clearance of toxic soluble misfolded intermediates, cells adopt another strategy, *i.e.*, autophagy-mediated clearance. In the current study, the deregulation and strong association of MGRN1 with expanded polyglutamine proteins, and therefore, a question rises, "how does MGRN1 respond to expanded polyglutamine proteins and target them for elimination?" Coexpression of truncated N-terminal Huntingtin (EGFP-HDQ74) with wild-type MGRN1 or its catalytically inactive mutant MGRN1(AVVA) form, was performed. As shown in Figure 4.15A, it was observed that the overexpression of the wild-type MGRN1 promotes the ubiquitination of EGFP-HDQ74 polyglutamine intermediates.

To confirm this finding, cell lysates from various experiments were immunoprecipitated with GFP antibody, and blots were probed using an anti-Ubiquitin antibody. It was speculated

that, MGRN1 interaction with expanded polyglutamine proteins could induce their ubiquitination. To validate this assumption, immunoprecipitation was performed from lysates of cells expressing expanded polyglutamine proteins using GFP antibody and blots were probed with Ubiquitin antibody.

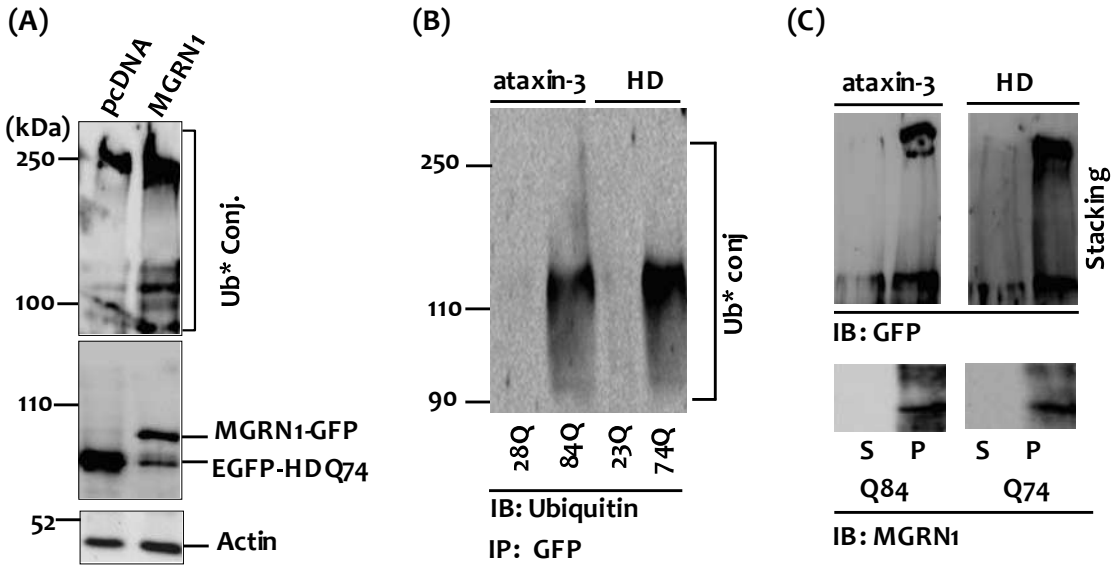

Figure 4.15 : *MGRN1 overexpression induces the ubiquitination of expanded polyglutamine proteins:* (A) Cos-7 cells were transiently co-transfected with MGRN1-GFP and EGFP-HDQ74 constructs. After 48h, the cells were subjected to immunoblotting analysis by using anti-ubiquitin, anti-GFP and anti-actin. (B) Cells were transfected with normal (EGFP-HDQ23) and polyglutamine-expanded (EGFP-HDQ74) constructs and lysates were processed for IP by GFP antibody and blot was developed with anti-ubiquitin. Cell lysates obtained from expanded polyglutamine proteins were separated in supernatant and pellet fractions and performed immunoblotting with anti-GFP and anti-MGRN1 antibodies (C).

Cell lysates obtained from expanded polyglutamine proteins were centrifuged at 10,000×g at room temperature for 5 minutes and the separated pellet and supernatant fractions were analyzed by immunoblotting with antibodies against GFP and MGRN1. In pellet samples, higher molecular protein complex of expanded polyglutamine proteins were retained in stacking gel (Figure 4.15B). Figure 4.15 shows that overexpression of wild-type MGRN1 caused a significant increase in the accumulation of ubiquitinated derivatives of EGFP-HDQ74 proteins. Bafilomycin, an autophagy inhibitor, induces the accumulation of ubiquitinated EGFP-HDQ74 proteins (Figure 4.16, left panel). Bafilomycin treatment caused massive aggregate formation and the accumulation of high-molecular-weight complexes of the expanded polyglutamine proteins, which suggests that polyglutamine aggregation is also cleared by the autophagy pathway (Figure 4.16, upper panel). As earlier reported that MGRN1 enhances the proteasome-independent ubiquitination of tumor-susceptibility gene 101 (TSG101). ITCH is another E3 Ubiquitin ligase, which induces the degradation of Deltex (DTX) via lysosomal pathway and colocalizes with endocytic vesicles.

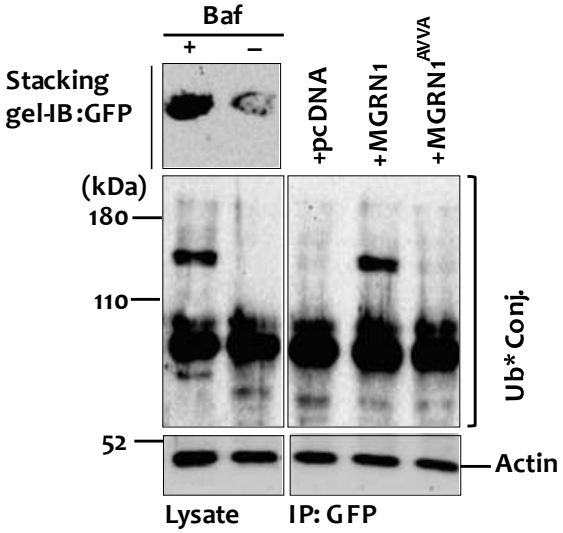

Figure 4.16 : *Expanded polyglutamine ubiquitination is promoted by MGRN1:* The Cos-7 cells were co-transfected with pcDNA-eGFP, MGRN1-GFP or MGRN1 (AVVA)-GFP mutant plasmid and the EGFP-HDQ74 construct as indicated above. Some cells were treated with or without 50 nM Bafilomycin (Baf) for 12h and subjected to immunoblotting analysis with GFP antibody. Some Cell lysates were subjected to immunoprecipitation (IP) using GFP antibody, and blots were probed with GFP, Ubiquitin and actin antibodies.

Nedd4 (neuronal precursor cell-expressed, developmentally down-regulated gene 4) is a HECT domain Ubiquitin ligase, which enhances the degradation of α-synuclein by the endo-lysosomal pathway. These studies also support the hypothesis that probably, MGRN1 can promote the ubiquitination of expanded polyglutamine and targets through autophagy pathway. As shown in Figure 4.17A and 4.17B, transiently transfected overexpressed wild-type MGRN1 overexpression decrease the levels of EGFP-HDQ74 expanded polyglutamine proteins, which can be prevented upon the addition of the autophagy inhibitor Bafilomycin (Figure 4.18A and 4.18B). Figure 4.17C and Figure 4.18C represents the plots showing significant decrement in expanded polyglutamine proteins' half lives in respective condition with MGRN1 overexpression. Earlier, it has been shown that improper sequestration of MGRN1 with cytosolic prion proteins contributes in neurodegeneration and MGRN1 interacts with Hsp70 chaperone. Therefore, it may be possible that MGRN1 targets expanded polyglutamine proteins anchored with Hsp70 chaperone and promotes their clearance from the dense cellular pool; however, for better understanding in near future, it needs further study in detail.

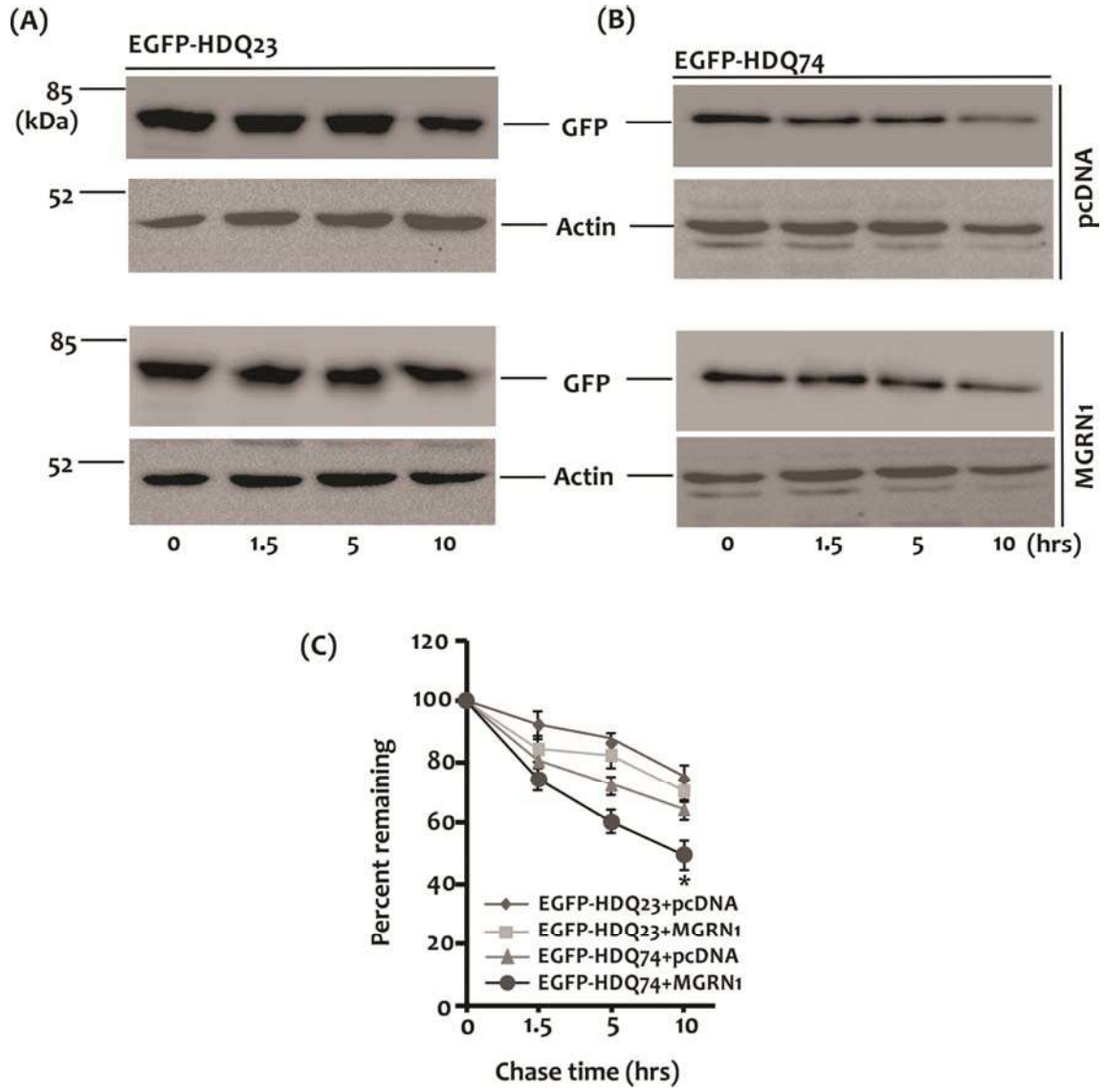

Figure 4.17 : *MGRN1 promotes degradation of expanded Huntingtin polyglutamine proteins:* (A-B) Cos-7 cells were transiently cotransfected with EGFP-HDQ23 and EGFP-HDQ74 constructs with MGRN1. At 48h after transfection, the cells were treated with cycloheximide (15 µg/ml) and chased for different periods. Blots were detected with GFP and actin antibodies. (C) Quantitation of the blot band intensities from three independent experiments was performed using NIH Image analysis software. Values are the mean ± S.D.

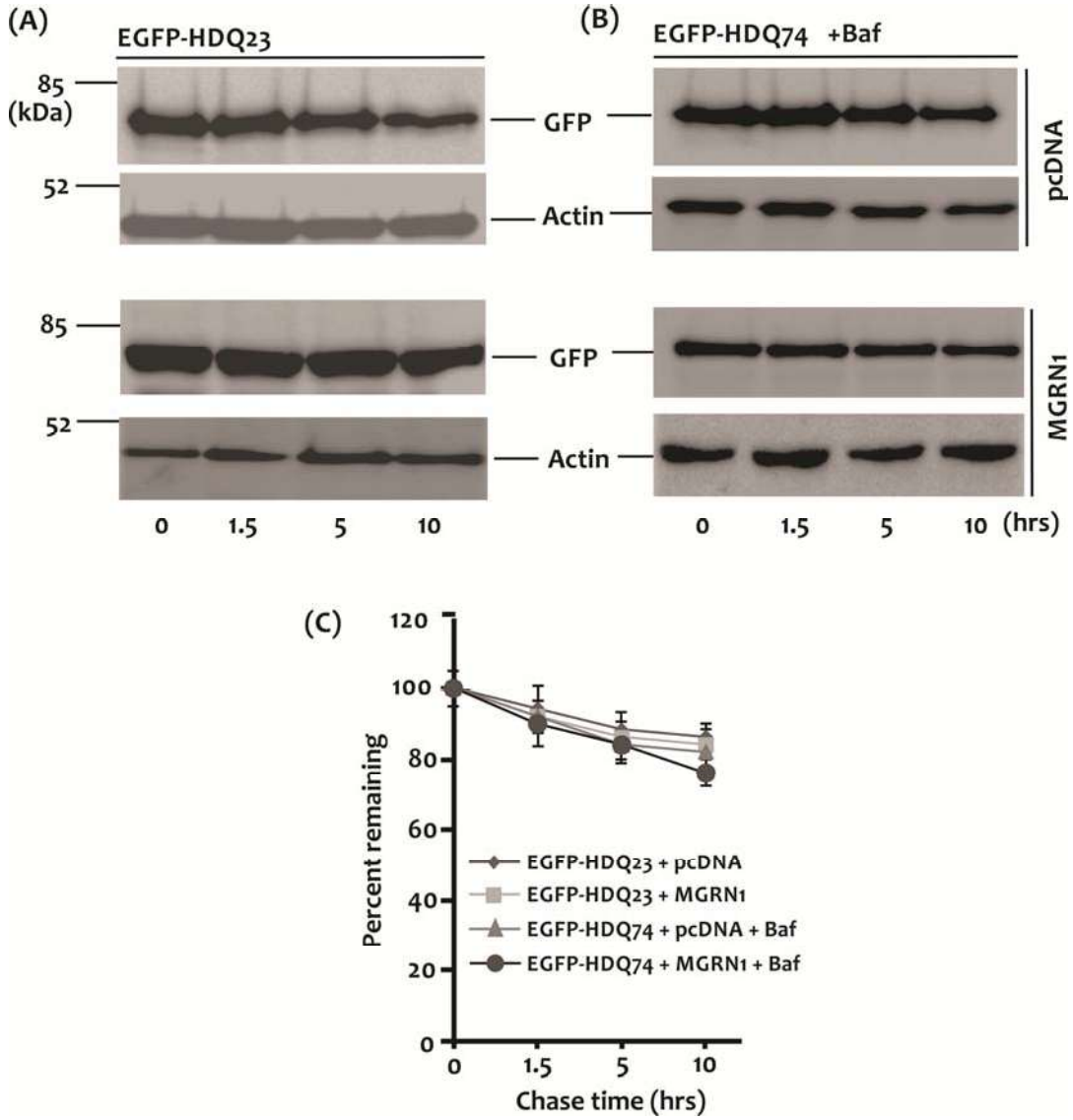

Figure 4.18 : *Degradation of expanded polyglutamine protein by MGRN1 is suppressed by autophagy inhibition*: As described in Figure 4.17, similar experiments were performed with treatment of 50 nM Bafilomycin (Baf). Blots were detected with GFP and actin antibodies (A-B). (C) The line graph shows the quantitation of the blot band intensities from three independent experiments performed using NIH Image analysis software. Values are the mean ± S.D. *, $P < 0.05$ compared with control.

4.1.6 MGRN1 Suppresses Aggregate Formation and Polyglutamine-induced Cell Death

As it was observed that MGRN1 enhances the ubiquitination and elimination of expanded polyglutamine proteins, question raised that whether MGRN1 could affect expanded polyglutamine protein aggregation and cell death. To explore, co-transfection of Huntingtin (EGFP-HDQ74) construct alongwith MGRN1 or MGRN1-siRNA were performed. As shown in Figure 4.19, aggregate formation was decreased by the overexpression of MGRN1. However, the number of aggregates increased in cells transfected with MGRN1-siRNA. The overexpression of MGRN1 enhances the clearance of expanded polyglutamine proteins, and down regulation promotes the accumulation of polyglutamine proteins (Figure 4.20).

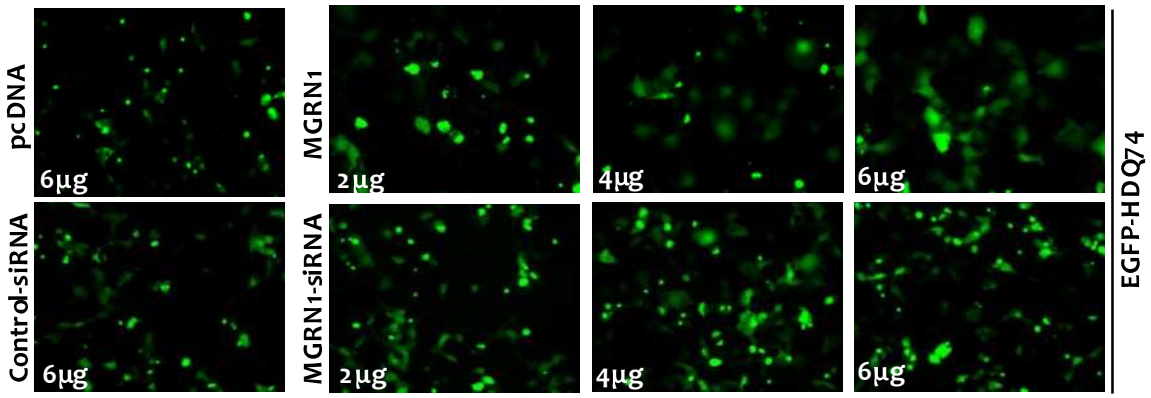

Figure 4.19 : *MGRN1 reduces aggregation of expanded polyglutamine protein:* EGFP-HDQ74 expanded-polyglutamine-expressing cells were co-transfected with MGRN1 and MGRN1-siRNA along with control (pcDNA) plasmid and control siRNA oligonucleotides. After 72h of transfection, the cells were subjected to quantitative immunofluorescence analysis and immunoblot analysis with MGRN1 and actin antibodies.

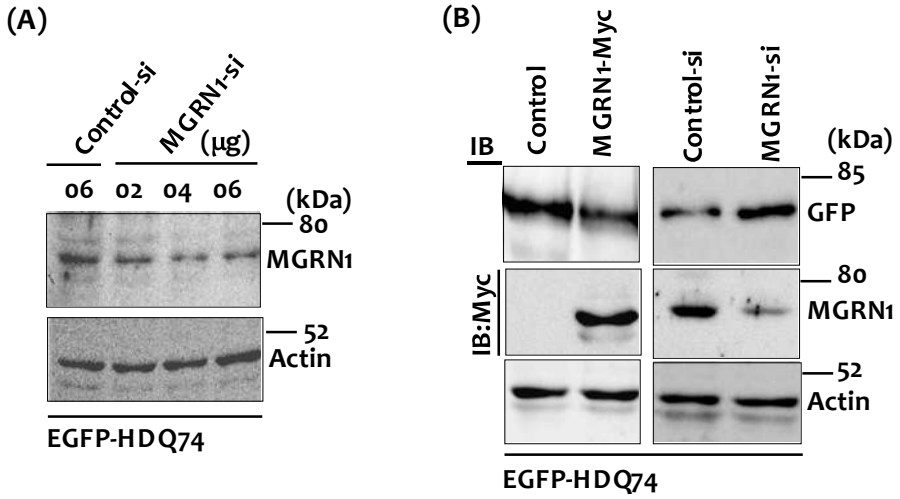

Figure 4.20 : *Representative blots to confirm knockdown of MGRN1:* As described in Figure 4.19, the same cells were collected and processed for immunoblotting analysis using anti-GFP, anti-Myc, anti-MGRN1 and anti-actin (A-B).

The overexpression of MGRN1 significantly suppresses aggregate formation in a concentration-dependent manner (Figure 4.21A). Interestingly, the suppression of aggregate formation and cytoprotective affect against ER and heat stress was more pronounced when MGRN1 was overexpressed along with Hsp70 (Figure 4.22A and 4.22C). Quantitative data from cells transfected with MGRN1-siRNA in a concentration-dependent manner showed a dose-dependent increase in the rate of aggregate formation, confirming the effect of MGRN1 on polyglutamine aggregate formation (Figure 4.21B). The knockdown of the endogenous levels of MGRN1 also significantly induced death in polyglutamine (74Q)-expressing cells (Figure 4.22B). The cumulative cytoprotective role of MGRN1 and Hsp70 under various stress conditions was further confirmed through exposing cells with ER and heat stress conditions which led to the observation that Hsp70 overexpression alleviates cytotoxicity under different stress conditions (Figure 4.22D).

Taken together, the data suggest that MGRN1 reduces the formation of aggregates of expanded polyglutamine proteins and protects cells from death mediated by the proteotoxic effects of polyglutamine proteins.

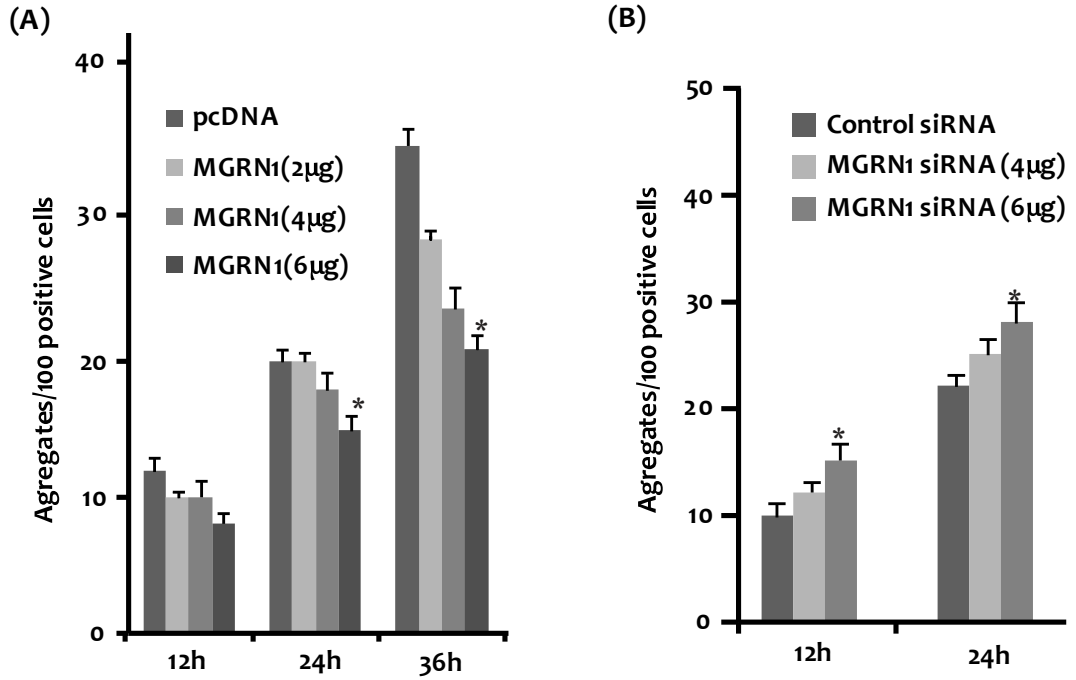

Figure 4.21 : *MGRN1 suppresses aggregation of Huntingtin expanded polyglutamine protein:* EGFP-HDQ74 expanded-polyglutamine expressing cells were transiently transfected with Myc-MGRN1 (A) plasmid and MGRN1-siRNA (B) oligonucleotides including controls; aggregate formation was counted under a fluorescence microscope at the different times indicated in the figure. The results are the mean ± S.D. of three independent experiments, each performed in triplicate. *, $p < 0.05$ compared with controls

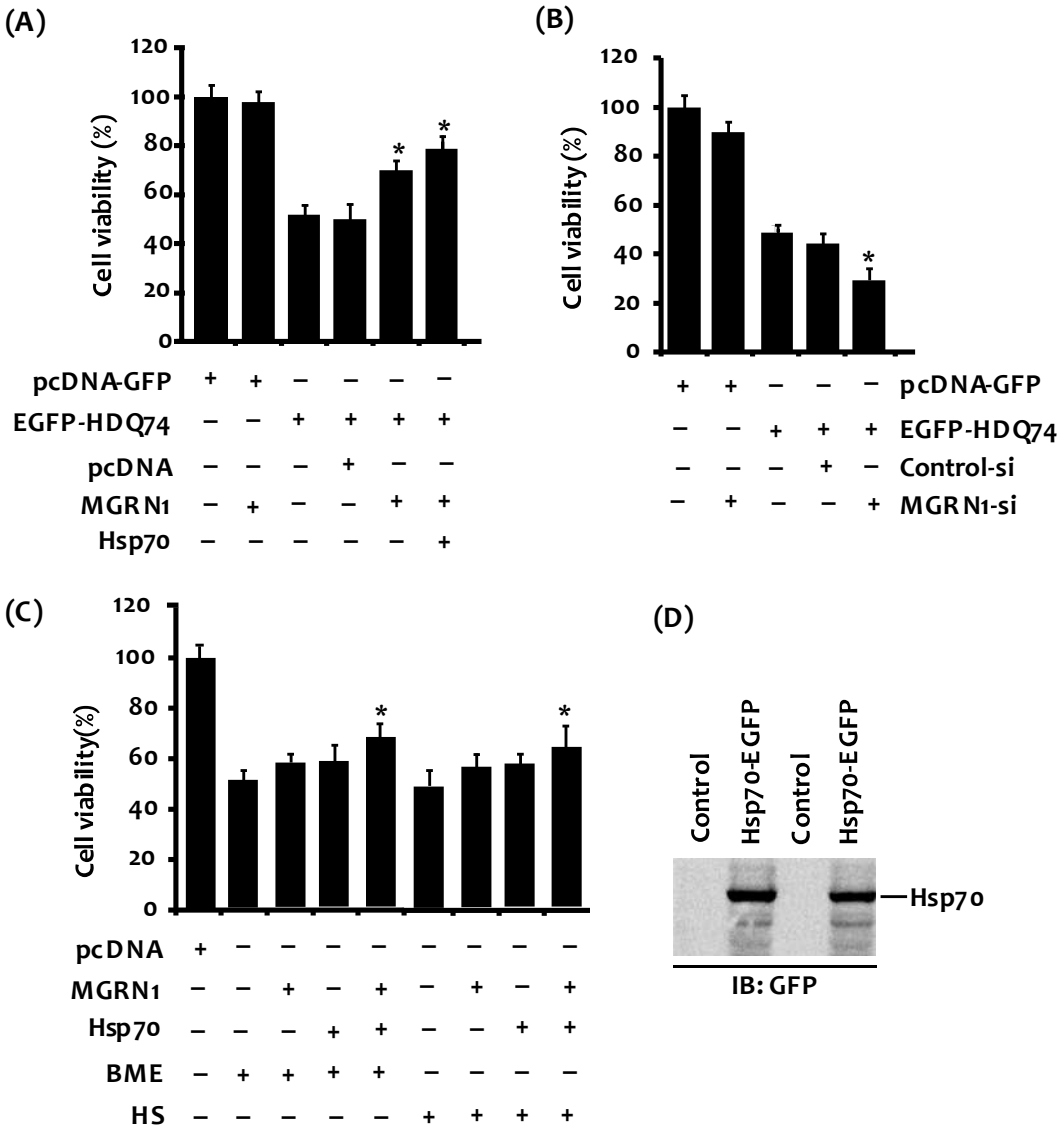

Figure 4.22 : *MGRN1 suppresses the expanded polyglutamine aggregation mediated cytotoxicity:* (A) The toxicity of EGFP-HDQ74 in cells co-expressing Myc-MGRN1 and Hsp70 constructs. Cells were harvested and replated in 96-well tissue-cultured plates. Cell viability was measured by MTT assay. Values are the mean ± S.D. of two independent experiments, each performed in triplicate. *, $p < 0.05$ compared with control (EGFP-HDQ74 transfected samples) (B) MGRN1 siRNA and control siRNA oligonucleotides were transiently transfected in EGFP-HDQ74 expanded polyglutamine expressing cells. Cell viability was measured by MTT assay. Values are the mean ±SD of two independent experiments, each performed in triplicate. * $p < 0.05$ compared with the control siRNA co-transfected with EGFP-HDQ74 plasmid. (C) Cells were treated with 10mM BME (β-mercaptoethanol) and exposed to heat stress (HS) 43°C for 45 minutes, after treatment cell viability was measured by MTT assay. Values are the mean ±SD of two independent experiments, each performed in triplicate.*, $p < 0.05$ compared with the control (stress-inducing treated) experiment. Cell lysates were also processed for immunoblotting with GFP antibody (D).

4.2 DISCUSSION

Cells eliminate abnormal proteins with the help of a protein quality control mechanism (QC). Expanded polyglutamine proteins induce multifactorial deleterious events in cells. Numerous E3s, such as Gp78, E6-AP, CHIP, Parkin, Hrd1 and Malin play crucial role in the clearance of polyglutamine proteins, reducing their aggregate formation and alleviating poly

(Q)-proteotoxicity [Tsai *et al.,* 2003; Jana *et al.,* 2005; Yang *et al.,* 2007; Mishra *et al.,* 2008; Garyali *et al.,* 2009; Ying *et al.,* 2009]. Here, it is found that mahogunin ring finger-1 (MGRN1), a putative E3 Ubiquitin ligase, interacts with misfolded and ubiquitinated expanded polyglutamine proteins in a cellular model of HD. Additionally, the recruitment and co-localization of MGRN1 Ubiquitin ligase were observed in various brain regions in the R6/2 transgenic mouse model of HD. Finally, the study shows that overexpression of MGRN1 facilitates the elimination of misfolded polyglutamine proteins via autophagy and reduces aggregate formation and cell death in expanded polyglutamine-expressing cells.

The overburden of misfolded or non-native protein aggregation impairs the function of the Ubiquitin proteasome system [Bence *et al.,* 2001; Bennett *et al.,* 2005]. However, how the accumulation or aggregation of polyglutamine proteins impairs the function of the Ubiquitin proteasome system and contributes to the pathomechanism of the disease, is still controversial [Bett *et al.,* 2006; Ortega *et al.,* 2007; Maynard *et al.,* 2009; Ortega *et al.,* 2010; Schipper-Krom *et al.,* 2012]. Previous studies indicate that the aggregation of polyglutamine proteins significantly impairs the activity of the proteasome against stress-induced protein aggregation. However, it is unknown how cells survive the proteasome dysfunction caused by massive polyglutamine protein aggregation. Thus, it is important to understand what other cellular factors or pathways are involved in cell survival and the mechanisms that inhibit polyglutamine-mediated cellular toxicity. Reports suggest that to avoid these noxious conditions and to eliminate the misfolded protein load, the cellular protein quality control mechanism (QC) adopts another survival strategy, *i.e.*, autophagy-mediated clearance [Mizushima *et al.,* 2008; Kon and Cuervo, 2010; Menzies *et al.,* 2011]. Neural precursor cells that express both developmentally downregulated protein 4 (NEDD4) and atrophin-interacting protein 4 (AIP4)/Itch E3 Ubiquitin ligases promote the clearance of melanocytic transmembrane proteins (*i.e.*, Melan-A/MART-1) by lysosomes [Levy *et al.,* 2005]. MGRN1 is a ring finger-1 E3 Ubiquitin ligase that is involved in spongiform neurodegeneration encephalopathy. Recently it has been observed that MGRN1 overexpression and loss of function do not influence prion disease; possibly functional sequestration of MGRN1 may cause cytosolic PrP linked neurotoxicity [Silvius *et al.,* 2013]. MGRN1 facilitates the ubiquitination and degradation of tumor-susceptibility gene 101 (TSG101) via the endolysosomal pathway [Jiao *et al.,* 2009].

Earlier, it has been proposed that cytosolic prion protein exposure disrupts the function of MGRN1 that contributes in neurodegeneration [Chakrabarti and Hegde, 2009]. In the current study, it has also been observed that MGRN1 mRNA and protein levels were reduced in the poly (Q) expanded Huntingtin- and Ataxin-3 fragment-expressing cells. Why were endogenous MGRN1 levels depleted in polyglutamine-expressing cells? Previous studies elaborate that the interaction of polyglutamine aggregates interrupts the normal function of various transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), CREB binding protein (CBP), TATA box binding protein (TBP) and specificity protein 1 (Sp1) [Shimohata *et al.,* 2000; Dunah *et al.,* 2002; Li *et al.,* 2002; Schaffar *et al.,* 2004; Goswami *et al.,* 2006]. Most probably sequestration and depleted function of these transcriptions contribute in the molecular pathomechanism of polyglutamine diseases. Overall, these studies also support our current speculation that MGRN1 interacts with polyglutamine aggregated intermediate species, for their clearance. It is possible that, this interaction leads to the co-aggregation and sequestration of MGRN1 with the existing polyglutamine protein aggregates, and this prolonged mislocalization may lead to the depletion of MGRN1, most likely contributing to the pathogenesis of polyglutamine diseases. In the present study, the interaction of MGRN1 with expanded polyglutamine proteins via a detailed co-immunoprecipitation study was confirmed. Colocalization of MGRN1 with cellular aggregates of poly(Q)-expanded Huntingtin and Ataxin-3 protein was also observed. Previously, malin E3 Ubiquitin ligase was shown to associate with ubiquitinated Lafora bodies, and the loss of malin function most likely

contributes to the pathogenesis of Lafora Disease [Rao *et al.,* 2010]. The malin E3 Ubiquitin ligase study supports our current findings; to validate this novel interaction, triple immunofluorescence analysis was used which established the co-localization of MGRN1 with p62 and Ubiquitin-positive polyglutamine aggregates in cells.

Based on the interaction and colocalization of MGRN1 with expanded polyglutamine proteins in the cellular model of HD disease, MGRN1 recruitment to the neuronal aggregates in R6/2 transgenic mice was observed. In this mouse model of HD disease, significant number of aggregates were detected that were positive for MGRN1, p62 and Ubiquitin staining and a clear redistribution of MGRN1 from the cytoplasm to the aggregates in the cerebellar Purkinje cells and cortical neurons was observed. These results prompted us to investigate the functional role of MGRN1 in the elimination of expanded polyglutamine proteins and the consequent effect of this degradation on aggregate formation.

4.3 CONCLUDING REMARKS

This study shows that autophagy inhibition induces the ubiquitination of expanded polyglutamine proteins. Induced autophagy retains an ability to reduce the toxicity of expanded polyglutamine proteins. Current observations demonstrate that MGRN1 overexpression specifically stimulates the rate of degradation of the polyglutamine protein expansions and reduces aggregate formation, providing a cytoprotective response against polyglutamine-induced toxicity. This result was confirmed by the depletion of MGRN1 levels by siRNA, which increased the accumulation of expanded polyglutamine protein aggregates and enhanced the poly (Q) expansion-induced cell death. Interestingly, it is noted that the protective response was more prominent when MGRN1 and the Hsp70 chaperone were co-expressed.

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