

Original Paper

Resveratrol Promotes LRSAM1 E3 Ubiquitin Ligase-Dependent Degradation of Misfolded Proteins Linked with Neurodegeneration

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Key Words

Resveratrol • LRSAM1 • E3 Ubiquitin Ligase • Proteasome • Neurodegeneration

Abstract

Background/Aims: Cells require regular maintenance of proteostasis. Synthesis of new polypeptides and elimination of damaged or old proteins is an uninterrupted mechanism essential for a healthy cellular environment. Impairment in the removal of misfolded proteins can disturb proteostasis; such toxic aggregation of misfolded proteins can act as a primary risk factor for neurodegenerative diseases and imperfect ageing. The critical challenge is to design effective protein quality control (PQC) based molecular tactics that could potentially eliminate aggregation-prone protein load from the cell. Still, targeting specific components of the PQC pathway for the suppression of proteotoxic insults retains several challenges. Earlier, we had observed that LRSAM1 promotes the degradation of aberrant proteins. Here, we examined the effect of resveratrol, a stilbenoid phytoalexin compound, treatment on LRSAM1 E3 ubiquitin ligase, involved in the spongiform neurodegeneration. **Methods:** In this study, we reported induction of mRNA and protein levels of LRSAM1 in response to resveratrol treatment via RT-PCR, immunoblotting, and immunofluorescence analysis. The LRSAM1-mediated proteasomal-based clearance of misfolded proteins was also investigated via proteasome activity assays, immunoblotting and immunofluorescence analysis. The increased stability of LRSAM1 by resveratrol was demonstrated by cycloheximide chase analysis.

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Results: Here, we show that resveratrol treatment induces LRSAM1 E3 ubiquitin ligase expression levels. Further, our findings suggest that overexpression of LRSAM1 significantly elevates proteasome activities and improves the degradation of bona fide heat-denatured luciferase protein. Exposure of resveratrol not only slows down the turnover of LRSAM1 but also effectively degrades abnormal proteinaceous inclusions, which eventually promotes cell viability. **Conclusion:** Our findings suggest that resveratrol facilitates LRSAM1 endogenous establishment, which consequently promotes the proteasome machinery for effective removal of intracellular accumulated misfolded or proteasomal-designated substrates. Altogether, our study proposes a promising molecular approach to specifically trigger PQC signaling for efficacious rejuvenation of defective proteostasis via activation of overburdened proteolytic machinery.

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Introduction

Proteins are imperative for normal cellular functions and survival. The regular maintenance of protein turnover mechanisms fulfills the routine course of assigned cellular functions to achieve proteostasis [1, 2]. Mutant or abnormal protein accumulation can trigger an alarm to quickly eliminate their aggregation from different cellular compartments. Failure in the degradation mechanisms of aggregation-prone proteins can cause critical neurodegenerative diseases and develop clinical symptoms of imperfect ageing [3, 4]. The removal of misfolded proteins from the different cellular compartments adds an extra metabolic burden on the cell. Under such circumstances, cells initially prefer their folding, utilizing molecular chaperones, prior to designating them for degradation [5]. Depletion in the chaperone capacity promotes the occurrence of neurodegeneration and ageing [6]. However, a complete understanding of protein folding mechanisms is still truant, and therefore it is challenging to come up with molecular strategies that can assist in improving chaperone functioning for better folding of aberrant proteins. Furthermore, the molecular mechanisms by which cells can specifically target the removal of abnormal or poorly folded proteins from the intracellular pool are unknown. It is also important to find out strategies based on small molecules that can improve the cellular PQC mechanisms for the effective clearance of damaged proteins to maintain overall proteostasis.

Cells continuously monitor the folding mechanism of damaged proteins; dysfunction in protein folding activates cellular proteolytic mechanisms [6-8]. Ubiquitin Proteasome System (UPS) is the primary selective proteolytic process in cells that removes aberrant client proteins [9]. Cells can recognize and separate the aberrant proteins for their removal with the help of E3 ubiquitin ligase enzymes of the UPS [10]. In cells, utilization of a few E3 ubiquitin ligases that specifically participate in cellular PQC mechanisms generates the first line of cellular defense against proteotoxic insults [11, 12]. Previous findings suggest that mutations or dysfunctions of E3 ubiquitin ligases contribute to neurodegenerative and neurodevelopmental disorders [13-15]. In cells under stress conditions, altered functions of quality control E3 ubiquitin ligases contribute to the proteostasis imbalance leading to proteotoxic insults [10].

Depleted molecular functions of PQC-E3 ubiquitin ligases consequently result into the build-up of aggregation-prone proteins affecting cellular health [16]. Over accumulation of misfolded protein inclusions can inhibit the proteasome system and may activate the autophagic pathway for the removal of unwanted aggresomes [17, 18]. Such findings suggest that upregulation of PQC-E3 ubiquitin ligases may ameliorate proteotoxicity and support the re-establishment of healthy proteostasis. However, the major challenge is to find out the molecular strategy by which we can activate or upregulate the functions of perturbed PQC-E3 ubiquitin ligases against aggregation-prone proteins associated with cellular damage.

Earlier studies have suggested that few natural compounds demonstrate strong anti-aggregation capabilities, and the use of such compounds may suppress the accumulation of proteins, which is likely an approach to minimize cellular damages [19-22]. Resveratrol

is found in raspberries and grapes, belonging to stilbene compounds. Interestingly, dietary supplement consumption of resveratrol decreases plaques synthesis in an animal model of Alzheimer's disease [23, 24]. Moreover, resveratrol treatment in early Parkinson's disease patients demonstrates improved chaperone networks along with enhanced protein degradation and redox balance restoring cytoprotection in their skin fibroblasts [25]. Such findings gave us a clue that resveratrol likely exhibits an inductive potential to improve the functions of PQC mechanisms against the abnormal proteinaceous inclusions. But, still, the mechanism by which resveratrol reduces the overburden of misfolded protein accumulation and destabilizes amorphous protein aggregates is unknown.

In our present investigation, we found that exposure of resveratrol elevates the endogenous levels of LRSAM1 E3 ubiquitin ligase as well as slows down its intracellular turnover; earlier, it has been reported that mutations in LRSAM1 cause spongiform neurodegeneration [26] and it also exhibits PQC competence, which suppresses misfolded proteins aggregation [10]. We observed that resveratrol treatment efficiently inhibits aggregation of bona fide mutant proteins and also encourages the clearance of thermally misfolded luciferase inclusions. Our current findings imply that the use of resveratrol may offer promising cytoprotective molecular strategies, which can alter the functions of PQC-components that, consequently induces the degradation of aberrant proteins. Altogether our results suggest that most likely, in the future we can develop a molecular strategy to prevent the loss of proteostasis via the removal of over accumulated toxic aggregates to overcome the impairment of PQC mechanisms.

Materials and Methods

Materials

Resveratrol, chloroquine, cycloheximide, 2-mercaptoethanol (BME), lactacystin, MG132, TRIzol, and all reagents for cell culture were procured from Sigma. OptiMEM and Lipofectamine® 2000 were acquired from Life Technologies. Luciferase antibody, anti-ubiquitin, anti-LRSAM1, anti-β actin, anti-GAPDH, anti-c-myc, and anti-actin antibodies were purchased from Santa Cruz Biotechnology. Anti-Lamp2 antibody was obtained from Thermo Fisher Scientific, anti-LC3 antibody was procured from Pierce antibodies, anti-p62/SQSTM1 antibody was procured from Sigma, and LRSAM1 antibody was obtained from Cell Signaling Technology. iScript™ One-Step RT-PCR Kit along with SYBR® was procured from Bio-Rad Laboratories. Proteasome-Glo™ assay reagents and Dual-luciferase reporter gene assay kits were obtained from Promega. Fluorescein isothiocyanate-conjugated (FITC) IgG anti-mouse and anti-rabbit, Rhodamine-conjugated IgG anti-mouse and anti-rabbit and horseradish peroxidase (HRP) conjugated IgG anti-mouse and anti-rabbit antibodies were acquired from Vector Laboratories. Luciferase-pcDNA3 (Addgene 18964) and pcDNA3-cMyc (Addgene 16011) were procured from Addgene, pcDNA™ 3.1 was obtained from Life Technologies, LRSAM1ΔRING-Myc was synthesized by digesting LRSAM1-Myc plasmid with BglII for 3 hours at 37°C and further T4 DNA ligase mediated self-ligation was performed overnight at 37°C; LRSAM1-specific siRNA oligonucleotides were obtained from Life Technologies.

Cell Culture Experiments

A549 and COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) comprising 10% fetal bovine serum (heat-inactivated) along with streptomycin (100 µg/ml)/penicillin (100 U/ml) antibiotic. Just before transfection, different culture plates were seeded with a sub-confluent density of cells. Transient transfection with different plasmid constructs was achieved using Lipofectamine® 2000 reagent following the instructions provided by the manufacturer. Post 24 h, cells were exposed to different chemical molecules and were prepared for immunoblotting or immunofluorescence staining. To assess the influence of resveratrol on cell morphology, COS-7 cells were subjected to different concentrations of resveratrol for diverse time intervals and visualized under a bright-field microscope.

Immunofluorescence Technique

COS-7 cells were plated into the 2-well chamber slides and then briefly transfected with different constructs and treated with control or resveratrol or MG132 or lactacystin. Post this, the slides were treated with phosphate-buffered saline (PBS) thrice, and fixation with 4% paraformaldehyde solution (PBS) for 15-20 minutes was done. They were then treated with 0.5% Triton X-100 for cell membrane permeabilization (5 mins), thoroughly washed with PBS for 3-4 times, and incubated in 2% rabbit serum solution (PBS) for a minimum of 30 mins. Post this, the cells were incubated overnight with different primary antibody (1:500 dilution) at 4°C, and afterward thoroughly washed with PBS for three times (5 mins each) and treated with secondary antibody conjugated either with rhodamine or fluorescein isothiocyanate for 1-4 hours. Afterward, the cells were mounted with an antifade solution and 4',6'-diamidino-2-phenylindole (DAPI) to stain nuclei as described earlier [27]. Then the prepared slides were observed under a fluorescence microscope.

Immunoblotting Technique

Transient transfection was performed with different plasmids on the cells maintained in 6-well plates and treated with control or resveratrol or MG132. Post exposure, whole cell lysates were made and used for performing SDS-PAGE (Bio-Rad Laboratories). Electrophoresed samples were transported onto the nitrocellulose membrane. Blocking was done using 5% non-fat milk in Tris-buffered saline Tween 20 (TBST) [50 mM Tris; 0.15 M NaCl, 0.1% Tween 20, pH 7.4] for not less than an hour, then were treated overnight at 4°C with suitable primary antibody solution in TBST (1:1000 dilution). After thoroughly washing with TBST for 3-4 times (5 mins each), the nitrocellulose membrane was treated with a secondary antibody conjugated with HRP enzyme (1:10000) for not less than 30 minutes. Development of blots was then carried out with Luminata Crescendo Western HRP substrate (EMD Millipore).

Cycloheximide Chase Experiment and Reporter Gene Assay

Resveratrol or control-treated COS-7 cells into 6-well plates were further treated with cycloheximide (15 µg/ml) for multiple time points. Total protein isolation was done, and the immunoblotting experiment was performed with anti-LRSAM1 and anti-GAPDH primary antibodies. 6-well plates having COS-7 were used for transient transfection with LRSAM1-Myc and luciferase plasmids; some wells were subjected to either MG132 or chloroquine or resveratrol. Post this, the cells were kept for 30 minutes at 43°C and re-incubated under general incubation environment for 2 hours and then advanced to immunoblotting experiment with anti-luciferase and anti-β-Actin antibodies. Similar experimental setup was utilized for analyzing luciferase activity via using Dual-luciferase reporter gene assay kit (Promega) following the maker's protocol.

Reverse Transcriptase-Polymerase Chain Reaction Analysis (RT-PCR)

A549 cells (primer compatible) were maintained onto 6-well culture plates and subjected to either control or resveratrol with different concentrations and different periods. Post-treatment, cells were harvested to isolate whole-cell RNA with the help of TRIzol. Further RT-PCR was performed for analyzing LRSAM1 and β-actin using the primer sequence; LRSAM1F, 5'-AACGCTGGAGTACCAGATG-3', LRSAM1R, 5'-AACCTTGATGGTTGCCAGAC-3', β-actinF, 5'-ATCGTCCACCGCAAATGCTTCTA-3', β-actinR, 5'-AGCCATGCCAATCTCATCTTGT-3'. The complementary DNA (cDNA) was first synthesized utilizing the total cell RNA with the help of iScript™ cDNA synthesis kit (Bio-Rad Laboratories) and amplified using SsoFast™ EvaGreen® Supermix and MyiQ™ Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories). The parameters for carrying out 37 cycles of PCR with LRSAM1 primers were 94°C 30s, 55°C 30s, 72°C 30s and 72°C 2 min (final extension). The reaction conditions for 25 cycles with β-actin primers were 94°C 30s, 55°C 45s, 72°C 30s, and 72°C 2 min (final extension).

Proteasome Activity Assay and Aggregate Counting

COS-7 cells were transiently transfected by LRSAM1-Myc or LRSAM1ΔRING-Myc or LRSAM1-siRNA and utilized to carry out proteasome activity assay with Proteasome-Glo™ systems (Promega) based on the protocol of the manufacturer. All the experiments were done in triplicates to analyze changes in proteasome activity. Brief transfection of COS-7 cells was performed for 48 h with pd1EGFP, followed by control or MG132 or resveratrol treatment to quantify misfolded d1EGFP aggregates. Cells with greater than one aggresome were marked as one prominent inclusion, and fluorescence microscopy (~450 transfected cells in both cases) was utilized to visualize and quantify the misfolded d1EGFP aggregates.

Results

Effects of Resveratrol on the Endogenous Level of E3 Ubiquitin Ligase LRSAM1 Linked With Cellular Protein Quality Control Mechanism

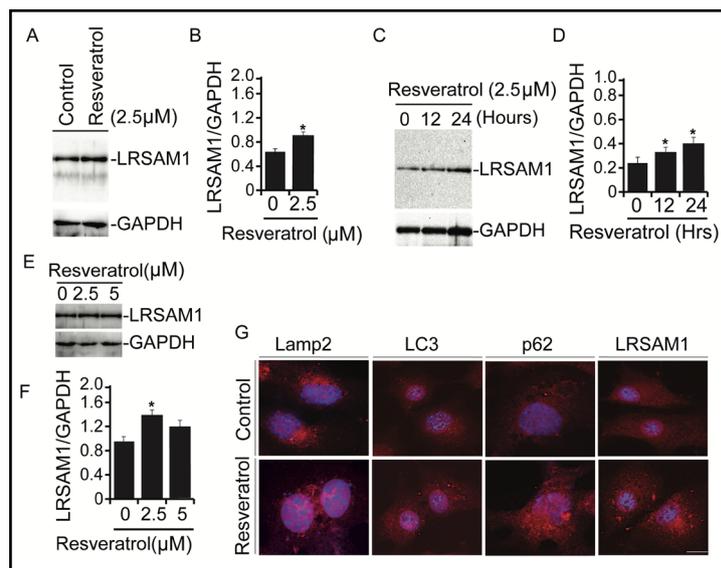
Polyphenol resveratrol, a natural compound, retains antioxidant properties. To elucidate the molecular characteristics of resveratrol against abnormal proteins, we conducted the analysis of cellular PQC components following resveratrol treatment. As shown in Fig. 1A-1B, we surprisingly observed that LRSAM1 endogenous level was increased after exposure of cells to resveratrol. Preliminary results suggested that treatment of resveratrol induces LRSAM1 levels. To further confirm these findings, similar experiments were performed at different time periods (Fig. 1C-1D) and in a concentration-dependent (Fig. 1E-1F) manner [28] and noticed similar effects of resveratrol as observed in the earlier analysis.

The elevated levels of LRSAM1 in response to resveratrol treatment suggest the relevant anti-aggregation molecular cope-up strategy of cells against intractable misfolded protein aggregates. To investigate the cytoprotective prospect of resveratrol over abnormal proteins via PQC components modulation, we performed an immunofluorescence analysis with or without resveratrol. As shown in Fig. 1G micrographs, exposure of resveratrol marginally upregulated various PQC components such as Lamp2, LC3, and p62 proteins. But in a similar experiment, treatment of resveratrol significantly induced the endogenous levels of LRSAM1 E3 ubiquitin ligase. These results indicate that resveratrol treatment can affect the levels of PQC components up to a considerable extent, which may generate cytoprotection against proteotoxic insults.

Expression of LRSAM1 Can Elevate Intracellular Proteasome Activities

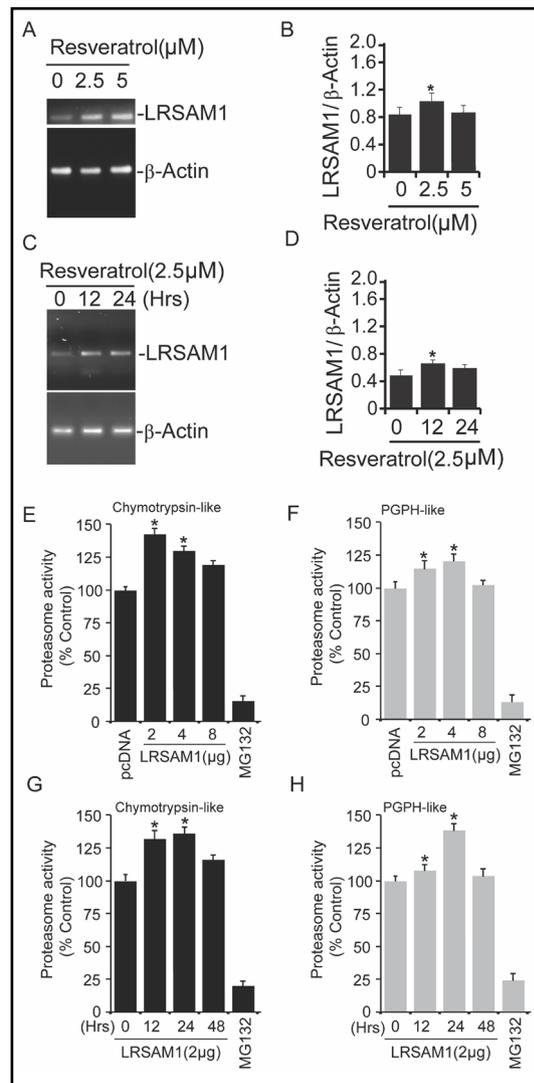
Previous findings suggest that resveratrol induces proteasomal degradation of Nanog via p53 activation [29]. In our current findings, we have noticed the elevation of LRSAM1 mRNA levels after resveratrol treatment in both concentration (Fig. 2A-2B) as well as time-dependent (Fig. 2C-2D) manner. Our earlier study demonstrates that LRSAM1 can target misfolded proteins for their intracellular degradation [10]. These findings prompt us to

Fig. 1. Resveratrol Increases the Levels of LRSAM1 in Cells. (A) and (B) COS-7 cells were treated with control and resveratrol (2.5 μ M); after 24 hours, cells were prepared for immunoblotting analysis with anti-LRSAM1 and anti-GAPDH antibodies, and their band intensities were quantified. (C) and (D) Cells were exposed in a time-dependent fashion (Resveratrol 2.5 μ M) and (E) and (F) at different concentrations (Resveratrol 24 h); cell lysates were prepared for immunoblotting with anti-LRSAM1 and anti-GAPDH antibodies; subsequent band intensity quantification (concentration and time) was done; for all the experimental set



up the band intensities were procured from three autonomous experiments and analyzed through NIH image analysis software; GAPDH was utilized to normalize the total protein amounts of samples in different experiments. (G) Similarly, resveratrol (2.5 μ M for 24 h) and control treated COS-7 cells on 2-well chamber slides were subjected to immunofluorescence analysis with anti-Lamp2, anti-LRSAM1, anti-p62, and anti-LC3 antibodies and visualized under fluorescence microscope; DAPI was used for staining nuclei. Scale bar, 20 μ m.

Fig. 2. Enhanced Expression of LRSAM1 Increases Proteasome Function in Cells. A549 cells were exposed with varying concentrations (Resveratrol 24h) (A) and for diverse time intervals (Resveratrol 2.5 μ M) (C). The total cell RNA was isolated and utilized to perform RT-PCR with LRSAM1 and β -actin (internal control) specific primers; all the reactions were done three times independently and included negative control; their respective band intensities (concentration (B) and time (D)) were quantified and analyzed through NIH image analysis software. (E-H) COS-7 cells were transiently transfected by diverse concentrations (24 h) and for diverse time interval (2 μ g) with LRSAM1 or only pcDNA expression constructs; some cells were subjected to MG132 (10 μ M for 12 h), a putative proteasomal inhibitor and later processed to quantify chymotrypsin-like (E (concentration) and G (time)); PGPH-like (F (concentration) and H (time)) proteasome functions. The values represented are mean \pm SD and acquired from a group of triplicate experiments performed three times autonomously. *P<0.05 when in comparison of control.



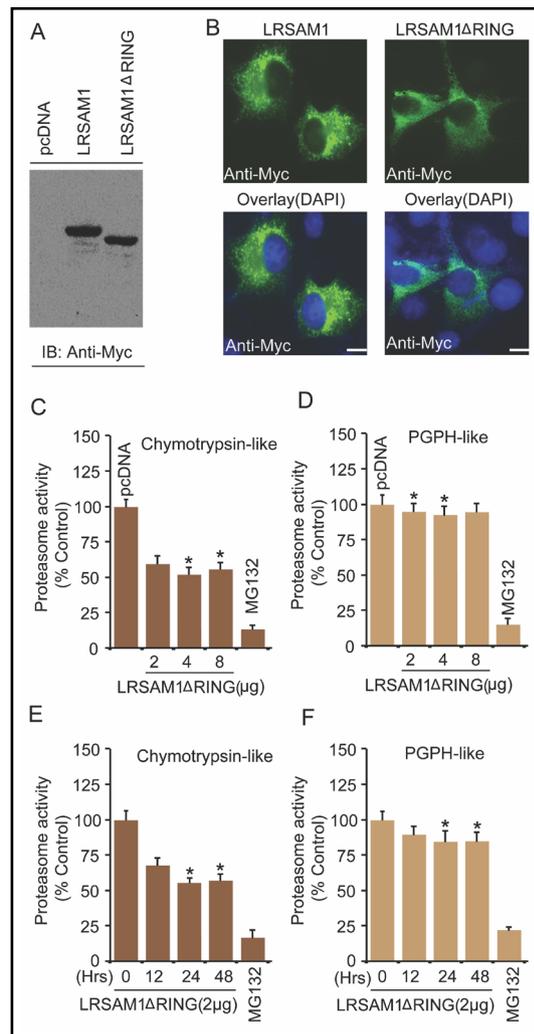
further characterize the detailed modulatory effects of LRSAM1 E3 ubiquitin ligase on intracellular protein destruction mechanism. Therefore, we have briefly transfected COS-7 cells with wild-type LRSAM1 plasmids in different concentration profiles. Post-transfected cell extracts were used for proteasome assay as described in the methods section.

We have observed elevation in both chymotrypsin-like (Fig. 2E) as well as post-glutamyl peptide hydrolase (PGPH)-like (Fig. 2F) protease activities of the proteasome after transient transfection of LRSAM1 construct at various concentrations. In a similar way, to further confirm the effect of LRSAM1 at different time intervals, we next performed other sets of proteasome assays with both chymotrypsin-like (Fig. 2G) PGPH-like protease activities (Fig. 2H), exogenous expression of LRSAM1 also induced the protease activities of proteasome at different time points. These results suggest that the upregulation/overexpression of LRSAM1 can modulate proteasome activities at varying concentrations and times.

Inactive Form of LRSAM1 Did Not Induce Proteasome Activities

Several intracellular regulatory systems respond to abnormal protein aggregation problems. Certain E3 ubiquitin ligases function specifically for PQC and finally target aberrant proteins for clearance. Previously, our study suggests that LRSAM1 also recognizes misfolded proteins for their degradation [10]. To further validate the function of LRSAM1 E3 ubiquitin ligase in moderating the proteasome function, cells were briefly transfected with

Fig. 3. RING Domain of LRSAM1 Is Necessary for Its Function to Enhance Proteasome Activity. (A) COS-7 cells were transfected with different plasmid constructs; pcDNA or LRSAM1-Myc or LRSAM1 Δ RING-Myc for 24 h; then, the cells were prepared to carry out immunoblotting analysis with anti-Myc antibody. (B) Similarly, COS-7 cells from 2-well chamber slides were transfected with LRSAM1-Myc or LRSAM1 Δ RING-Myc plasmid constructs for 24 h; and further proceeded for immunofluorescence analysis with anti-Myc antibody staining; DAPI was used for counterstaining the nucleus. Scale bar, 20 μ m. (C-F) Transfection of COS-7 cells was performed with diverse concentrations (24 h) and for various time intervals (2 μ g) with LRSAM1 Δ RING-Myc plasmid, and some cells exposed to MG132 (10 μ M for 12 h); treated cells were used to quantify chymotrypsin-like (C (concentration) and E (time)); PGPH-like (D (concentration) and F (time)) proteasome functions. The values represented are mean \pm SD and acquired from a triplicate set of experiments done three times independently. *P<0.05 when in comparison of control.

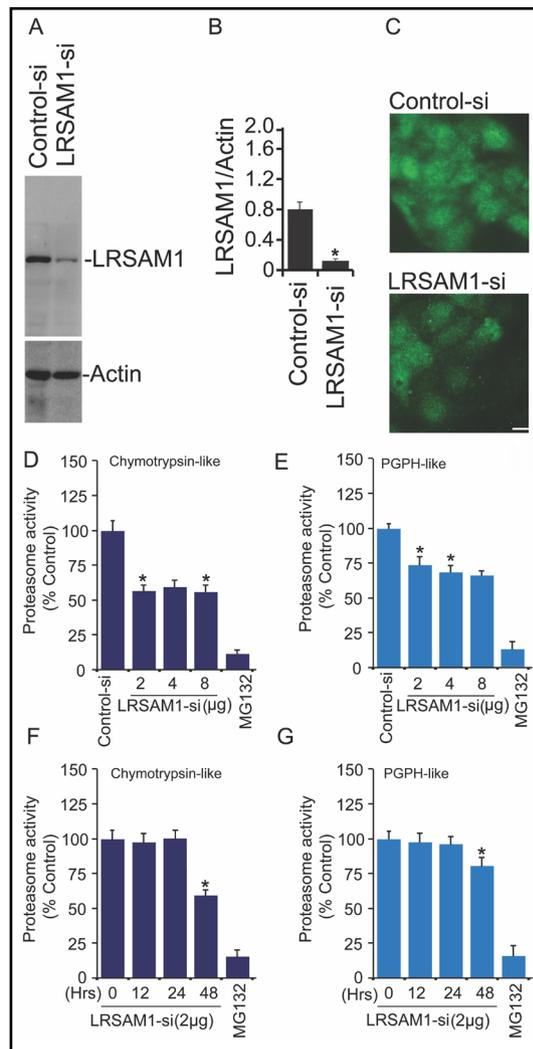


LRSAM1 Δ RING mutant, the catalytically inactive form of the full-length LRSAM1 construct. The expression of catalytically inactive LRSAM1 Δ RING mutant was confirmed by both immunoblotting and immunofluorescence analysis, as shown in Fig. 3A and 3B, respectively. We then examined the effects of LRSAM1 Δ RING on both chymotrypsin-like and PGPH-like protease activities. Next, we detected that exogenous expression of LRSAM1 Δ RING mutant suppressed both proteasome chymotrypsin-like (Fig. 3C) and PGPH-like protease (Fig. 3D) activities in a concentration-dependent fashion. A similar effect of LRSAM1 Δ RING mutant was found on the above-described proteasome chymotrypsin-like (Fig. 3E) and PGPH-like protease (Fig. 3F) activities when cells were transiently transfected at various time intervals.

Depletion of LRSAM1 Cannot Induce Proteasome Activities

Because we have observed the induction of proteasome activities after exogenous expression of normal LRSAM1 in comparison to inactive LRSAM1 Δ RING form, it encouraged us to examine the consequence of LRSAM1 depletion on various proteasome activities. To test this question, we knock down the endogenous levels of LRSAM1 by using the siRNA transfection approach. Transient transfection of A549 cells with control and LRSAM1-siRNA was performed and post-transfected cell extracts were used for the verification of siRNA-mediated LRSAM1 depletion via immunoblotting (Fig. 4A-4B) and immunofluorescence analysis as depicted in Fig. 4C micrographs. Next, to address the above question, we performed detailed proteasome assays in the presence of different concentrations of

Fig. 4. LRSAM1 Knockdown Diminishes the Proteasome Function. (A) A549 cells were cultured and briefly transfected with Control or LRSAM1-siRNA for 24 h; afterward, cells were harvested, and total protein was analyzed with anti-LRSAM1 and anti-Actin antibodies using immunoblotting protocol; Actin protein amounts were utilized to normalize the protein content in each sample, and the quantified band intensities (B) from NIH image analysis software were plotted for three independent experiments. (C) Similar experimental setup was used to carry out the immunofluorescence analysis using anti-LRSAM1 antibody, visualized under fluorescence microscope; scale bar, 20 μ m. (D-G) A549 cells were briefly transfected by different concentrations (24 h) and for a different time interval (2 μ g) with LRSAM1-siRNA or Control-siRNA; some cells were exposed to MG132 (10 μ M for 12 h) and advanced as described in the method section to quantify the chymotrypsin-like (D (concentration) and F (time)) and PGPH-like (E (concentration) and G (time)) proteasome activity. The values represented are mean \pm SD and acquired from triplicate set of experiments done three times independently. *P<0.05 when in comparison of control.



LRSAM1-siRNA. Proteasome assay analysis revealed that both chymotrypsin-like (Fig. 4D) and PGPH-like (Fig. 4E) protease activities of proteasome were suppressed when LRSAM1 was partially depleted from the cells. Similar results were obtained when LRSAM1-siRNA were transfected at different time intervals, and post-transfected cells were utilized for measuring chymotrypsin-like (Fig. 4F) as well as PGPH-like (Fig. 4G) protease activities. Altogether, this set of results shows that depletion of LRSAM1 can marginally suppress proteasome activities.

Resveratrol Treatment Reduces the Aggregation of Aberrant Proteins

Several neurodegenerative diseases are a result of the over-build-up of aberrant proteins. Thus, it is crucial to check the outcome of resveratrol exposure on aggregation propensity of mutant bona fide misfolded proteins. COS-7 cells were transiently transfected with GFP-wtCAT (Fig. 5A and 5B) and GFP- Δ 9CAT (Fig. 5C and 5D) constructs. Certain sets of post-transfected cells were exposed to resveratrol and putative proteasome inhibitor MG132 (Fig. 5A-5D). Exogenous GFP- Δ 9CAT expression produced intracellular aggregates in comparison to GFP-wtCAT protein. Exposure of resveratrol dramatically reduced the number as well as the size of misfolded GFP- Δ 9CAT protein aggregates (Fig. 5C and 5E); the addition of MG132 resulted in further accumulation of distinct perinuclear GFP- Δ 9CAT aggresomes (Fig. 5D and 5F). Consistent with our preliminary findings, here we observed that resveratrol treatment induces the clearance of aberrant protein accumulation, probably via influencing the function of cellular PQC machinery and its molecular components.

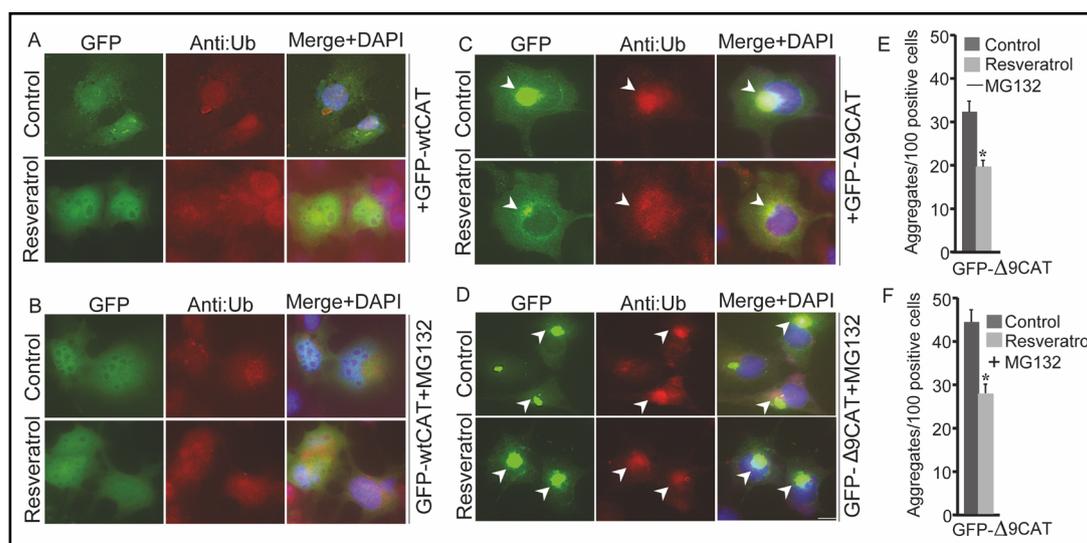


Fig. 5. Resveratrol Treatment Helps in the Removal of Mutant Bona Fide Misfolded Proteins. (A-D) Transient transfection of COS-7 cells was performed for 24 h with GFP-wtCAT (A, B) and GFP-Δ9CAT (C, D); later incubated with either control or Resveratrol (2.5 μM) for 24 h. Certain sets of the same experiment were performed with MG132 (10 μM for 12 h). Afterward, the cells were processed for immunocytochemistry analysis with anti-ubiquitin antibody. The accumulation of misfolded GFP-Δ9CAT in the cytoplasmic perinuclear region was confirmed along with the co-localization of ubiquitin (arrows) through an immunofluorescence microscope. The nucleus location was confirmed with the help of DAPI (blue) staining. Scale bar, 20 μm. The quantification of GFP-Δ9CAT aggregates was done in the presence of resveratrol without (E) or with (F) MG132. The results are represented as mean ±SD of triplicate set of three autonomous experiments, *P<0.05 in comparison of control.

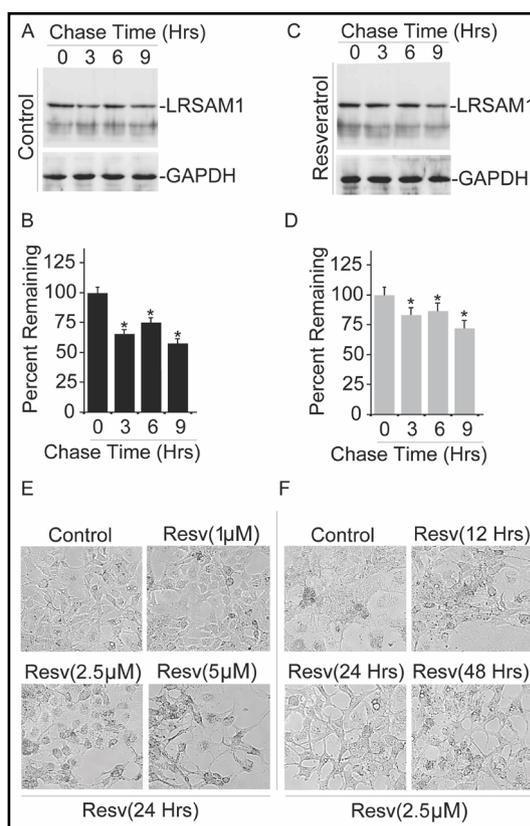
Resveratrol Stabilizes Endogenous Level of LRSAM1 Protein

To determine how the treatment of resveratrol influences the intracellular amounts of LRSAM1 E3 ubiquitin ligase, we determined whether the half-life of LRSAM1 is affected in the presence of resveratrol. We carried out a cycloheximide chase analysis to quantify the half-life of LRSAM1, with or without resveratrol (Fig. 6A-6D). In the chase experiment, synthesis of new proteins had been blocked by cycloheximide; collected cell lysates of chase-experiment at different time points were used for immunoblotting analysis via using LRSAM1 antibody. As shown in Fig. 6C and 6D, protein amounts of LRSAM1 were significantly stabilized after the treatment of resveratrol as compared to the control experiment (Fig. 6A and 6B). Simultaneously, it is important for us to determine whether resveratrol addition affects overall cellular viability or not; therefore, cells were subjected to resveratrol treatment at different concentrations and time intervals, and bright-field images were acquired as shown in Fig. 6E and 6F, respectively. In our current observation, no significant adverse effects of resveratrol were noticed on cell viability. Notably, the turnover rate of LRSAM1 protein was more stabilized when exposed to resveratrol, suggesting that the exposure of resveratrol to the LRSAM1 protein turnover is specific and does not affect cell viability.

Resveratrol Promotes Heat-Denatured Luciferase Protein Degradation and Protects Against Cellular Stresses

Proteotoxic insults associated with cell death are majorly caused by the build-up of misfolded proteins. Despite this, we know that resveratrol may retain the molecular capabilities to improve the functions of PQC mechanism, but it is critical for us to detect the outcome of resveratrol exposure on the levels of various model-misfolded proteins. In order to examine this, cells were briefly transfected with a luciferase-containing plasmid construct. Post-transfected cells were subjected to 43°C for 30 minutes, and recovery

Fig. 6. Resveratrol Stabilizes LRSAM1 Level in Cell. (A-D) COS-7 cells were given resveratrol treatment (2.5 μ M for 24 h) and then with cycloheximide for different time intervals; post-treatment, the cells were prepared for immunoblotting experiment with anti-LRSAM1 and anti-GAPDH antibodies (A (control) and C (resveratrol)); their respective band intensities were quantified (B (control) and D (resveratrol)) from three autonomous experiments via using NIH image analysis software; GAPDH was utilized to normalize the total protein for each sample. COS-7 cells were grown onto a 6-well plate, and resveratrol was given in concentration- (Resveratrol 24 h (E)) and time-dependent (Resveratrol 2.5 μ M (F)) manner or with control and visualized by bright-field microscope as represented in the micrographs.

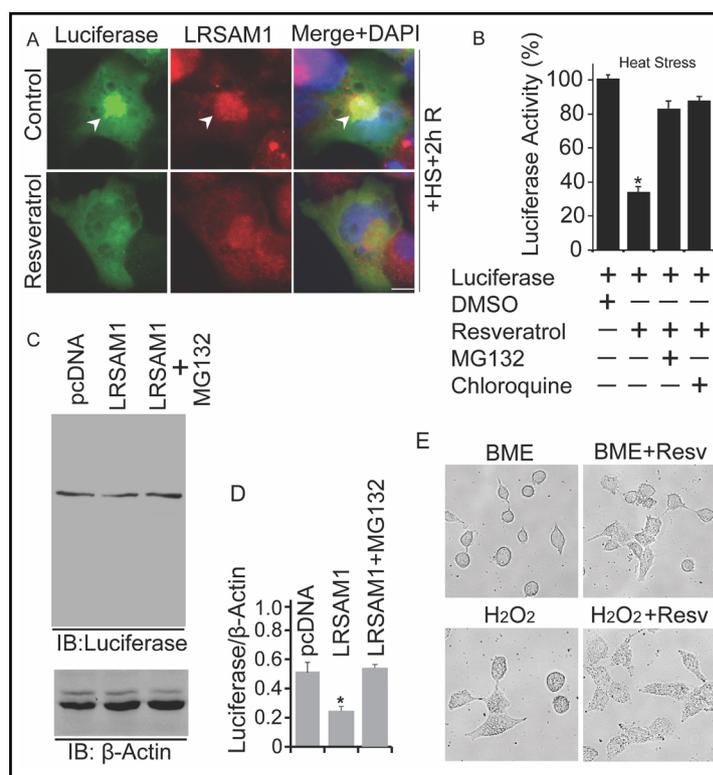


was attained at 37°C for 02 hours. Similarly, some cells were treated with resveratrol and used for immunofluorescence analysis using anti-LRSAM1 antibody (Fig. 7A). Treatment of resveratrol elevated the cellular levels of LRSAM1 and promotes the degradation of aberrant heat-distorted luciferase inclusions. As described above, for luciferase assay, identical sets of cells were used (Fig. 7B). Furthermore, compared to the control (DMSO), the addition of resveratrol induces the clearance of thermally-misfolded luciferase, and the effect of resveratrol was suppressed by the treatment of MG132 and chloroquine. Immunoblot analysis (Fig. 7C and 7D) also confirmed that exogenous expression of LRSAM1 promotes the clearance of heat-denatured proteins and was stopped by MG132 addition. Accumulation of misfolded proteins can cause various types of stress, such as oxidative and ER stress conditions [26]. As demonstrated in Fig. 7E, treatment of resveratrol possesses a cytoprotective response against ER (2-Mercaptoethanol; BME) and oxidative stress (H₂O₂) inducing agents.

Resveratrol-Based PQC Moderation Capabilities Alleviates Cytotoxicity

Polyubiquitinated proteins are generally degraded by proteasome machinery. Inhibition of proteasome may lead to the build-up of aberrant proteins, causing proteotoxic insults in cells [30]. To test the notion of how resveratrol can affect overall aggregation propensity, we transiently transfected a model proteasome substrate pd1EGFP plasmid that retains proline, glutamic acid, serine, and threonine signal sequence that can be used for specifically targeting for proteasome degradation. As shown in Fig. 8A and 8C, inhibition of proteasome by MG132 forms ubiquitinated d1EGFP inclusions, and the addition of resveratrol reduces the formation of those abnormal inclusions. To further confirm the above results, next, we used lactacystin, another putative proteasome inhibitor; as demonstrated in Fig. 8B and 8D, treatment of lactacystin induces the abundance of d1EGFP aggregates, which can be reduced by the exposure of resveratrol. Because treatment of resveratrol decreases the number of aggregates, we thought to use this as a reliable strategy to further validate the

Fig. 7. Effect of Resveratrol on Removal of Heat-Denatured Luciferase and Other Proteotoxic Stress. (A) COS-7 cells were cultured onto 2-well chamber slides and temporarily transfected with firefly luciferase expression plasmid (24 h). The cells were subjected to control or resveratrol (2.5 μ M for 24 h) and later to heat-stress at 43°C for 30 mins and re-incubated at 37°C for 2 h (recovery); the cells were then prepared for immunofluorescence analysis using anti-luciferase (green) and anti-LRSAM1 (red) antibody visualized under a fluorescence microscope. The arrowheads denotes the perinuclear accumulation of the misfolded luciferase co-localized with LRSAM1; DAPI was utilised for staining nuclei; scale bar, 20 μ m. Similar experimental setup in 6-well culture plates was used to carry out the luciferase activity assay (B); where



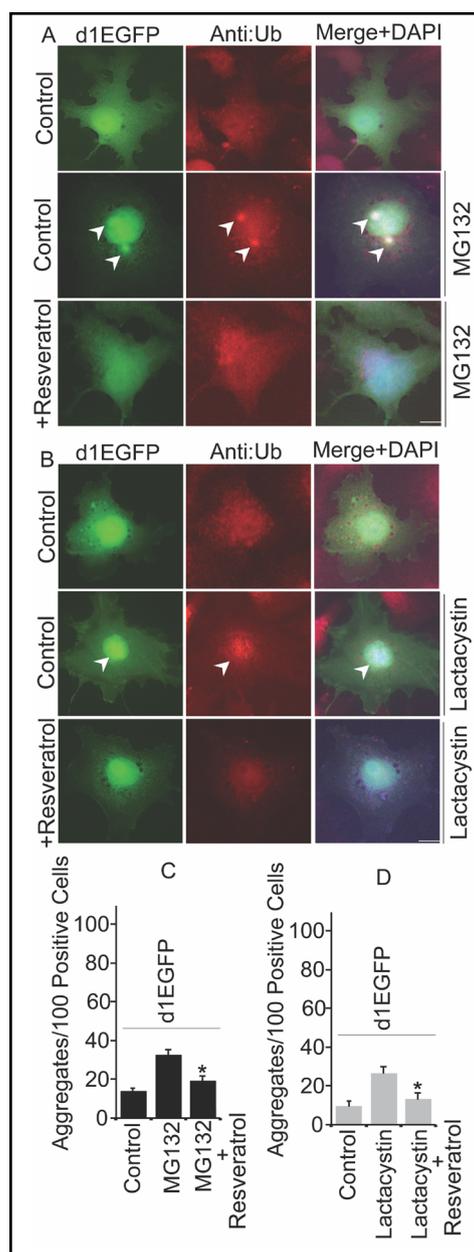
some cells were subjected to MG132 (10 μ M) or chloroquine (20 μ M) for 12 hours. COS-7 cells with transient transfection of luciferase were then briefly overexpressed with LRSAM1 constructs for 24 h, and the cell lysate was used for immunoblotting examination with anti-luciferase and anti- β actin antibodies (C); β -actin was compared to normalize the total protein levels of each samples and the band intensities were quantified and plotted (D) from a group of three independent experiments and analyzed through NIH image analysis software. (E) COS-7 cells were treated either with Resveratrol (2.5 μ M for 24 h) and 5 mM BME for 3 h or only BME or Resveratrol (2.5 μ M for 24 h) and 0.5 mM H_2O_2 for 3 h or only H_2O_2 , and visualized under normal bright-field microscope as demonstrated in micrograph (representative of triplicates experiments).

cytoprotective nature of resveratrol based on the elevation of LRSAM1 levels. Collectively, our results suggest that resveratrol exhibits cytoprotective PQC moderation capabilities, and most likely; it targets LRSAM1 E3 ubiquitin ligase for clearing over accumulated or misfolded proteins.

Discussion

Cells acquire an intracellular defensive PQC mechanism against the accumulation of misfolded proteins [31, 32]. It is feasible to expect that elevation of PQC mechanisms predominantly targets over-accumulated damaged or old proteins. Prevention of such clearance may lead to pathogenic events specifically linked with the post-mitotic cells or neurodegenerative disorders [33, 34]. Despite the fact that the clinical indication is an aggregation of abnormal inclusions in protein conformational disorders, what molecular strategies can we design that can help ameliorate overall cellular health and fitness? Previous findings suggested that natural compounds might generate a better therapeutic strategy to ameliorate proteotoxicity associated with neurodegenerative diseases and imperfect ageing [35-37]. Our current findings suggest that resveratrol, a stilbenoid belonging to a natural phenol group, may alter the PQC mechanism through specific modulations in the levels of LRSAM1 E3 enzyme associated with spongiform neurodegeneration.

Fig. 8. Resveratrol Treatment Alleviate Misfolded Protein Aggregation. Brief transfection of COS-7 cells with pd1E-GFP construct was done for 24 h and later treated with control or Resveratrol (2.5 μ M for 24 h). Some cells were subjected to either MG132 (10 μ M for 12 h) (A) or Lactacystin (10 μ M for 12 h) (B); and further processed for immunofluorescence analysis with anti-ubiquitin antibody and visualized using fluorescence microscope. The nuclear localization was confirmed through DAPI staining; the arrowheads represent the perinuclear misfolded d1EGFP aggregates; Scale bar, 20 μ m. In a similar experimental setup, the transiently transfected pd1EGFP cells were subjected to either control or resveratrol (2.5 μ M for 24 h), and some of the cells to MG132 (10 μ M for 12 h) (C) or Lactacystin (10 μ M for 12 h) (D) and the quantification of d1EGFP misfolded aggregates was done. The results are represented as mean \pm SD of triplicate set of three autonomous experiments, *P<0.05 in comparison of control.



Among the primary observations, we have explicitly noticed the precise effects of resveratrol on the intracellular LRSAM1 E3 enzyme levels associated with the cellular PQC pathways. Additionally, we also noticed that LRSAM1 expression elevates intracellular proteasome activities, and the inactive form of LRSAM1 did not induce proteasome activities. Our data shows that resveratrol treatment induces the LRSAM1 endogenous levels under both concentration and time-dependent manner. Earlier our findings clarify the cytoprotective nature of LRSAM1 against aberrant protein accumulation [10, 38]. Current results implicate, for the first time, an opportunity that resveratrol can induce intracellular LRSAM1 E3 ubiquitin ligase; such contribution can clear misfolded proteins from the cell and open novel prospects for improved understanding of proteinopathies at molecular levels.

The observation that the LRSAM1 level is induced in the presence of resveratrol leads to the question how proteasome machinery can clear the elevated LRSAM1-mediated degradation load. Previous findings suggest that induced proteasomal activities can efficiently degrade abnormal proteins [39, 40]. These studies provide a clue that probably

elevated LRSAM1 can alter proteasome functions; since the effects of LRSAM1 on proteasome activities retain critical consequences for the degradation of ubiquitinated proteins. To answer this question, we overexpressed LRSAM1 at various concentration and time-based studies and used samples for proteasome activities analysis. Surprisingly, we noticed that exogenous expression of LRSAM1 elevated proteasome activities, which can be compromised when LRSAM1 Δ RING mutant inactive form is expressed. Also, LRSAM1 partial depletion via the siRNA knock-down approach confirmed the specific effects of LRSAM1 on proteasome activities. These results suggest that treatment of resveratrol most likely elevates LRSAM1 levels, which further indirectly leads to a cellular signal to induce proteasome functions for better degradation of ubiquitinated proteins. Our findings are also supported by earlier studies which demonstrated that resveratrol induces PQC components such as the autophagy mechanism [28].

We observed for the first time the elevation of LRSAM1, a component of PQC molecular machinery, after resveratrol treatment. Accumulation of misfolded proteins can inhibit proteasome functions [18], yet it is challenging to design a molecular approach by which cells can efficiently clear aggregation-prone aberrant proteins and avoid the blocking of the proteasome. To test whether treatment of resveratrol can promote the degradation of ubiquitinated misfolded proteins, here we executed an immunofluorescence experiment with bona fide wild-type chloramphenicol-acetyltransferase (wtCAT) and mutant Δ 9CAT proteins [41] both with and without resveratrol. Δ 9CAT generates intracellular aggregates and aggresome-like structures around the nucleus.

Notably, degradation of GFP- Δ 9CAT aggregates was significant in the presence of resveratrol as compared to control. The present findings open experimental avenues for further analysis and clarified the effects of resveratrol on LRSAM1 half-life. Under the cycloheximide chase experiment, we observed that resveratrol exposure slows down the degradation of LRSAM1 and induces its intracellular stabilization, which also maintains overall cellular health and viability.

Next, to demonstrate how resveratrol targets misfolded proteins, we used heat-denatured luciferase proteins in our experiments to find out the relevance of resveratrol treatment on modelled misfolded proteins. Resveratrol has anti-aggregation potential to mitigate the deposition of thermally denatured inclusions. Further consequences of these findings were also confirmed by luciferase assay and immunoblotting analysis of exogenously expressed luciferase protein obtained after heat shock and recovery experiments.

Conclusion

Our results demonstrate that use of resveratrol promotes proteasomal-model substrate d1EGFP inclusions clearance, which were formed after the inhibition of proteasome by using its putative inhibitors MG132 and lactacystin. Resveratrol treatment suppressed aggregation of misfolded proteins, and previous studies also suggest that decreased aggregates retention improves cell viability [42]. Understanding the anti-aggregation nature of this compound, we observed its potential as a cytoprotective agent. Using distinct combinatorial cellular proteotoxic stress conditions, we observed that resveratrol exhibits rescue effects against disturbed proteostasis and promotes cellular survival. Recent reports reveal that activated PQC responds to various cellular stress conditions [43] and prevents the accumulation of misfolded proteins, which may ameliorate proteinopathies [44, 45]. An increased understanding of specific elimination of damaged or old proteins via selective activation of PQC mechanism may open new therapeutic interventions and effective treatment against protein conformational disorders such as neurodegeneration and imperfect ageing.

Abbreviations

CAT (Chloramphenicol acetyl transferase); ER (Endoplasmic reticulum); GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); IgG (Immunoglobulin G); Lamp2 (Lysosome-associated membrane protein 2); LC3 (Microtubule-associated proteins 1A/1B light chain 3B); LRSAM1 (Leucine Rich Repeat and Sterile Alpha Motif Containing 1); p62/SQSTM1 (Sequestosome-1); PGPH (post-glutamyl peptide hydrolase); siRNA (Small inhibitory RNA).

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Author Contributions

A.R.D, R.M, N.S, Y.A.J, P.K, S.K and A.C performed the designed experiments and implemented the research. H.C.J, A.P and R.K.G verified the analytical methods and helped in generation of framework of experiments and perform critical analysis of manuscript. A.M overall design various experiments. Also contributed to the analysis of the results and perform the writing of the manuscript. All authors discussed the results and contributed to the final manuscript.

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Disclosure Statement

The authors declare that no conflicts of interest exist.

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