Video Article

Assays for the Degradation of Misfolded Proteins in Cells

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Abstract

Protein misfolding and aggregation are associated with various neurodegenerative diseases. Cellular mechanisms that recognize and degrade misfolded proteins may serve as potential therapeutic targets. To distinguish degradation of misfolding-prone proteins from other mechanisms that regulate their levels, one important method is to measure protein half-life in cells. However, this can be challenging because misfolding-prone proteins may exist in different forms, including the native form and misfolded forms of distinct characteristics. Here we describe assays to examine the half-life of misfolded proteins in mammalian cells using a highly aggregation-prone protein, Ataxin-1 with an extended polyglutamine (polyQ) stretch, and a conformationally unstable luciferase mutant as models. Cycloheximide chase is combined with cell fractionation to examine the turnover rate of misfolding-prone proteins in various cellular fractions. We further depict a fluorescence-based assay using an enhanced green fluorescence protein (EGFP)-fusion of the luciferase mutant, which can be adapted for high throughput screening on a microplate-reader.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54266/>

Introduction

Proteins are the most abundant macromolecules in cells, and they play an essential role in virtually all biological processes. The biological activity of most proteins requires their folding into, and maintaining, the native three-dimensional structures. Proteins with aberrant conformations not only lose their normal functions, but also frequently form soluble oligomeric species or aggregates that impair the functions of other proteins and are toxic to cells^{1,2}. To counteract protein misfolding, cells employ both molecular chaperones, which assist unfolded or partially folded polypeptides to reach their native conformation, and degradation pathways, which eliminate misfolded proteins³. Given the complexity and stochastic nature of the folding process, protein misfolding is inevitable, and it may not be reversed in the case of mutations, biosynthetic errors, and post-translational damages¹. Hence, cells ultimately rely on degradation pathways to maintain their protein quality.

The importance of cellular protein quality control (PQC) systems is underscored by the prevalence of protein-misfolding diseases, including cancer, diabetes, and many neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease (HD), and spinocerebellar ataxias (SCA)^{4,5}. For example, mutations in the tumor suppressor p53 is the single most frequently genetic lesion in tumors, associated with ~50-70% of all cases⁶. A substantial fraction of p53 mutations are missense mutations that alter the conformation of p53, leading to the formation of aggregates⁷. Furthermore, proteins with expanded polyQ stretches are genetically and pathologically associated with HD and SCA. These progressive and often fatal diseases manifest when the length of the polyQ stretch in the affected proteins exceeds a certain threshold, and becomes increasingly severe as the length of the polyQ stretch prolongs⁸.

An attractive approach for treating these diseases is to therapeutically bolster cellular PQC systems, especially the degradation pathways. However, the pathways involved in the degradation of defective proteins, particularly those in mammalian cells, remain poorly understood. Although it has been recognized that the proteasome is critically important for the degradation of misfolded proteins, a vital issue remains undefined: how misfolded proteins are specifically recognized and targeted for degradation. Moreover, although PQC systems have been identified in cellular compartments including the cytoplasm, the endoplasmic reticulum, and the mitochondrion, the PQC systems in the nucleus remain unclear².

Recent studies by our lab have identified a system that recognizes and degrades a variety of misfolded proteins in the nucleus of mammalian cells⁹. This system is comprised of the promyelocytic protein (PML), a nuclear protein and a member of the tripartite motif-containing (TRIM) protein family, and RNF4, a RING-domain containing protein. PML, and several other TRIM proteins, possess SUMO (small ubiquitin-like modifier) E3 ligase activity, which facilitates the specificity and efficiency of protein SUMOylation¹⁰. RNF4 belongs to a small group of SUMO-targeted ubiquitin ligases (STUBL), which contain one or more sumo-interacting motifs (SIMs) in addition to the RING domain that affords them the ubiquitin ligase activity¹¹. We found that PML specifically recognizes misfolded proteins through discrete substrate recognition sites that can discern distinct features on misfolded proteins. Upon binding, PML tags misfolded proteins with poly-chains of SUMO2/3, two nearly identical

mammalian SUMO proteins that can form poly-chains due to the existence of an internal SUMOylation site. SUMOylated misfolded proteins are then recognized by RNF4, which leads to their ubiquitination and proteasomal degradation. We further demonstrated that the PML-RNF4 system is important for protection against neurodegeneration, as deficiency in PML exacerbates the behavioral and neuropathological defects of a mouse model of SCA type 1 (SCA1)⁹.

To distinguish protein degradation from other cellular mechanisms that may regulate protein levels, the rates of protein turnover was measured⁹. Among the most frequently used methods to determine protein turnover are pulse chase and cycloheximide (CHX) chase. These two methods examine over time, respectively, the radioisotope-labeled proteins of interest in translation-proficient cells and total preexisting proteins of interest in translation-inhibited cells. However, a major challenge for studying pathogenic and misfolding-prone proteins is that the half-life of these proteins can be extremely long. For example, Ataxin-1, Ataxin-7, Huntingtin, α -synuclein, and TDP-43 all have half-lives of more than 12 to 24 hr^{9,12-16}. The slow turnover rates of these proteins preclude the use of the CHX chase analysis because the cells harboring these proteins may not survive prolonged translation inhibition, especially because the misfolded proteins themselves can be highly toxic to cells. The pulse-chase analysis with isotopic labeling may also be challenging for proteins that are highly aggregation-prone. Most pulse-chase assays rely on immunoprecipitation to separate the protein of interest from all the other proteins that are also radioactively labeled. This procedure normally includes lengthy immunoprecipitation and wash procedures, during which SDS-insoluble aggregates can form, making the analysis with SDS-PAGE electrophoresis inaccurate.

Here a protocol to analyze nuclear misfolded proteins with a slow turnover rate is described⁹. A pathogenic form of Ataxin-1 (Atxn1) that contains a stretch of 82 glutamines (Atxn1 82Q) is used for this purpose⁸. When expressed in cells, an enhanced green fluorescent protein (GFP) fusion of Atxn1 82Q forms microscopically visible inclusions in the nucleus (**Figure 1A**). Pulse chase analysis reveals that the half-life of Atxn1 82Q is over 18 hr⁹. Atxn1 82Q is composed of species with misfolded conformations of different characteristics, as well as species with native conformation. It is likely that these species are degraded at different rates, and thus should be analyzed separately. Lysates from Atxn1 82Q-GFP-expressing cells are fractionated into NP-40-soluble (soluble or NS, likely representing native proteins or misfolded monomeric/oligomeric proteins) and NP-40-insoluble (NI, aggregated/misfolded) portions. The latter can be further divided into SDS-soluble (SS; likely disordered aggregates) or SDS-resistant (SR; likely amyloid fibrils) fractions (**Figure 1B**). NS and SS fractions can be analyzed by SDS-PAGE followed by western blotting, whereas the SR fraction can be detected by filter retardation assays followed by immunoblotting. CHX chase is combined with the detergent fractionation method, and discovered that the half-life of SS Atxn1 82Q is much shorter than that of NS Atxn1 82Q and total Atxn1 82Q (**Figure 2A**), indicating that the SS fraction can be readily recognized and degraded in cells⁹. Thus, this method provides a powerful tool to study the dynamics of misfolded proteins and to compare their degradation pattern.

We also describe a method that is suitable for high throughput screening for identifying macromolecules or small compounds that can modulate the degradation of misfolded proteins. This method is based on a conformationally destabilized mutant of firefly luciferase (LucDM)¹⁷, a model chaperone substrate. We have fused LucDM to a nuclear localization signal (NLS) to probe the PQC systems in this cellular compartment, and GFP (NLS-LucDM-GFP) for convenience of detection. NLS-LucDM-GFP forms microscopically visible nuclear aggregates in a small percentage of cells (**Figure 3A**). Similar to Atxn1 82Q, NLS-LucDM-GFP but not its wild-type counterpart NLS-LucWT-GFP is modified by SUMO2/3 and regulated by the PML-RNF4 pathway⁹. NLS-LucDM-GFP also forms SS and SR species, although the relative amounts of SS and SR species are minimal compared to NS, using the conditions described in protocol 3 below. To simplify the assay, we only analyze SDS soluble LucDM (including both the NS and SS fractions) by SDS-PAGE and western blot. Importantly, CHX chase assay showed that the half-life of SDS-soluble NLS-LucDM-GFP is much shorter than that of its wild-type counterpart (**Figure 3B**), suggesting that LucDM is a specific substrate for the system that recognizes and degrades misfolded proteins.

The degradation of LucDM-GFP causes a significant drop in overall fluorescence signal. Therefore, we have also developed a protocol for real-time detection of cellular LucDM-GFP using microplate fluorescence-based assay. Many high-throughput screen (HTS) systems are developed for drugs or genes modifying cellular aggregates and cellular viability caused by aberrant proteins¹⁸⁻²⁰. However, very few HTSs are specifically designed for targeting degradation in mammalian cells. This protocol serves as a robust system for rapid and large-scale analysis of the effects of protein expression, knockdown, and drug treatment on cellular misfolded protein degradation. Using HeLa cells as example, below we describe the protocols for the analysis of these two misfolded proteins. The assays can also be applied to other cell lines, although transfection conditions and time course may need to be optimized for individual cell lines.

Protocol

1. Preparation of Reagent

1. Prepare cell lysis buffer (50 mM Tris, pH 8.8, 100 mM NaCl, 5 mM MgCl₂, 0.5% NP-40). Supplement 2 mM DTT, 1x complete protease cocktail, and 250 IU/ml benzonase before use.
2. Prepare pellet buffer (20 mM Tris, pH 8.0, 15 mM MgCl₂). Supplement 2 mM DTT, 1x complete protease cocktail and 250 IU/ml benzonase before use.
3. Prepare 3x boiling buffer (6% SDS, 20 mM Tris, pH 8.0). Supplement 150 mM DTT before use.
4. Prepare low-fluorescence DMEM medium for assays using microplate fluorescence reader. Mix 25 mM glucose, 0.4 mM glycine, 0.4 mM arginine, 0.2 mM cysteine, 4.0 mM glutamine, 0.2 mM histidine, 0.8 mM isoleucine, 0.8 mM leucine, 0.8 mM lysine, 0.2 mM methionine, 0.4 mM phenylalanine, 0.4 mM serine, 0.8 mM threonine, 0.078 mM tryptophan, 0.4 mM tyrosine, 0.8 mM valine, 1.8 mM CaCl₂, 0.81 mM MgSO₄, 5.33 mM KCl, 44.0 mM NaHCO₃, 110 mM NaCl, 0.9 mM NaH₂PO₄. Adjust pH of solution using HCl or NaOH to pH 7.4. Sterilize the medium through filtration.
NOTE: This medium contains components of the standard DMEM medium with high glucose except that Fe(NO₃)₃, vitamins and Phenol Red are omitted. Phenol Red, riboflavin and pyridoxal in regular DMEM culture medium significantly interfere with the detection of fluorescence signal²¹. Culture medium without those components is crucial for successful live cell GFP imaging. The medium is stable for 12 months when stored refrigerated.

2. Degradation Assay of Atxn1 82Q GFP

1. Plate approximately 3×10^5 HeLa cells into 35 mm plates with DMEM medium supplemented with 10% fetal bovine serum (FBS), so that after O/N culturing cells grow to a confluence of 40-60% at the time of transfection.
NOTE: The number of plates needed is based on the number of time points and treatments described below.
2. Transfect HeLa cells with 0.3 μ g of Atxn1 82Q-GFP/pRK5 plasmid into each well using transfection reagent according to manufacturer's instruction⁹. Make a master transfection mix containing DNA and transfection reagent and aliquot it for each well.
3. 4-5 hr post transfection, examine the live cells under an inverted fluorescent microscope for GFP expression with excitation wavelength 450-490 nm. Return cells back to incubator after imaging.
NOTE: Observe both diffused GFP signals and small speckles of GFP signals in nucleus at this time.
4. Remove the medium by vacuum aspiration and add 2 ml fresh DMEM containing 50 μ g/ml CHX. Treat cells for 0, 4, 8, 12 and 16 hr with CHX before harvesting. To examine proteasomal degradation, include proteasome inhibitor MG132 (10 μ M) in one plate treated for 16 hr as a control.
5. Harvest one plate of cells at each time point. Remove the medium by vacuum aspiration and wash the plates twice with 3 ml ice-cold 1x phosphate buffered saline (PBS). Snap-freeze the plate on dry ice.
6. After the last time point (16 hr), scrape the frozen cells (from all time points) into 150 μ l of ice-cold cell lysis buffer and incubated on ice for 30 min.
7. Centrifuge the cell lysates in a benchtop centrifuge at 17,000 x g for 15 min at 4 °C.
8. Transfer the supernatant, which contains NP-40-soluble (NS) proteins, to another tube using a pipette.
NOTE: Use supernatants for measuring protein concentrations by Bradford assay if needed.
9. Rinse the pellets by gently adding approximately 200 μ l 1x PBS to the tubes without disturbing the pellets. Carefully remove PBS by vacuum aspiration or pipette. Re-suspend them in 150 μ l ice-cold cell pellet buffer, followed by 15-30 min incubation on ice.
NOTE: The resuspended pellet fraction contains NP-40 insoluble (NI) proteins. See **Figure 1B** for a diagram of detergent fractionation.
10. Add 75 μ l of 3x boiling buffer into NS fractions and NP-40 insoluble (NI) fractions resuspended from the pellets. Heat the samples at 95 °C on a heat block for 5 min.
NOTE: Clumps in the NP-40 insoluble fractions are dissolved after heating and samples will become clear.
11. Add SDS-gel loading buffer to an aliquot of boiled NS and NI. Load equal volume of samples collected from all time points to SDS-PAGE gel. The volume loaded corresponds to approximately 20 μ g of NS from sample collected at Time 0. NOTE: NS, as well as SDS-soluble (SS) protein from NI fraction, can be resolved by SDS separating gel. In contrast, SDS-resistant (SR) aggregates from NI fraction are stuck in the gel loading wells (**Figure 1B**). To improve western blot detection, double volume of NI can be loaded.
12. Detect NS and SS Atxn1 82Q-GFP by western blot using anti-GFP antibody and enhanced chemiluminescence (ECL).
NOTE: Atxn1 82Q in the SS fraction is generally less compared to the NS fraction when using this protocol. Longer ECL exposure is needed for optimal signals.
13. Examine SR Atxn1 82Q from the pellet fraction by filter retardation assays using a dot-blot apparatus (**Figure 1B**). Briefly, set up a dot-blot apparatus holding a 0.2 μ m cellulose acetate membrane. Load 80-120 μ l of boiled NI into each well of the dot-blot apparatus.
NOTE: As only small amounts of SR aggregates are formed in cells, for the dot blot assay, load a volume of the SR fraction that is approximately 10-15 times of the volume of the NS fraction used for western blot analysis.
 1. After filtering the samples through the membrane by vacuum, Atxn1 82Q-GFP aggregates stuck on the membrane can be detected by anti-GFP immunoblotting^{9,12,22}.
NOTE: See previous reports^{9,12,22} for detailed description of filter retardation assay. This step is optional because SR Atxn1 82Q is minimal compared to SS and NS fractions following the short transfection described in this protocol. In addition, the levels of SR Atxn1 82Q largely remain the same over CHX chase (**Figure 2B**). However, it is still crucial to examine whether the amounts of SS Atxn1 82Q are affected over time for any drug treatment or gene expression in initial experiments. SS protein species stay in SDS-PAGE gel loading wells can be detected by western blot. However, this method is less sensitive and generates more variable results (**Figure 1B**).

3. Degradation Assay of NLS-luciferase-GFP Using SDS-PAGE and Western Blot

1. Plate approximately 3×10^5 HeLa cells into 35 mm plates with DMEM medium supplemented with 10% fetal bovine serum (FBS). After O/N culturing, a confluence of 40-60% is reached at the time of transfection. The number of plates needed is based on the number of time points and treatments described below.
2. Transfect HeLa cells with 1.0 μ g NLS-luciferase-GFP/pRK5 plasmid into each well using transfection reagent according to manufacturer's instruction⁹. Make a master transfection mix containing DNA and transfection reagent for aliquot of each well.
3. After O/N transfection (around 15 hr), examine the live cells under an inverted fluorescent microscope for GFP expression with excitation wavelength 450-490 nm. Return cells back to incubator after imaging.
NOTE: Diffused nuclear GFP signal can be observed in the majority of the cells (70%). A small percentage (5%) of cells have nuclear aggregates.
4. Remove the medium by vacuum aspiration and add 2 ml fresh DMEM containing 50 μ g/ml CHX. Treat cells for 0, 1.5, 3, 4.5 and 6 hr with CHX before harvesting. To examine proteasomal degradation, include additional proteasome inhibitor MG132 (10 μ M) in one plate treated for 6 hr as control.
5. Harvest one plate of cells at each time point. Remove the medium by vacuum aspiration and wash the plates twice with 3 ml ice-cold phosphate buffered saline (PBS). Snap-freeze the plate on dry ice.
6. After the last time point (6 hr) is finished, cells frozen at all-time points are scraped into 150 μ l of ice-cold cell lysis buffer and incubated on ice for 30 min.
7. Add SDS gel-loading buffer to whole cell lysates for a final concentration of 2% SDS and 50 mM DTT. Incubate samples on a heat block at 95 °C for 5 min.
8. Analyze NLS-luciferase-GFP by SDS-PAGE and western blot using anti-GFP antibody. Load equal volume of samples collected from all time points to SDS-PAGE gel. The volume loaded corresponds to approximately 20 μ g of whole cell lysates from sample collected at Time 0.

4. Real-time Degradation Assay of NLS-luciferase-GFP Using Fluorescence Microplate Reader

1. Seed approximately 1×10^4 HeLa cells into black 96-well tissue culture plates with transparent bottom. After O/N culturing, a confluence of 50-70% is reached at the time of transfection.
NOTE: 60 μ l of medium containing completely suspended HeLa cells are seeded directly into each well. Do not add additional medium or rock plate back and forth after seeding, otherwise cells may be distributed unevenly.
2. Transfect HeLa cells with 0.05-0.1 μ g of NLS-luciferase-GFP/pRK5 plasmid⁹ into each well using transfection reagent according to manufacturer's instruction. Make a master transfection mix containing DNA and transfection reagent and aliquot it for each well. Three wells with cells are not transfected with DNA, which serves as control for background fluorescence signal for each treatment condition.
NOTE: An alternative way to reduce transfection variation is to transfect cells before seeding. However, a large portion of cells transfected with misfolded proteins fail to attach or show reduced viability using this method. It is likely that some cells may not stand the stress caused by trypsin digestion when expressing toxic misfolded proteins. As a result, the overall fluorescent signal is significantly reduced.
3. 20-24 hr after transfection, examine the live cells under an inverted fluorescent microscope for GFP expression with excitation wavelength 450-490 nm.
4. Remove the medium by vacuum aspiration. Add approximately 200 μ l 1x PBS to each well and then aspirate it to remove residual amount of DMEM medium.
5. Add 60 μ l of low-fluorescence DMEM medium with 5% FBS and 50 μ g/ml CHX. To examine proteasomal degradation, include additional proteasome inhibitor MG132 (10 μ M) in one set of samples. Set triplicate of wells for each treatment/condition.
NOTE: MG132 treatment is included as controls for proteasomal degradation because it is crucial to rule out other factors that can cause drop of fluorescence signal, including cell death and fluorescence quenching.
6. Measure the fluorescence signal of GFP immediately on a fluorescent plate reader after adding CHX.
NOTE: The software measurement settings are shown in **Table 1**.
7. Read the plate every hour for up to 8-10 hr. After reading, return the plate back to cell culture incubator.
8. Export the data as spreadsheet file. Use mean value of multi-reads for each well as fluorescence intensity. Normalize values of each data point to the mean values of Time 0 in the respective groups. Plot the normalized fluorescence intensity over time as shown in **Figure 4A** and **4B**, and **Figure 5**, right panels.
9. Perform statistical analysis of degradation rates between two conditions using two-way ANOVA with repeated measures²³.
NOTE: In this analysis, "fluorescence intensity" is the dependent variable whereas "treatment conditions" and "time" are the two factors. Instead of comparing data at individual time points, two-way ANOVA with repeated measures analyzes the difference between the two treatment groups over the entire time course.

Representative Results

In a steady state analysis, microscopically visible Atxn1 82Q-GFP nuclear aggregates can be observed in 30 - 50% of HeLa cells 20 hr after transfection (**Figure 1A**). Western blot analysis of NS and SS fractions using anti-GFP antibody shows a distinct band of Atxn1 82Q-GFP between 100 kDa and 150 kDa markers, corresponding to the protein's molecular weight (**Figure 1B**). Atxn1 82Q-GFP in the SR fraction can be detected either by filter retardation assay, or by SDS-PAGE and western blot as species at the top of the stacking gel (**Figure 1B**). For half-life analysis using protocol described above, CHX chase starts 4-5 hr after initial transfection before any large nuclear aggregates are formed. The half-life of Atxn1 82Q-GFP in the SS fraction is around 5 hr, in contrast to a slight or no decrease of Atxn1 82Q-GFP in the NS fraction over 16 hr (**Figure 2A**). The degradation of Atxn1 82Q-GFP is partially inhibited by treating cells with MG132 (**Figure 2A**). We previously showed that PML stimulates the degradation of misfolded protein by promoting their SUMOylation. Here we use PML knockdown as an example of altered degradation pathway. PML siRNA treatment prolonged the half-life of Atxn1 82Q-GFP in the SS fraction (**Figure 2A**). No obvious changes in SR Atxn1 82Q-GFP are detected in either control or PML siRNA-treated cells (**Figure 2B** and **2C**).

Twenty hr after transfection, NLS-LucDM-GFP aggregates become microscopically visible in 5 - 15% of HeLa cells, but this can vary depending on the amounts of DNA that are transfected (**Figure 3A**). The half-life of SDS soluble NLS-LucDM-GFP is 2 - 3 hr (**Figure 3B** and **3C**). The fluorescence intensity of transfected cells also decreases over time upon CHX treatment (**Figure 4A** and **4B**). Six hr after CHX treatment, the fluorescence intensity reaches a stable stage and stops decreasing (**Figure 4A** and **4B**). At the same time point, soluble NLS-LucDM-GFP is largely degraded (**Figure 3B** and **3C**), suggesting that the remaining fluorescence is generated from aggregated species resistant to degradation. Consistently, aggregated, but not diffused, GFP remains in cells 9 hr after CHX treatment (**Figure 4C**). Interestingly, transfection using a higher amount of NLS-LucDM-GFP plasmid generates more nuclear aggregates as well as higher intensity of remaining fluorescence, although the degradation rate is not affected (**Figure 4A** and **4B**). Using either SDS-PAGE followed by western blot or fluorescence reading, we are able to detect the inhibition of NLS-LucDM-GFP degradation by MG132 treatment or PML siRNA (**Figures 3C**, **4A**, **4B**, and **5**).

Figure 1. Detection of Atxn1 82Q-GFP by fluorescent microscopy and detergent fractionation. (A) HeLa cells were transfected with Atxn1 82Q-GFP and stained with DAPI. Individual and merged images are shown. Scale bar = 10 μ m. (B) A diagram showing the detergent fractionation of Atxn1-82Q expressing cells as described in protocol 2. Molecular weight markers are indicated on the left of western blot images of Atxn1-Atxn 82Q. [Please click here to view a larger version of this figure.](#)

A

B

C

Figure 2. Degradation assay of Atxn1 82Q-GFP using detergent fractionation. HeLa cells were previously treated with control (-) or PML siRNA (A and C) or un-treated (B). Cells were transfected with Atxn1 82Q-GFP and treated with CHX in the absence or presence of MG132. Cell lysate fractions were analyzed by SDS-PAGE followed by western blot (A, for NS and SS fractions) or filter retardation assay (B and C, for SR fraction) using anti-GFP antibody. [Please click here to view a larger version of this figure.](#)

A

B

C

Figure 3. Analysis of NLS-LucDM-GFP degradation by fluorescence microscopy and western blot. (A) HeLa cells transfected with NLS-LucDM-GFP were stained with DAPI. Individual and merged images are shown. Scale bar = 25 μ m. Arrowhead indicates a cell with nuclear aggregates. (B and C) HeLa cells were transfected with NLS-LucDM-GFP and the wild-type luciferase fusion protein NLS-LucWT-GFP (B), or transfected with control or PML siRNA, followed by transfection with NLS-LucDM-GFP (C). Cells were treated with CHX and MG132 as indicated. Whole cell lysates were analyzed by western blot using anti-GFP antibody. Image is modified from previous publication with permission⁹. [Please click here to view a larger version of this figure.](#)

Figure 4. Fluorescent microplate-based assay for NLS-LucDM-GFP degradation. HeLa cells seeded on 96 well plates were transfected with 0.05 µg (**A**) or 0.1 µg (**B** and **C**) NLS-LucDM-GFP and treated with CHX in the presence or absence of MG132. (**A** and **B**) Fluorescence intensities of wells were measured at the indicated time points (Mean values $\pm$ standard deviations, $n = 3$). Panels on the left show the original fluorescence reading, whereas the panels on the right show the values normalized to readings at Time 0 in the respective treatment groups. The difference between the groups treated with and without MG132 was analyzed by two-way ANOVA with repeated measures. P values between the two treatment groups are indicated. (**C**) Wells were examined before or after 9 hr CHX treatment, or after CHX and MG132 treatment, by fluorescence microscope. To better show both cells with bright aggregated NLS-LucDM-GFP and those with dim diffused protein, gamma value of 1.5 was applied to all the images. Scale bar = 100 µm. [Please click here to view a larger version of this figure.](#)

Figure 5. A reduced degradation rate of NLS-LucDM-GFP in PML knockdown cells. HeLa cells previously treated with control or PML siRNA were seeded into 96 well plates. Degradation of NLS-LucDM-GFP was analyzed as described in Figure 4 (two-way ANOVA; $p < 0.0001$). [Please click here to view a larger version of this figure.](#)

Figure 6. Fluorescence of Atxn1 82Q-GFP remains unchanged over CHX chase. HeLa cells were transfected with Atxn1 82Q-GFP. 24 hr post-transfection, degradation of Atxn1 82Q-GFP was analyzed as described in Figure 4. The figure shows the original fluorescence reading. [Please click here to view a larger version of this figure.](#)

Figure 7. Fluorescent microplate-based assay for degradation of cytoplasmic LucDM-GFP. HeLa cells seeded on 96 well plates were transfected with 0.1 μ g LucDM-GFP treated with CHX in the presence or absence of MG132. The results were analyzed as described in Figure 4 (two-way ANOVA; $p < 0.0001$). [Please click here to view a larger version of this figure.](#)

Plate definition file		GRE96fb
Measurement mode		Fluorescence Intensity
		Bottom
Wavelength	Excitation wavelength	485 nm
	Excitation bandwidth	9 nm
	Emission wavelength	535 nm
	Emission bandwidth	20 nm
Number of flashes		5
Gain		Manual 200
Integration time		20 μ s
Multiple reads per well	Type	Circle (filled)
	Size	5x5
	Border	400 μ m

Table 1.: Measurement settings on fluorescence microplate reader.

Discussion

Mechanisms that regulate the degradation of misfolded proteins are essential for maintaining the homeostasis of cellular proteins, and they likely represent valuable drug targets for treating neurodegenerative disorders and other protein-misfolding diseases. Here, assays that examine the degradation of misfolded proteins are described, using a pathogenic Atxn1 protein (Atxn1 82Q) and a nuclear localized luciferase mutant (NLS-LucDM) as examples.

To examine the degradation Atxn1 82Q, which has a half-life over 18 hr, we introduced a novel method by combining cell fractionation with classical CHX⁹. This method revealed a much quicker clearance of the misfolded species of Atxn1 82Q over time. In addition, the introduction of LucDM provides a model misfolded substrate to study general degradation mechanisms of misfolded proteins. To address the need for an HTS system for detecting misfolded protein degradation in mammalian cells, we developed a fast degradation assay for LucDM using fluorescence microplate reader. The system serves as a powerful tool for chemical and genetic screens.

The Atxn1 82Q protein is present in NS, SS, and SR fractions of cell lysates. The SR fractions become increasingly prevalent during prolonged culture after transfection, suggesting that it is in a form that is difficult to be reversed or degraded, e.g., the amyloid fibrils. Generation of toxic misfolded species or aggregates that cannot be readily degraded may block protein degradation pathways, impeding the analysis of endogenous cellular machinery controlling aberrant proteins^{24,25,26}. To avoid formation of a large amount of SR, we start CHX chase 4-5 hr after initial transfection. We have found that this is critical for the analysis of cellular conditions that may delay the degradation of the SS fraction. In addition to transfection time, it is also crucial not to overwhelm cells by expressing Atxn1 82Q protein at very high levels. If no or very slow degradation is observed for all fractions, less amount of Atxn1 82Q plasmid should be used for transfection. Ideally, the assay should be finished within 12 hr as apoptotic cells are noticed beyond this time point. In **Figure 2A**, the amount of SS Atxn1 82 in control cells is strikingly different from that in PML siRNA-treated cells as short as 8 hr. Caution needs to be taken if a difference in the treated group is only observed beyond 12 hr, as it may be caused by other factors that affect cell viability rather than protein degradation.

Compared to NS Atxn1 82Q, Atxn1 82Q in the SS fraction is eliminated rapidly, indicating that it represents a misfolded species that can be readily degraded by the proteasome (**Figure 2A**). In contrast, the levels of SR Atxn1 82Q remain unchanged over the same time course (**Figure 2B**), confirming that it is an un-degradable species. Still, analysis of the SR fraction should be important for analyzing mechanisms that directly removes cytoplasmic aggregates (e.g., autophagy) or affect the formation of these aggregates. Noticeably, MG132 appears to be less effective in preventing Atxn1 82Q decrease in SS fraction. This may be caused by elevation in the ROS levels upon proteasome inhibition²⁷, which could promote the aggregation of Atxn1 82Q.

A great advantage of LucDM-GFP assay is that it can be adapted to HTS analysis using fluorescence plate reader. We initially attempted to develop a HTS system for Atxn1 82Q. However, because only a small fraction of overall Atxn1 82Q protein is degraded over time, the fluorescence of cells expressing GFP-Atxn1 82Q remains largely unchanged over the course of CHX chase (**Figure 6**). Other misfolded proteins with overall long half-life would have similar problems with real-time fluorescent assay. In contrast, a marked decrease of overall NLS-lucDM-GFP protein is observed (**Figures 3 and 4**). This feature, together with its short half-life (2-3 hr), enables this model chaperone substrate to be readily detected by fluorescence spectroscopy. In previous studies, GFPu, GFP protein fused to a constructive degradation signal (CL-1), has been widely employed as a reporter for the functionality of the ubiquitin-proteasome system^{28,29}. It is also a prominent surrogate misfolded protein in cell and animal models³⁰. Compared to GFPu, one advantage of the degradation assay described here is that misfolded luciferase is an extensively used model chaperone substrate. By examining luciferase activity, one can easily assess the native, denatured, and refolded conformations of luciferase *in vitro* and *in vivo*. Thus, our system serves as a highly useful platform to connect protein binding and folding assays with cellular degradation system using the same substrate⁹.

Through real-time fluorescence measurement the following values can be obtained: The starting fluorescence intensity, stable remaining fluorescence intensity, and protein half-life. These values are correlated respectively with the amount of overall protein before chase, amount of proteins resistant to degradation, and the rate of protein turnover. Therefore, it is possible to use real-time fluorescence assay to make a comprehensive analysis of the levels and dynamics of different misfolded protein species. Occasionally, a relatively large variation of the starting fluorescence among replicates is observed (e.g., **Figure 5**, left panel). This is likely to be caused by variability in cell seeding or transfection. However, despite the difference in the starting fluorescence intensity, the signal of all replicates drops at a very similar rate. Normalization of signals from each time points to the intensity at time 0 can reduce the well-to-well variation and more accurately reflects the degradation rates (**Figure 4A, 4B and Figure 5**, left vs. right panels).

In addition to the nuclear form, we also analyzed cytoplasmic LucDM-GFP. Cytoplasmic LucDM-GFP has a similar degradation pattern except higher percentage of remaining fluorescence was observed when the same experimental procedure was applied as described in Protocol 4 (**Figure 7**). The degradation could be completely inhibited MG132, indicating this assay can be used to monitor cytoplasmic protein quality

control through proteasomal degradation. For future studies, it would be interesting to explore whether LucDM-GFP can be applied to other cellular compartments, e.g., mitochondria or endoplasmic reticulum, when fused to specific cellular compartment localization signals.

As noted in the protocol for fluorescent-based assay, it is crucial to use low-fluorescent medium to reduce fluorescent background and to use master mix of DNA and transfection reagent to reduce well-to-well transfection variation. The gain control can be adjusted on some fluorescent plate readers. It is important to set the gain to manual instead of optimized and use the same value throughout the entire assay (**Table 1**). The value of the gain can be adjusted to maximize the reading from the cellular GFP fluorescence as long as the reading does not go beyond the limit of detection.

At the current stage, we are still relying on transfection to express LucDM. To reduce variation and simplify the assay for HTS, a future goal is to establish a stable cell line with inducible expression of LucDM. However, as microplate and culture medium both have background fluorescence, it is crucial to have GFP signal significantly higher than background. Unfortunately, the stable lines we established recently all have low expression of LucDM. Thus, the development of a stable line with higher protein expression will significantly improve the screening efficiency.

The degradation assays described here can also be applied to other misfolded proteins. Due to the dynamic feature of misfolded proteins, these assays need to be coupled with other analysis to uncover the in-depth mechanisms of protein quality controls. These analyses include measuring the steady state levels of aggregates and their partitions in different cell fractions (**Figure 1**) and analyzing proteins folding or aggregation using purified recombinant proteins. These assays will help to distinguish direct or indirect effects of drug or gene expression on protein degradation or aggregation.

Disclosures

The authors declare that they have no competing financial interests.

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