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‘Trim’ming PolyQ proteins with engineered PML

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Abstract

Protein abnormalities are the major cause of neurodegenerative diseases such as SCA (Spinocerebellar Ataxia). Protein misfolding and impaired degradation leads to the build-up of protein aggregates inside the cell, which may further cause cellular degeneration. Reducing levels of either the soluble misfolded form of the protein or its precipitated aggregate, even marginally, could significantly improve cellular health. Despite numerous pre-existing strategies to target these protein aggregates, there is considerable room to improve their specificity and efficiency. In this study, we demonstrated the enhanced intracellular degradation of both monomers and aggregates of mutant Ataxin1 (Atxn1 82Q) by engineering an E3 ubiquitin ligase enzyme, PML (Promyelocytic Leukemia protein). Specifically, we showed enhanced degradation of both soluble and aggregated Atxn1 82Q in mammalian cells by targeting this protein using PML fused to Single Chain Variable Fragments (scFvs) specific for monomers and aggregates of the target protein. The ability to solubilize Atxn1 82Q aggregates was due to the PML-mediated enhanced SUMOylation of the target protein. This ability to reduce the intracellular levels of both misfolded forms of Atxn1 82Q may not only be useful for treating SCA, but also applicable for the treatment of other PolyQ disorders.

Keywords

Spinocerebellar Ataxia; Degradation; PML; Monomer; Aggregate

Introduction

Protein folding is complex and stochastic, making it an error prone process. The errors introduced by genetic mutations and post-translational damages are irreversible (Dobson, 2003; Goldberg, 2003). In order to maintain proper protein folding and prevent protein aggregation inside a cell, the protein quality control (PQC) system – consisting of several classes of molecular chaperones, co-chaperones, and the degradation machinery – either helps amend protein misfolding or degrades misfolded proteins (Hartl, Bracher, & Hayer-Hartl, 2011; Sin & Nollen, 2015). If left unmanaged, levels of misfolded proteins can build up and pose a serious threat to the health of the cell.

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A group of pathologies associated with elevated levels of abnormally folded proteins in affected cells are called proteinopathies (Sin & Nollen, 2015). In neurons, the misfolding and aggregation of proteins with varying expansions of glutamine (PolyQ) leads to a set of neurodegenerative diseases, collectively known as PolyQ disorders (Khare, Ding, Gwanmesia, & Dokholyan, 2005; Temussi, Masino, & Pastore, 2003). The disease manifestation is due to the expansion of CAG repeats (which encode a PolyQ stretch) and the severity of disease is directly proportional to the length of the expansion beyond a threshold length. Wild-type Atxn1 contains 6 to 44 PolyQ repeats in healthy humans, while the mutant Atxn1 contains an expanded PolyQ stretch containing up to 83 repeats of glutamine (Zoghbi & Orr, 2009). Such PolyQ expansions in Atxn1 lead to spinocerebellar ataxia type 1 (SCA1) (Martins Junior et al., 2018). Similarly, an expansion of CAG repeats in the exon1 of the HTT gene, leads to Huntingtin disease (Harding & Tong, 2018). There are several ways in which these misfolded PolyQ proteins can alter cellular function. They can do so in their monomeric form by interacting promiscuously and hampering normal cellular interactions, or in the form of aggregates, where the insoluble precipitate hampers cellular function leading to cellular degeneration (Gatchel & Zoghbi, 2005; Takeuchi & Nagai, 2017).

Degradation is the last cellular option to try to prevent toxicity arising from misfolded aggregates which cannot be rescued by the folding pathway. Autophagy and Ubiquitin-proteasomal pathways are the two possible pathways to degrade these protein aggregates. While these pathways are not yet completely understood, they are being investigated extensively in recent years (Dantuma & Bott, 2014).

TRIM (Tripartite Motif) proteins are intriguing members of the protein degradation machinery. They constitute a class of E3 ubiquitinase enzymes, usually containing a conserved RING (Really Interesting New Gene) domain, B box domain/s and a Coiled Coil (CC) domain. The RING domain mediates the conjugation of ubiquitin or small ubiquitin like modifiers (SUMO) to the target proteins (Patil & Li, 2019). The C-terminus of the TRIM proteins contains conserved motifs like PRYSPRY, and often determines their binding specificity (Ozato, Shin, Chang, & Morse, 2008). The target diversity of this superfamily of proteins ranges from viral capsid proteins, and bacterial antigens to pathogenic oligomeric/aggregated proteins (Guo et al., 2014; Ozato et al., 2008). TRIM proteins have been shown to regulate innate immunity and/or provide anti-viral activity. As an example, TRIM5 α functions as a pattern recognizing assembly that blocks HIV-1 by targeting the viral capsid after entry (Black & Aiken, 2010). Another fascinating TRIM member, TRIM21, functions as an intracellular Fc receptor and mediates proteasomal degradation of intracellular antibodies. TRIM21 has been shown to intercept assemblies of misfolded tau protein and facilitate their degradation (McEwan et al., 2017). Promyelocytic Leukemia protein (PML; TRIM19) is another well-studied member of the TRIM family, which has recently been shown to bind to PolyQ proteins through their CC domain and to SUMOylate the aggregates. RNF4 is a ubiquitin E3 ligase with four tandem SUMO-interacting motifs. PML-assisted SUMOylation promoted the RNF4 (TRIM31)-mediated ubiquitination of Atxn1 82Q, which in turn aided target degradation by the proteasomal pathway. Levels of the SDS resistant fraction of Atxn1 82Q were reduced upon co-transfection of cells with PML and RNF4, suggesting that PML functioned by binding to aggregates and facilitating their

solubilization and degradation. However, the levels of the soluble fraction of Atxn1 82Q remained unchanged in the presence of PML (Guo et al., 2014) and might still pose a major threat to cellular health as mentioned before.

We hypothesized that adding a protein-recognition domain to PML might enhance the specificity and efficiency of target protein solubilization and degradation. We note that there has been an extensive interest in systems that promote the targeted intracellular degradation of proteins for applications ranging from new therapeutics to chemical biology tools (Caussinus, Kanca, & Affolter, 2011; Lai & Crews, 2017; Portnoff, Stephens, Varner, & DeLisa, 2014; Sakamoto et al., 2001). For instance, PROteolysis TArgeting Chimeras (PROTACs), bifunctional molecules composed of a moiety that binds to a target protein linked to a moiety that recruits an E3 ubiquitin ligase, promote the proteosomal degradation of a target protein (Lai & Crews, 2017; Sakamoto et al., 2001). Engineered proteins that combine the enzymatic activity of an E3 ubiquitin ligase with a domain that binds to a target protein have also been shown to promote the degradation of target proteins (Caussinus et al., 2011; Portnoff et al., 2014). We reasoned that adding a protein recognition domain to a SUMOylating enzyme, PML, might similarly enhance the SUMOylation of a target protein.

In this study, we demonstrate the enhanced intracellular degradation of both soluble and aggregated Atxn1 82Q by fusing PML to PolyQ-specific scFvs. We characterized the mechanism of enhanced degradation of Atxn1 82Q and also showed that the results could be extended to another PolyQ protein – the Huntingtin protein (htt 46Q). This ability to reduce the intracellular levels of misfolded proteins may be broadly useful for the treatment of SCA and other PolyQ disorders.

Materials and Methods

DNA construction

All genes were codon optimized for mammalian expression. DNA encoding PML-IRES2-RNF4 was synthesized by Gene Universal Inc. (Newark, DE) and cloned between BglII and BstBI sites in the mcs2 of pvitro2-hygro-mcs vector (InvivoGen, San Diego, CA). PML sequence was derived from UniProt # P29590–5 and RNF4 sequence from UniProt # P78317. All PML-based constructs had a CFP (cyan fluorescent protein) marker cloned between the AgeI and XbaI sites in the mcs1 of the pvitro2-hygro-mcs vector. DNA encoding Atxn1 82Q-GFP was synthesized by Gene Universal Inc. (Newark, DE) and cloned between BglII and BstBI sites in the mcs2 of pvitro2-hygro-mcs vector (InvivoGen, San Diego, CA). In addition, another version of Atxn1 82Q-GFP was designed with DsRed cloned between the AgeI and XbaI sites in mcs1, serving as a secondary marker. Further, constructs for p53-GFP and htt 46Q-GFP in pvitro2-hygro-mcs vector with a DsRed marker were designed by replacing the Atxn1 82Q gene by mammalian optimized DNA encoding p53 (UniProt # Q761V2) and htt 46Q (Krobitsch & Lindquist, 2000)(derived from Addgene plasmid #1178). PML SRS2-IRES2-RNF4 was designed by deleting the substrate recognition site 2 (SRS2) region near the C-terminus of PML (aa 571–633) (Guo et al., 2014). Fusions of various scFvs with PML were made by replacing the region coding for SRS2 with a sequence encoding the scFv. scFvs used for this study include MW1 (De Genst, Messer, & Dobson, 2014; Legleiter et al., 2009), 6E (Kvam et al., 2009), and a control scFv

– FRZ – that binds to Frizzled-7 (Fzd7). MW1 (De Genst et al., 2014; Legleiter et al., 2009) and 6E (Kvam et al., 2009) bind to soluble and fibrillar forms of PolyQ proteins, respectively. The anti-Fzd7 scFv (FRZ) was derived from the monoclonal antibody OMP-18R5 (that binds to the extracellular domain of Frizzled-7) and used as a control (Gurney et al., 2012).

The vector pTRE3G-mCherry (631347, Clontech, Mountain View, CA) was modified to replace the DNA coding for mCherry and IRES2, with DNA codon-optimized for mammalian expression and encoding CFP and the 2A peptide (Szymczak et al., 2004), respectively. This modified vector was referred to as pTRE3G-CFP-2A, and was synthesized by Gene Universal Inc (Newark, DE). DNA encoding PML-IRES2-RNF4 was cloned in the modified pTRE3G-CFP-2A vector between the EcoRV and BamHI sites. In addition, variants of the PML-IRES2-RNF4 cloned in pTRE3G-CFP-2A vector were made with scFvs 6E and MW1 replacing the SRS2-coding region near the C-terminus of PML.

Cell culture and transfection

Human Embryonic Kidney (HEK 293T) cells were purchased from American Type Culture Collection (ATCC CRL-11268) and cultured in Eagle Medium (High-glucose DMEM; Gibco 11995065) containing 10% HI-FBS (Heat-Inactivated Fetal Bovine Serum; Gibco 10438018) (hereon referred to as D-10 media) in a humidified incubator at 37 °C with 5% CO₂. DNA transfections were carried out with Lipofectamine 2000 using the manufacturer's protocol.

Co-transfections were carried out with one aggregation-prone target DNA (Atxn1 82Q-GFP/htt 46Q-GFP/p53-GFP) and another targeting member (PML/PML-scFv fusions) in a 1:4 ratio. For all the western blot-based assays, transfected cells were treated with a 20 µM solution of the proteasomal inhibitor MG132 (Sigma-Aldrich; M7449) for 4–5 hours before cell lysis.

Ataxin-1 aggregation kinetics assay

HEK293T cells were cultured in a 24-well plate (Corning CLS3527) at a seeding density of 40,000 cells/well. After 24 hours, cells attained 80% confluence and were transiently transfected with Atxn1 82Q-GFP and a PML/PML-scFv plasmid (PML, PML SRS2, PML-6E, PML-MW1, or PML-FRZ). After 24 hours of incubation, cells were trypsinized and resuspended in Dulbecco's Phosphate-Buffered Saline (DPBS; Gibco 11995065) containing 0.5 mM EDTA (Millipore 20–158). The cell suspension was passed through the strainer cap of a FACS tube (Nunc 0877123) to remove agglomerated cells. Three thousand cells from each tube were sorted by a Fusion cell sorter (BD, Biosciences, CA) into a 96-well plate containing D10. After incubating for 2–3 hours at 37 °C, the number of visible aggregates per cell were counted using an epifluorescence microscope (Zoe, BioRad) at 10× magnification. Counting was repeated at fixed intervals until 48 hours post-seeding.

Flow cytometry

Cells were co-transfected with respective plasmids in a 24-well plate and trypsinized 24 or 48 hours after transfection. Trypsinized cells were resuspended in DPBS containing 0.5 mM

EDTA and 30,000 cells were analyzed using a FACS Fortessa (BD Biosciences). Doubly transfected cells containing both target and PML/PML-scFv were gated using CFP and DsRed channels. Median fluorescence intensity (MFI) was analyzed for each sample.

Cell lysate fractionation and western blotting

Cells were transiently transfected in 6-well plates with respective plasmids and after 24 hours of transfection, cells were lysed in NP-40-containing buffer (2 % NP-40, 50 mM Tris pH 8.8, 100 mM NaCl, 5 mM MgCl₂, 2 mM DTT, Benzonase 250 IU/ml, 1 mM PMSF, 20 mM N-ethylmaleimide (NEM)) and centrifuged at 13000 rpm for 10 mins. Lysate was then fractionated into NP-soluble supernatant and pellet. The pellet was later solubilized in 2% SDS containing buffer (20 mM Tris pH 8.0, 15 mM MgCl₂, 50 mM DTT, 250 IU/ml Benzonase, 1 mM PMSF, 20 mM NEM, 2% SDS) and incubated at 95 °C for half an hour. Samples from both fractions were further denatured in LDS buffer and resolved by western blot in denaturing conditions. Primary antibodies against the following proteins were used at the dilutions indicated. GFP (GSN, 1:500; Sigma HPA054026), GAPDH (1:3000; Abcam ab8245). The HRP conjugated anti-mouse (1:3000; Cell Signaling 7076P2) antibody was used for secondary probing.

Immunofluorescence of cultured cells

Cells were cultured in four chambered glass bottom dishes (Nunc D35C4-20-0-N). After 24 hours of transfection, cells were fixed with 4% paraformaldehyde for 30 minutes and permeabilized with 70% methanol for an hour at -20 °C. Cells were blocked with 1% BSA, and incubated with primary anti-PML antibody, 15 µg (Abgent P29590). Cy3-conjugated anti-mouse antibody (1:50) (Vector labs CY 2300) was used as the secondary antibody. For nuclear staining, cells were stained with DAPI (Sigma-Aldrich D9542) for 5 min at room temperature. Cells were mounted using antifade mounting medium from Invitrogen (P36961). Images were acquired with a 40× objective lens of Spinning Disc Confocal microscope. Volocity software was used to process images.

Results and Discussions

SCA1, a rapidly progressive PolyQ disorder, is caused by a mutation in the gene encoding Atxn1, resulting in a PolyQ expansion in Atxn1 beyond a threshold (Martins Junior et al., 2018; Zoghbi & Orr, 2009). This extended PolyQ tract affects Atxn1 folding and renders it prone to aggregation in the nucleus. Lack of an efficient degradation machinery leads to accumulation and anchoring of this misfolded mutant Atxn1 in the nucleus, affecting its function (Fan et al., 2014; Tsai et al., 2004).

One of the E3 ubiquitin ligase enzymes, PML (TRIM19), has been shown to solubilize Atxn1 82Q by SUMOylation (Guo et al., 2014). Once SUMOylated, the misfolded Atxn1 82Q was shown to be subsequently ubiquitinated by RNF4, thereby targeting the misfolded proteins for proteasomal degradation. Although, this system was efficient at clearing aggregates of Atxn1 82Q, the levels of soluble monomeric Atxn1 82Q (precursors to aggregates) were unaffected (Guo et al., 2014). We have attempted to upgrade PML

SUMOylation ability by improving its target efficiency by fusing it to scFvs specific for both aggregate and monomeric soluble forms of Atxn1 82Q.

We generated plasmids encoding PML and a version of PML with the substrate recognition site 2 (SRS2) near the C-terminus deleted (PML Δ SRS2). We fused PolyQ-specific scFvs – 6E (Kvam et al., 2009) and MW1 (Legleiter et al., 2009) – to PML Δ SRS2. As a control, we also designed a version of PML fused to a non-PolyQ-specific scFv – FRZ (that binds to the extracellular domain of Fzd7) – to evaluate the benefit of targeting PML to the PolyQ protein -Atxn1 82Q. The PML and PML variants were co-expressed with RNF4 spaced by an internal ribosome entry site 2 (IRES2). This strategy allowed for the expression of both proteins (PML-scFv and RNF4) under a single promoter. Additionally, CFP was expressed using the same vector as a marker for PML and RNF4 expression. Atxn1 82Q-GFP was cloned in a vector with a DsRed marker: GFP served as a marker to track cellular Atxn1 aggregates and DsRed served as a marker for Atxn1 82Q expression.

Targeting both soluble and insoluble fractions of Atxn1 82Q

HEK 293T cells were co-transfected with plasmids encoding Atxn1 82Q-GFP along with a plasmid encoding either Vector control (VC; pvitro2-hygro-mcs), PML, PML Δ SRS2, or a PML-scFv fusion protein (PML-MW1, PML-6E, or control PML-FRZ) in a 24-well plate. After incubating for 24 hours, the total fluorescent Atxn1 82Q-GFP protein level was quantified using FACS expressed as median fluorescent intensity (MFI). To ensure that the analyzed cell populations expressed both Atxn1 82Q-GFP and PML/PML-scFvs, cells were gated for both DsRed and CFP markers on the respective plasmids. The relative MFI (RMFI) was calculated by normalizing the MFI of PML/PML-scFv fusion protein-treated cells relative to that for cells co-transfected with the vector control. As seen from the RMFI results in Fig. 1A (after 24 hrs of incubation), the total Atxn1 82Q-GFP levels in cells transfected with PML Δ SRS2 were comparable to that in cells transfected with PML, suggesting no specific role of that region (SRS2) in PML-mediated Atxn1 82Q-GFP degradation. Notably, cells co-transfected with PML had 2-fold higher MFI than cells co-transfected with PML-MW1 (Figure 1A). Thus, increasing the specificity by fusing a PolyQ-binding scFv to PML Δ SRS2 resulted in enhanced protein degradation and lower Atxn1 82Q-GFP levels in PML-MW1 expressing cells. When incubated for 48 hours, the effect of PML-MW1 was enhanced even further, with cells co-transfected with PML having 2.6-fold higher MFI than cells co-transfected with PML-MW1 (Figure 1B). Atxn1 82Q-GFP levels in cells expressing a fusion of PML with another PolyQ-specific scFv (PML-6E) were also lower than those in PML-expressing cells at both 24 and 48 hours (Figures 1A, B). In contrast, enhanced degradation was not observed in control PML-FRZ-expressing cells (Figures 1A, B).

The flow-cytometric results indicating enhanced Atxn1 82Q-GFP degradation by PML-MW1 and PML-6E relative to that with PML alone were further confirmed using western blotting (Figures 1C, S1A). As above, cells were co-transfected with plasmids encoding Atxn1 82Q-GFP along with a plasmid encoding either Vector control, PML, PML Δ SRS2, PML-MW1, PML-6E, or PML-FRZ, and lysed 24 hours after transfection using SDS buffer

(2%) to ensure total protein solubility. As seen in Fig. 1C (left panel), Atxn1 82Q-GFP-total protein levels were the lowest for cells expressing PML-MW1 or PML-6E.

Next, we fractionated the total protein lysate into NP 40 soluble (NS) and NP 40 insoluble or SDS soluble (SS) fractions based on the lysate solubility in NP 40 or SDS buffer respectively (Guo et al., 2014). Levels of Atxn1 82Q-GFP in the SS fraction for PML-treated cells were lower than those for cells treated with the vector control (Fig. 1C), consistent with the results of Guo et al. Levels of Atxn1 82Q-GFP in the SS fraction for PML-MW1-treated and PML-6E-treated cells were comparable to those for PML-treated cells. Excitingly, levels of Atxn1 82Q-GFP in the NS fraction were significantly lower for PML-MW1-treated and PML-6E-treated cells compared to PML-treated cells (Figure 1C, S1B). Similar trends were seen in fractionated samples from cells lysed 48 hours after co-transfection (Figure S2). The results mentioned above, where total and NS levels of Atxn1 82Q-GFP were significantly lowered in the presence of PML-MW1, were very encouraging and prompted us to explore further the mechanism by which these PML fusion proteins influence Atxn1 82Q aggregation.

Mechanism of enhanced degradation with PML-MW1

In order to further characterize the enhanced solubilization and degradation of Atxn1 82Q-GFP caused by PML-MW1, cells were co-transfected with Atxn1 82Q-GFP and PML/PML-scFv fusion proteins and imaged using confocal microscopy. Nuclear staining using DAPI and staining with a PML-specific antibody confirmed that PML-scFv fusion proteins, like PML, were localized in the perinuclear space (Figure 2A, middle column, rows 2–5). The solubilized Atxn1 82Q-GFP in cells co-transfected with PML-MW1 was present in the form of diffuse protein (Figure 2A, 3rd column, 4th row) in contrast to the compact aggregates observed in cells co-transfected with the vector control (Figure 2A, 3rd column, 1st row) or with PML-FRZ (Figure 2A, 3rd column, 5th row).

In order to investigate whether the enhanced solubilization and degradation of Atxn1 82Q-GFP in PML-MW1-treated cells was a result of enhanced SUMOylation of the target protein, we performed western blot analysis (Figure 2B). Cell lysates from co-transfected cells (co-transfected with the same plasmid pairs as above) were probed using an anti-SUMO2/3 antibody and the intensity of the smear above the expected Atxn1 82Q-GFP band (at 122 kDa) indicated the level of SUMO-modified Atxn1 82Q-GFP (Guo et al., 2014). As seen in Figure 2B, the increased intensity of the smear for PML-MW1-treated cells confirmed the enhanced SUMOylation of the Atxn1 82Q-GFP target by PML-MW1 relative to that with PML alone (Figure 2B).

Atxn1 82Q Aggregation Kinetics regulated by PML fusion proteins

Having tested the influence of various PML-scFv fusion proteins on steady state levels of Atxn1 82Q-GFP, next we investigated their influence on the kinetics of protein aggregation. To that end, HEK 293T cells were co-transfected with plasmids encoding Atxn1 82Q-GFP and PML/PML-scFv fusion proteins. After an incubation period of 24 hours, a FACS cell sort was run based on the FITC channel and cells were sorted into the wells of 96-well plate. After incubating the cells for another 2 hours, the number of cells with visible protein

aggregates were counted using an epifluorescence microscope at a 10× magnification periodically over 48 hours (Figure 3). While cells co-transfected with the vector control, PML, and PML SRS2 accumulated Atxn1 82Q-GFP aggregates at a comparable rate, the rate of aggregation was significantly lower in cells expressing PML-MW1 or PML-6E, with the fraction of cells with aggregates being essentially constant over the 48 hour period for cells expressing PML-MW1 (Fig. 3). Moreover, the first time point in the figure (2 hours post seeding) represents the fraction of cells without aggregates 2 hours after co-transfection; the fraction of cells without aggregates is highest (closest to 1) for cells expressing PML-MW1.

Pre-formed Aggregates and PML

Next, we wanted to probe the mechanistic basis for the enhanced degradation seen using PML-MW1. The enhanced effect could be explained by two hypotheses. One possible explanation involves direct action of PML-scFvs on the aggregates, with the SUMOylation of aggregates facilitating the solubilization and further degradation by RNF4. We also explored a second possibility, inspired by the enhanced degradation of NP-soluble Atxn1 82Q-GFP upon treatment with PML-MW1 (Figure 1C, top right panel), wherein, PML-MW1 could SUMOylate and lead to the enhanced degradation of the soluble/monomeric form of Atxn1 82Q-GFP. A reduction in levels of monomer would be expected to inhibit aggregation.

To distinguish between these two hypotheses, we investigated the influence of PML and PML-scFv fusion proteins on Atxn1 82Q-GFP in the presence of pre-formed Atxn1 82Q-GFP aggregates. We reasoned that if PML-MW1 was primarily acting on aggregates, then the presence of preformed aggregates should not cause any change in the previously observed trends. In contrast, if the enhancement was based on enhanced degradation of soluble forms of Atxn1 82Q-GFP, then the difference between the extent of PML-mediated and PML-scFv-mediated degradation would now be diminished.

To enable the expression of PML and the PML-scFv fusion proteins to be turned on after the formation of Atxn1 82Q-GFP aggregates, PML, PML-MW1, and PML-6E were cloned in a Doxycycline-inducible pTRE3G plasmid. HEK 293T cells were co-transfected with Atxn1 82Q-GFP and either PML, PML-6E, or PML-MW1, and aggregates were allowed to form for 48 hours. PML/PML-scFv expression was then induced by treating cells with Doxycycline (1µg/ml) for the next 24 hours. The levels of Atxn1 82Q-GFP were then analyzed using flow-cytometry. When aggregates were first allowed to form for 48 hours before inducing PML/PML-scFv expression, there was no difference in the RMFI values for PML or PML-scFv treated Atxn1 82Q-GFP (Figure 4A). The comparable RMFI values for cells expressing PML and PML-MW1 in the presence of pre-formed aggregates (Figure 4A) suggested that the enhanced degradation seen with PML-MW1 (Figure 1A) was not due to its direct action on Atxn1 82 Q-GFP aggregates. We further characterized the aggregation kinetics of Atxn1 82Q-GFP following the Doxycycline-induction of PML, PML-6E, or PML-MW1 for 24 hours. The initial level of aggregates and their progression along the time course of 24 hours post-sorting was comparable for cells co-transfected with the Vector control or with one of the three PML variants. These results supported the second hypothesis

wherein the enhanced degradation seen with PML-MW1 was primarily due to its action on soluble Atxn1 82Q-GFP.

Other PML target

PML has a wide variety of targets and has previously been shown to SUMOylate other PolyQ proteins such as Huntingtin protein (Htt) (Guo et al., 2014). Since the MW1 and 6E scFvs can recognize other PolyQ proteins, we next investigated if PML-6E/MW1 (in combination with RNF4) could enhance the degradation of another PolyQ protein, relative to that seen with PML. PML-scFv fusion protein was able to degrade Htt 46Q-GFP (Figure 5A) but not the control proteins p53 (Figure 5B) or eGFP (Figure 5C), suggesting the specific targeting of PolyQ proteins.

The PQC system determines the health of a cell (Sin & Nollen, 2015). Being able to manage the levels of improperly folded or aggregated proteins via degradation is a check point for avoiding promiscuous cellular interactions or cellular degeneration (Takeuchi & Nagai, 2017). In this study, we have tried to boost the activity of a naturally occurring E3 ligase enzyme, PML, by fusing it to specific scFvs, which increased the target specificity. Previously, PML has been shown to solubilize Atxn1 82Q aggregates by SUMOylation, which were subsequently degraded by RNF4. Here, the target protein was both monomeric as well as the aggregated form of Atxn1 82Q-GFP. We demonstrated a decrease in the levels of both monomeric and aggregated forms of the protein by fusing PML to target-specific scFvs. We observed that reducing levels of the soluble form of an aggregation-prone protein could have a significant impact on the rate of aggregation. It would be interesting to explore therapeutic applications of this strategy. One possible approach to implementation would be based on gene therapy, in which a transgene encoding a PML-scFv fusion protein could be delivered to a target cell. Another possible approach would be conceptually similar to the strategy used in designing PROTACs, in which a small molecule with specificity for the target protein could be fused to a moiety that recruits PML. A small but targeted enhancement in the rate of degradation of aggregation-prone proteins could help significantly in improving cellular health and this strategy has potential use for treating PolyQ disorders as well as other protein misfolding diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Characterization of the influence of PML-scFv fusion proteins on levels of Atxn1 82Q-GFP.

A) Characterization of levels of Atxn1 82Q-GFP in HEK 293T cells using flow cytometry 24 hours after co-transfection with plasmids encoding Atxn1 82Q-GFP and vector control/ PML/PML-scFv fusion proteins. GFP intensity was measured as median fluorescence intensity. RMFI= MFI (SAMPLE)/MFI (VECTOR CONTROL). Values represent mean $\pm$ sd, n=3. B) Characterization of levels of Atxn1 82Q-GFP by flow cytometry, 48 hrs after co-transfection with plasmids encoding Atxn1 82Q-GFP and vector control/ PML/PML-scFv fusion proteins. Values represent mean $\pm$ sd, n=3. C) Characterization of levels of Atxn1 82Q-GFP in HEK 293T cells by Western Blotting. HEK 293T cells were co-transfected with Atxn1 82Q-GFP and 1-VC, 2-PML, 3- PML Δ SRS2, 4-PML-6E, 5-PML-MW1, 6-PML-FRZ. For total cell lysate, cells were processed with 2% SDS buffer and probed by western blotting with an anti-GFP antibody (1:500). For fractionation, cells were processed in NP 40 buffer and the soluble fraction in this buffer was labelled as NS. Pellet was dissolved in SDS buffer, resulting in the SS fraction. VC: Vector Control.

Figure 2. Solubilization and SUMOylation by PML/PML-scFv fusion proteins.

A) HEK 293T cells were co-transfected with Atxn1 82Q-GFP and VC/PML/PML-scFvs (PML-MW1, PML-6E, PML-FRZ) in four-chambered glass bottom cover-glass. Cells were processed for DAPI staining and incubated with PML-specific mouse antibody, followed by further incubation with a Cy3-conjugated-Anti-mouse secondary antibody. A Spinning-Disc Confocal microscope was used to acquire images at 40x. Left column shows the DAPI staining overlaid with Bright field image in order to show nuclear staining. The images in the middle column represent an overlay of DAPI and Red channel, where Cy3-stained PML/PML-scFvs are seen as red specks localized around the nucleus (marked using yellow arrows in the middle column). The images in the right column represent an overlay of DAPI, Red, and Green channels, where along with PML/PML-scFv expression, Atxn1 82Q-GFP is

seen either as intense green dots (Red arrow) for cells co-transfected with VC/ PML-FRZ (First and 5th row), as partially diffuse green dots for cells expressing PML (2nd row) and almost completely solubilized Atxn1 82Q-GFP for cells expressing PML-MW1 (4th row). Images were processed using Volocity software. B) HEK 293T cells were co-transfected with Atxn1 82Q-GFP and VC/PML/PML-scFvs and after an incubation of 24 hours, total cell lysate was probed using anti-SUMO 2/3 antibodies in western blotting. The experiment was performed with three biological repeats for each construct, as grouped by the marked lines. The samples were run on two separate gels as shown by a line between the samples but both blots were imaged together. Sumo 2/3 conjugates appear as a smear above anticipated Atxn1 82Q-GFP band at 122 KDa. VC: Vector Control.

Figure 3. Aggregation kinetics of Atxn1 82Q-GFP.

HEK 293T cells were co-transfected with Atxn1 82Q-GFP and VC/ PML/ PML Δ SRS2/ PML-scFv fusion proteins. After 24 hours of incubation, cells were sorted and cells with Atxn1 82Q-GFP aggregates were counted using an epifluorescence microscope. The graph shows the fraction of HEK 293T cells without visible Atxn1 82Q-GFP aggregates as a function of time (hours). Vector control (green circle); PML (Red square); PML Δ SRS2 (brown triangle); PML-6E (inverted purple triangle); PML-MW1 (orange diamond); and PML-FRZ (black circle). The values represent mean $\pm$ sd, n=3. VC: Vector Control.

Figure 4: Characterization of the influence of PML-scFv fusion proteins in the presence of pre-formed Atxn1 82Q-GFP aggregates.

A) HEK 293T cells were co-transfected with Atxn1 82Q-GFP and PML/PML-6E/MW1 encoded in Doxycycline inducible pTRE3G plasmid. PML/PML-scFv expression was induced by Doxycycline treatment, 48 hours after co-transfection in half of the wells for each construct. The remaining uninduced wells formed the control groups, denoted as UI. Total Atxn1 82Q-GFP present after 24 hours of Doxycycline treatment was measured as MFI using flow cytometry. B) Aggregation kinetics were studied for cells expressing pre-formed aggregates of Atxn1 82Q-GFP co-transfected with VC, PML, or PML-6E/MW1. As explained for (A), after an incubation of 48 hours, cells were induced with Doxycycline. Twenty four hours after Doxycycline induction, cells were sorted and the number of cells

with visible protein aggregates were counted for the next 24 hours. The fraction of cells without aggregates was plotted against time post-seeding. The first data point is at 3 hours post seeding. Both plots represent mean $\pm$ sd, n=3

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Figure 5. Characterizing specificity of PML-scFvs.

HEK293T cells were co-transfected with VC/ PML/PML-MW1/PML-6E/FRZ and (A) Htt 46Q-GFP, (B) p53-GFP, and (C) eGFP. Twenty-four hours after co-transfection, cells were trypsinized and processed for flow-cytometric analysis. Target protein level was measure as MFI. All three plots represent mean $\pm$ sd, n=3 VC: Vector Control.