Annexure A
Materials and Methods

A. 1 MATERIALS

Tunicamycin, Myricetin, MG132, Cycloheximide, DMSO, chloroquine and all cell culture reagents were purchased from Sigma-Aldrich Corp. (St. Louis, MO). TRIzol, Lipofectamine® 2000, and OptiMEM were procured from Life Technologies (Carlsbad, CA). ProLong® Gold Antifade with DAPI was obtained from Thermo Fisher Scientific (Waltham, MA). TUNEL assay kit and Dual Luciferase® Reporter Gene Assay kit were procured from Promega. Lab chemicals were purchased from Sigma-Aldrich, Himedia and Sisco research laboratories (SRL). Reverse transcriptases PCR kits were obtained from Bio-Rad Laboratories (Hercules, CA). Plasmid constructs used in the paper were obtained from Addgene (Cambridge, MA).

Table A.1 List of Antibodies Used in the Study

Serial Number	Antibodies	Company	Catalogue Number	Host Animal
1	p27	Santa-Cruz	sc-528	Rabbit
2	p53	Santa-Cruz	sc-6243	Rabbit
3	p21	Santa-Cruz	sc-756	Rabbit
4	Gp78	Santa-Cruz	sc-166358	Mouse
5	GFP	Santa-Cruz	sc-8334	Rabbit
6	P-p27	Santa-Cruz	sc-16324	Goat
7	Cyclin E	Santa-Cruz	Sc-25303	Mouse
8	Cyclin D1	Santa-Cruz	sc-8396	Mouse
9	Cdk4	Santa-Cruz	sc-260	Rabbit
10	β-actin	Santa-Cruz	sc-81178	Mouse
11	E6-AP	Santa-Cruz	sc-25509	Rabbit
12	Hsp70	Santa-Cruz	sc-32239	Mouse
13	HSF-1	Santa-Cruz	sc-9144	Rabbit
14	Ubiquitin	Santa-Cruz	sc-9133	Rabbit
15	Luciferase	Santa-Cruz	sc-74548	Mouse
16	AMFR	Cell Signaling Technology	9590	Rabbit

Horseradish peroxidase-conjugated anti-mouse, anti-goat, anti-rabbit IgG, anti-rabbit and anti-mouse (IgG-fluorescein isothiocyanate and IgG-rhodamine), were purchased from Vector Laboratories (Burlingame, CA).

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

Serial Number	Construct Name	Source
1	pEGFP Gp78/JM27	Addgene (13310)
2	pCI-Neo-Gp78 R2M/JM21	Addgene (13304)
3	pcDNA3-EGFP	Addgene (13031)
4	p3868 HA-E6-AP	Addgene (8648)
5	pF141 pAcGFP1 SOD1WT	Addgene (26402)
6	pF148 pSOD1G37RAcGFP1	Addgene (26409)
7	pAAV asyn WT	Addgene (36055)
8	pAAV asyn S87A	Addgene (36056)
9	Luciferase-pcDNA3	Addgene (18964)
10	pEGFP-C1-Ataxin3Q28Q	Addgene (22122)
11	pEGFP-C1-Ataxin3Q84Q	Addgene (22123)
12	pEGFP-C1 GFP-wtCAT	Gifted
13	pEGFP-C1 GFP-Δ9CAT	Gifted
14	EGFP-HDQ23	Gifted
15	EGFP-HDQ74	Gifted

A1.1 Cell Lines

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

A.2 METHODS

Multiple molecular biology techniques and biochemical methods were used for finding role of Gp78 in cellular proliferation and Myricetin mediated suppression of misfolded proteins accumulation. Detailed description of important methods and techniques are given below in subsections.

A.2.1 Plasmid Isolation

Various plasmids isolated by Mdi Quanta Midi Kit, containing all the prepared solutions were used according to manufacturer's given protocol. Isolated plasmids were dissolved in Tris-acetate EDTA buffer and their concentration checked by nanodrop spectrophotometer.

A.2.2 Cell Culture, Transfection and Treatment

Dulbecco's modified Eagle's medium (DMEM) supplemented with 1X antibiotic antimycotic (Gibco) and 10% heat denatured fetal bovine serum was used to culture kidney derived fibroblast like Cos-7 and lung epithelial carcinoma A549 cells. The optimal culture conditions of 37 ^{o}C and 5% CO_2 were given to cells. For experiments, cells were plated either into six-well tissue culture plates or into two-well chamber slides.

On appropriate confluency, cells were given treatment or transfected with desired plasmids with lipofectamine® 2000 according to given manufacturer's protocol. Transfection efficiency of Cos-7 cells was about 80-90% and 60-70% for A549 cells. After 48 Hrs of transfection, cells were used for immunofluorescence, immunoblotting, and other experiments.

A.2.3 Immunoblotting and Cycloheximide Chase

Transient transfection was performed on Cos-7 cells different plasmids constructs and some samples were also treated with MG132. After 48 Hrs of transfection, or required treatment Hrs, cells were washed with phosphate buffer saline and collected. Lysates were processed for

SDS PAGE and further transferred onto nitrocellulose membranes. Skimmed milk (5%), prepared in Tris-buffered saline and Tween 20 (TBST) (50 mMTris; pH 7.4, 0.15M NaCl, 0.05% Tween), was used as blocking buffer; thereafter TBST was also used for 1:200 dilution of primary antibodies and overnight incubation at 4 °C. On the subsequent day, blots were washed three times with TBST and probed with appropriate horseradish peroxidase-conjugated secondary antibodies, and blots were developed using Luminata Crescendo Western horseradish peroxidase (HRP) substrate (EMD Millipore). Cos-7 cells were given treatment and transfected with different plasmids with or without MG132 for chase experiments and after 48 Hrs of post-transfection, cells were treated with cycloheximide (15 µg/ml). Cell lysates were collected and subjected to immunoblot analysis.

A.2.4 Protein Estimation, Agarose Gels and Immunoblots Quantification

Bradford working stock was prepared by diluting stock solution purchased from BioRad. Six dilution of bovine serum albumin stock solution (1 mg/ml) were used to prepare standard curve. Sample solution was mixed with 800 µl of standard in microcentrifuge tube and vortexed after addition of 200 µl of Bradford reagent. Tubes were incubated for 5 minutes and absorbance was measured at 595 nm. Concentrations of samples were calculated according to prepared standard curve. Quantification of band intensities of blots and agarose gels in images were analyzed by NIH ImageJ software.

A.2.5 Reporter Gene Assay and Docking Studies

Cells were transfected with luciferase plasmid and treated with Myricetin under normal 5% CO_2 condition. For 30 minutes, plates were kept at 43 ^oC and then again kept at 37 ^oC in CO_2 incubator for 2 Hrs recovery period. Collected samples from these cells were used for dual luciferase reporter gene assay performed according to the manufacturer's (Promega) protocol. For docking studies, E6-AP HECT catalytic domain structure was taken from protein databank ID: 1D5F. Similarly, the structure of Myricetin was obtained from PDB ID: 2O63. Autodock vina was used for docking study [Trott and Olson, 2010] with grid dimensions of 56 Å × 64 Å × 111 Å, that included entire HECT catalytic domain centred on 91.019, 90.02 and 49.614 coordinates. The depicted image in the figure was generated using Chimera tool [Pettersen *et al.*, 2004].

A.2.6 Bright Field Images, Immunofluorescence Staining and Aggregate Counting

Cells were grown into six-well tissue culture plates and given a required drug treatment. Post-treatment, images were obtained with bright field microscope. For immunofluorescence staining, cells were plated into chamber slides (two-well) at appropriate density. After transfection with appropriate plasmids or drug doses, cells were rinsed with phosphate buffer saline (PBS) twice. Cells fixing was performed with paraformaldehyde (4% in PBS) for 30 minutes and then rinsed with PBS twice. Permeabilization of cells was done by 0.5% Triton X-100, for the duration of 5 minutes and then again rinsed three times with PBS. Standard 2% horse serum in PBS was used for 30 minutes blocking. Appropriate primary antibody (1:200 dilution) was used for overnight incubation at 4 ^oC and then given 4 Hrs incubation at room temperature (RT) with the rodamine-conjugated secondary antibody.

Mounting of slides was performed by antifade with 4', 6-diamidino-2-phenylindole (DAPI) that gives stain to the nuclei. Slides were viewed for images under a fluorescence microscope and similar slides were also used to count aggregates manually under a microscope (approximately 400 transfected cells in each set). While counting, more than one aggregate in a single cell was considered as one big inclusion.

A.2.7 Reverse Transcriptase Polymerase Chain Reaction Analysis

RNA samples were extracted from A549 cells transfected with pcDNA-EGFP and Gp78-EGFP for overexpression and with control and AMFR-siRNA for knockdown studies, after 48

Hrs of transfection with TRIzol reagent, using manufacturer's protocol (OneStep RT-PCR kit Qiagen).

Table A.3. List of the Primer Sequences Used for RT-PCR Analysis

Serial Number	Primer Name	Primer Sequence
1	β-actin Forward	5'-ATCGTCCACCGCAAATGCTTCTA-3'
2	β-actin Reverse	5'-AGCC ATGCCAATCTCATCTTGTT-3'
3	AMFR Forward	5'-ACTCTCCTGTCCCTGGACCT-3'
4	AMFR Reverse	5'-TCATTGTTGACAGCCAGCTC-3'
5	p27 Forward	5'-AACGTGCGAGTGTCTAACGG-3'
6	p27 Reverse	5'-CCCTCTAGGGGTTTGTGATTCT-3'

The conditions used for RT-PCR for β-actin: 30 minutes at 50 °C for reverse transcription, followed by an initial denaturation step for 2 minutes at 94 °C and further Cycling at 94 °C for 30 seconds denaturation, 55 °C for 45 seconds annealing, 72 °C for 1 minute, a final step of extension at 72 °C for 2 minute and 4 °C to hold the reaction. The cycle number for β-actin is 23. The conditions used for AMFR: 94 °C for 30 seconds denaturation, 59 °C for 45 seconds annealing, 72 °C for 1 minutes for 35 cycles and for p27 is 95 °C for 15 seconds denaturation, 60 °C for 30 seconds annealing, 72 °C for 30 seconds for 37 cycles.

A.2.8 Statistical Analysis

All experiments were performed in triplicate. Statistical analyses in all experiments were done by student's t test, with $p < 0.05$ statistical significance and the values represents the mean ± SD in all experiments.

A.2.9 TUNEL Staining

In two well chamber slides, A549 cells were plated, and transfected with control and AMFR-siRNA. After 48 Hrs of transfection, cells were washed with phosphate buffer saline, 4% paraformaldehyde was used to fixed them and further permeabilized with 0.5% Triton X–100 for 5 minutes. After washing, cells were blocked with 5% horse serum, and used for TUNEL staining as per instructions of manufacturer (Promega). Images were obtained by fluorescence microscope and manually counted for TUNEL-positive cells.

A.2.10 Knockdown Experiments

In six well culture plates and two well chamber slides, A549 cells were seeded, followed by transfection with control siRNA and AMFR-siRNA, on the next day. After 48 Hrs of transfection, cells were processed for immunobloting, RT-PCR, and immunofluorescence. To study the effects of AMFR-siRNA in A549 cells, growth of the cells was monitored and cell number was counted at 24, 36, 48, and 96 Hrs post-transfection, in both Control and AMFR-siRNA transfected cells. p27 siRNA were transfected in six-well plate with or without Gp78-EGFP and cell proliferation was monitored by bright field images using microscope at 12, 24, 36, and 48 Hrs post-transfection.

A.2.11 Filter Trap Assay

Cos-7 cells were transfected with construct of ataxin-3(Q84) in presence or absence of Myricetin. Extracted protein samples from these cells were allowed to pass through nitrocellulose membrane under suction pressure from FTA equipment. Aggregates with their interacting proteins were detected by β-actin and GFP antibodies.