Annexure A

Materials and Methods

A.1 MATERIALS

Cycloheximide, Chloroquine, Tunicamycin, Thapsigargin, Beta-mercaptoethanol, Bafilomycin, TRIzol reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT), JC-1 (5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarbocyanine iodide), and all cell culture reagents were obtained from Sigma-Aldrich. Lipofectamine® 2000, optiMEM, MGRN1 siRNA and Universal Negative Control siRNA purchased from Santa Cruz and Sigma-Aldrich. General laboratory chemicals were purchased from Merck, Himedia and Sigma-Aldrich.

Table A.1: List of Antibodies Used in the Study

Serial Number	Antibodies	Company	Catalogue Number	Host animal
1.	MGRN1	Santa Cruz	sc-160518	Goat
2.	MGRN1	Sigma-Aldrich	HPA007653	Rabbit
3.	Hsp70	Santa Cruz	sc-32239	Mouse
4.	Luciferase	Santa Cruz	sc-74548	Mouse
5.	P62	Sigma-Aldrich	WH0008878M1	Mouse
6.	Huntingtin	Thermo	H7540	Rabbit
7.	Ubiquitin	Santa Cruz	sc-58448	Mouse
8.	GFP	Santa Cruz	sc-8334	Rabbit
9.	GFP	Roche	12600500	Mouse
10.	β-tubulin	Santa Cruz	sc-80011	Mouse
11.	Myc	Sigma-Aldrich	M5546	Mouse
12.	Actin	Sigma-Aldrich	A2066	Rabbit
13.	NeuN	Gifted	-	Mouse

Table A.2: List of Expression Plasmids Used in the Study

Serial Number	Construct Name	Source
1.	MGRN1-GFP	Gifted
2.	MGRN1^{AWA}-GFP	Gifted
3.	MGRN1-Myc	Gifted
4.	pcDNA-Luciferase	(Addgene 18964)
5.	pcDNA^{TM} 3.1	Life Technologies
6.	EGFP-HDQ23	Gifted
7.	EGFP-HDQ74	Gifted
8.	pEGFP-C1-Ataxin3Q28	(Addgene-22122)
9.	pEGFP-C1-Ataxin3Q84	(Addgene-22123)
10.	pEGFP-Hsp70	(Addgene-15215)
11.	pcDNA3-EGFP	(Addgene 13031)
12.	pcDNA3-cMyc	(Addgene 16011)

Goat anti-rabbit IgG-Rhodamine, goat anti-mouse IgG-FITC, AP-conjugated anti-mouse, anti-rabbit IgG, anti-goat IgG, as well as HRP conjugated anti-mouse, anti-rabbit and anti-goat secondary antibodies were purchased from Vector Laboratories. Triple staining was performed using Zenon®Alexa Fluor® kits.

A.1.1 Cell Lines

A549, 293T and Cos-7 cells were used in the experiments.

A.1.2 Huntington's Diseases (HD) Transgenic Mice Brain Samples

R6/2 transgenic mice (12-week-old male) and control male littermates (of same age) were used in the experiments. The CAG repeat sizes in the R6/2 transgenic mice were 134Q and 130Q. CAG repeat length of Htt for each mouse was routinely determined by the almost same method described previously [Mangiarini *et al.*, 1996] to exclude the deviated repeat samples due to the instability of CAG repeat. PCR was performed with FAM-labeled primer 31329 (ATGAAGGCCTTC GAGTCCCTCAAGTCCTTC) and primer 33934 (GGCGGCTGAG GAAGCTGAGGA) followed by capillary electrophoresis with ABI 3130xl (Applied Biosystems) and analysis with Genescan software (Applied Biosystems). In immunofluorescence staining experiments, 5 µm thick paraffin-embedded brain sections were used. All the mice experiments were approved by the Animal Experiment Committee of the RIKEN Brain Science Institute, Japan.

A.2 METHODS

Various molecular biology techniques and methods were used in the current study to explore the role of the E3 Ubiquitin ligase in protein quality control and neurodegeneration. The details of the important methods adopted are given below.

A.2.1 Large scale Plasmid Extraction

Different plasmid DNA and the positive cloned DNA constructs were extracted using QIAGEN midi prep kit as per instruction.

A.2.2 Cell Culture, Transfection, Cell Viability Assay and Counting of Aggregates

The A549, 293T and Cos-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and the antibiotics penicillin/streptomycin. Cells were transiently transfected with expression vectors using the Lipofectamine® 2000 reagent according to the manufacturer's instructions. The transfection efficiency was about 80–90%. After 24 or 48h of transfection, cells were used for immunofluorescence staining, co-immunoprecipitation, and immunoblotting. For the cell viability assay, cells were first transfected with different expression plasmids and/or treated with desired drugs or stress inducing agents as indicated in different experiments. The cell viability was measured by the MTT assay as described previously [Mishra *et al.*, 2008]. Statistical analysis was performed using the Student's *t* test, and $p < 0.05$ indicated statistical significance. Aggregate formation was manually counted under a fluorescence microscope (~500 transfected cells in each case), and cells containing more than one aggregate were considered to have a single aggregate.

A.2.3 Co-immunoprecipitation

Cos-7 cells were transiently transfected with desired plasmids. After 24h, cells were washed with cold PBS, scraped, pelleted by centrifugation, and lysed on ice for 30 minutes with NP-40 lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% NP-40 and complete protease inhibitor cocktail). Cell lysates were briefly sonicated, centrifuged for 10 minutes at 15,000×g at 4ºC and the supernatants (total soluble extract) were used for immunoprecipitation as described earlier [Jana *et al.*, 2005]. For each immunoprecipitation experiment, approximately 200 mg protein in 0.1 ml NP40 lysis buffer was incubated with 2.5-5 µg of various primary antibodies. After overnight incubation at 4ºC with rotation, 80 ml protein G-agarose beads were added, and incubation was continued at 4ºC for 5h. The beads were washed six times with NP-40 lysis buffer. Bound proteins were eluted from the beads with SDS sample buffer, vortexed, boiled for 5 minutes, and analyzed by immunoblotting. Most primary antibodies were used in 1:500 dilutions for immunoblotting.

A.2.4 Immunofluorescence and Immunohistochemical Techniques

In some experiments, Cos-7 cells were transiently transfected with Ataxin-3 constructs along with MGRN1 for 48h. For some experiments Cos-7 cells were transiently transfected with the full-length MGRN1-GFP or MGRN1-AVVA-GFP. Cells were then washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, permeabilized with 0.5% Triton X-100 in PBS for 5 minutes, washed extensively, then blocked with 2% horse/goat serum in PBS for 1h. The primary antibody incubation was carried out overnight at 4°C. After several washing steps with TBST, cells were incubated with secondary antibody (1:500 dilutions) for 1h, washed several times, and mounted. Samples were observed using a fluorescence microscope, and digital images were assembled using Adobe Photoshop. The appropriate FITC and rhodamine-conjugated secondary antibody was used to visualize expression and localization.

Antibodies dilutions were used in 1:500 to 1:1000 for immunohistochemical staining in the control and HD transgenic mouse brain sections, the paraffin-embedded brain sections were deparaffinized, subjected to antigen retrieval, and then processed for staining. The appropriate secondary antibodies were used to visualize expression and localization.

A.2.5 MTT Assay

Conversion of MTT to colored formazan catalyzed by the viable, but not the dead cells, was used for measurement of cell viability. Cells were plated in 96 well plates and cultured for specified time in culture medium. Treatments were done as described and 100µl of MTT reagent (5mg/ml in PBS) was added to the cell samples in 1ml of medium followed by incubation for 2-4 hours at 37ºC. After removal of culture medium, 100 µl of lysis buffer (acidic isopropanol) were added. The samples were immediately mixed to prepare formazan particles and form a solution. Then, 100 µl of each sample in triplicate were transferred to 96-well ELISA plates (in case of 24-well plates) and absorbance was measured at 595 nm in microplate reader. The results are presented as percent of the control.

A.2.6 JC-1 Staining

To measure mitochondrial membrane potential, cells were plated into 2 well chamber slides at subconfluent density. After 24h, some cells were transiently transfected with either MGRN1-siRNA or Control-siRNA oligonucleotides. Post-transfected cells treated with H_2O_2 (0.05 mM for 10 minutes) were probed with 5 mM JC-1 fluorescence dye for 45 minutes in the CO_2 incubator and extensively washed with prewarmed PBS at 37˚C. JC-1 stained cells were qualitatively observed under a fluorescence microscope using 568-nm filter.

A.2.7 Protein Estimation

Bradford reagent stock solution (BioRad) was diluted five times to prepare working stock. Protein estimation was done by using Bradford's microassay method [Bradford, 1976]. Standard curve was prepared using six dilutions of 1 mg/ml BSA stock. Dilutions of 1 µg, 2 µg, 6 µg, 8 µg and 10 µg were prepared in duplicate. 800 µl of standard and sample solution were added to different microfuge tubes. Then 200 µl of Bradford reagent was added, vortexed and incubated for 5 minutes. Absorbance was measured at 595 nm, standard curve was prepared and protein concentration calculated accordingly.

A.2.8 SDS-PAGE and Immunoblotting

SDS-PAGE was carried out with different acrylamide percentages

10% resolving gel (10ml):

Contents: dH_20 - 4.0 ml, 30% acrylamide-bis acrylamide mix - 3.3 ml, 1.5M Tris (pH 8.8) - 2.5 ml, 10% SDS - 0.1 ml, 10% ammonium persulfate - 0.1 ml, TEMED - 0.004 ml

5% stacking gel (10 ml):

Contents: dH_20-5.65 ml, 30% acrylamide- bis acrylamide mix - 1.65 ml, 1.0M Tris (pH 6.8) - 2.5 ml, 10% SDS - 0.1 ml, 10% ammonium persulfate - 0.1 ml, TEMED - 0.004 ml

After polymerization of resolving gel, stacking gel mix was poured between glass plates. The required comb (10 well or 15 well) was inserted between the plates. The gel was allowed to polymerize for 25-30 minutes. The samples prepared in sample loading dye was added (approx 10 µg) into the wells of 0.75mm thick moulds after setting up the casket and adding the electrode buffer (25mM tris, 190 mM glycine and 3.5mM SDS). The gel was run at a constant current of 30 mAmp until dye front reached the end of plate. After removing the gel, transfer was set up using nitrocellulose membrane and dry transfer was performed using transfer buffer (25 mM tris, 190 mM glycine and 3.5mM SDS and 20% methanol).

Transfer was set up at a constant current 150 mAmp for one and half hour. After transfer, the membrane was analyzed for equal protein loading using PonceauS stain and then incubated for 1hr in blocking buffer [5% skimmed milk prepared in TBST (50 mM Tris, pH 7.4, 0.15 M NaCl, 0.05% Tween)]. The membrane was then incubated in the primary antibody overnight at 4ºC, prepared in TBST. The membrane was washed for 5-10 minutes, 3-4 times (depending on the proteins being analyzed), and incubated in secondary antibody prepared in TBST, at a dilution of 1: 2000 for 30 minutes to 1 hour; the blots were again washed 3-4 times for 5-10 minutes each. Later the blots were developed using developer prepared in GB3 buffer (1 M NaCl, 0.1mM $MgCl_2$, 10 M tris) and NBT/BCIP (in DMSO) substrate for alkaline phosphatase detection (10 ml GB3, 68 µl NBT, 34 µl BCIP) or chemiluminescent detection using Luminata® Horseradish peroxidase substrate (Millipore).

A.2.9 RNAi Experiments

A549 cells were plated into six/ninety-six well tissue culture plates and the following day the cells were transiently transfected with either MGRN1-siRNA or control-siRNA oligonucleotides. After 24h, cells were collected and processed for RT-PCR, immunoblotting, Luciferase assays and aggregation count through fluorescence imaging.

To study the effect of MGRN1 deficiency on misfolded protein degradation, the A549 cells were transfected with either pcDNA-Luciferase plasmid or Huntingtin expanded polyglutamine constructs in culture plates, and on the following day, the cell viability was monitored using the MTT assay or Luciferase activitly was measured using a

Luciferase assay kit (Promega); in some experiments, protein aggregation was observed and aggregates were manually counted as well as collected proteins samples were used for immunoblotting in order to confirm the knockdown.

A.2.10 TUNEL Assay

A549 cells were plated into four well chamber slides and on the next day cells were transfected with control-siRNA and MGRN1-siRNA oligonucleotides. Post-transfected cells treated with H_2O_2 (1.5 mM for 4h). After treatment cells were processed for TUNEL staining according to manufacturer's (Promega) instructions**.** Images were taken through a fluorescence microscope and TUNEL-positive cells were counted manually.

A.2.11 Filter Trap Assay

The filter trap assay was perfomed using a nitrocellulose membrane, where the protein samples extracted from Cos7 cells transfected with normal- and expanded polyglutamine constructs, were allowed to pass through under suction pressure using a Filter Trap Assay equipment. The higher molecular weight aggregates along with their interactors were detected using immunoblotting with Anti-GFP, Anti-MGRN1 and Anti-Myc antibodies.

A.2.12 RT-PCR Analysis

The total RNA was extracted using TRIzol reagent (Sigma) and semi-quantitative RT-PCR was carried out with OneStep RT-PCR kit (Qiagen).

Table A.2.1: List of the Primer Sequences Used for RT-PCR Analysis

Serial Number	Primer Name	Primer Sequesnce
1.	MGRN1-F1	5'-ATGGGCTCCATTCTCAGC-3'
2.	MGRN1-R1	5'-GTTGCTGTTGTCGCTGTTCT-3'
3.	MGRN1-F2	5'-CGGGCCCTCCTGCAGATC-3'
4.	MGRN1-R2	5'-CTGGCAGGTAGATGTCAGCA-3'
5.	Actin-F	5'-ATCGTCCACCGCAAATGCTTCTA-3'
6.	Actin-R	5'-AGCCATGCCAATCTCATCTTGTT-3'

Table A.2.2: RT-PCR Conditions Used for RT-PCR Analysis

<table>
<tr><th>Step</th><th>Temperature</th><th>Time</th><th></th></tr>
<tr><td>Reverse transcription</td><td>50° C</td><td>30 minutes</td><td rowspan="2"></td></tr>
<tr><td>HotStar Taq DNA Polymerase activation</td><td>95° C</td><td>15 minutes</td></tr>
<tr><td>Denaturation</td><td>94° C</td><td>30 seconds</td><td rowspan="3">β-Actin (Human) 21-23 cycles
MGRN1 (Human) 34-37 cycles</td></tr>
<tr><td>Annealing</td><td>55° C</td><td>45 seconds</td></tr>
<tr><td>Elongation</td><td>72° C</td><td>01 minutes</td></tr>
<tr><td>Final extension</td><td>72° C</td><td>10 minutes</td><td rowspan="2"></td></tr>
<tr><td>Store</td><td>04° C</td><td>Hold</td></tr>
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A.2.13 Quantification of Agarose Gels and Immunoblots

Densitometric analysis of the band intensities of agarose gels and blots was done using NIH image analysis software Image J.

A.2.14 Statistical Analysis

All experiments were carried out in duplicate or triplicate. The standard curve was plotted using known concentrations of standards and the concentrations of the sample were calculated using linear regression. The test of significance was done using Student's t-test. The values were considered statistically significant from controls if $p < 0.05$ as described in the respective results.

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