The JAMM motif of human deubiquitinase Poh1 is essential for cell viability

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Abstract

Poh1 deubiquitinase activity is required for proteolytic processing of polyubiquitinated substrates by the 26S proteasome, linking deubiquitination to complete substrate degradation. Poh1 RNA interference (RNAi) in HeLa cells resulted in a reduction in cell viability and an increase in polyubiquitinated protein levels, supporting the link between Poh1 and the ubiquitin proteasome pathway. To more specifically test for any requirement of the zinc metalloproteinase motif of Poh1 to support cell viability and proteasome function, we developed a RNAi complementation strategy. Effects on cell viability and proteasome activity were assessed in cells with RNAi of endogenous Poh1 and induced expression of wild-type Poh1 or a mutant form of Poh1, in which two conserved histidines of the proposed catalytic site were replaced with alanines. We show that an intact zinc metalloproteinase motif is essential for cell viability and 26S proteasome function. As a required enzymatic component of the proteasome, Poh1 is an intriguing therapeutic drug target for cancer. [Mol Cancer Ther 2007;6(1):262–8]

Introduction

Directed proteolysis by the ubiquitin proteasome pathway controls a variety of cellular processes, including cell cycle, cell survival, signal transduction, and protein quality control (1). A cascade of E1, E2, and E3 enzymes covalently conjugates ubiquitin molecules to select proteins, targeting them for destruction by the 26S proteasome, a multicatalytic chambered protease (2, 3). The 26S proteasome is composed of a barrel-shaped proteolytic 20S core particle with one or both ends capped by 19S regulatory particles. 19S regulatory particles contain at least 17 distinct subunits, divided into base and lid subcomplexes (3). The base complex alone is competent to gate the channel of the 20S core particle for degradation of peptides. The base and lid complexes are jointly required for degradation of proteins conjugated with ubiquitin chains, functioning in substrate recognition, substrate unfolding, removal of ubiquitin, and substrate translocation into the 20S chamber (4).

Ubiquitination is a reversible modification due to the actions of deubiquitinating enzymes that catalyze the removal of ubiquitin conjugates (1, 5–8). It is the balance of ubiquitinating and deubiquitinating activities that maintains proper levels of proteins controlled by ubiquitin-dependent proteolysis. Deubiquitination of substrates before association with the proteasome can rescue proteins from irreversible degradation, whereas deubiquitination of proteasome-associated substrates can either promote or prevent degradation, depending on the timing and extent of deubiquitination in relation to substrate release or proteolysis (9–12). Multiple deubiquitinases are associated with eukaryotic proteasomes, including Poh1/Rpn11 (5, 10, 11), Usp14/Ubp6 (13, 14), and Uch37 (9, 15, 16).

Poh1/Rpn11 is a subunit of the 19S regulatory particle with the capacity to remove ubiquitin from proteasome substrates via hydrolysis of the proximal ubiquitin isopeptide bond to a substrate (4, 10–12, 17). Of the proteasome-associated deubiquitinases in yeast, only Rpn11 is essential (18, 19). Uch37/Uch2 nulls are viable in *Schizosaccharomyces pombe* (15), and Ubp6 nulls are viable in *Saccharomyces cerevisiae* (12). Nonlethal mutants of yeast Rpn11 cause multiple cellular defects, including severe growth impairment, increased pools of ubiquitinated proteins, and overreplication of DNA (18, 19). Poh1/Rpn11 contains a JAMM/MPN$^+$ motif sequence, $Ex_nHxHx_7Sx_2D$, resembling the active site of zinc metalloproteinases (19–21). The crystal structure of an archaebacterium JAMM-containing protein has been determined by two groups, confirming the prediction that a zinc ion is coordinated by the two histidines and aspartic acid of the JAMM motif (20–22). To evaluate the role of the Rpn11 JAMM motif in metal ion–dependent deubiquitination, various laboratories have mutated one or both conserved histidines to alanines and reintroduced the mutant protein into proteasomes for functional evaluation. Such studies have shown that mutations in JAMM motif histidines are lethal in yeast and fly, leading to elevated levels of ubiquitin conjugates (10, 11, 19, 23, 24). Additionally, purified yeast proteasomes containing Rpn11 point mutants are incompetent for deubiquitination and degradation of polyubiquitinated substrates (10).

Based on work in nonmammalian systems, we hypothesized that Poh1 would be an essential deubiquitinase affecting ubiquitin-dependent proteolysis in mammalian cells. Here, we tested the effect of small interfering RNAs

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(siRNA) targeting each mammalian proteasome–associated deubiquitinase on cell viability and further tested the effects of disrupting the Poh1 JAMM motif on cell viability, proteasome function, and assembly.

Materials and Methods

Antibodies and Reagents

The anti–Poh1 rabbit polyclonal antibody was purchased from Zymed Laboratories, Inc. (Invitrogen, Carlsbad, CA) The anti–V5 mouse monoclonal antibody was purchased from Invitrogen. The anti–ubiquitin mouse monoclonal antibody and the anti–actin goat polyclonal antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti–subunit α6 mouse monoclonal antibody was purchased from Biomol International (Plymouth Meeting, PA).

siRNAs were obtained from Dharmacon, Inc. (Boulder, CO) as annealed and purified duplexes. The custom Poh1 untranslated region (UTR) siRNA pool was composed of the following four siRNA sequences: (*a*) CAAGTTAAATCTAGCTCAA, (*b*) GCAAGACAAGGGTCCATAT, (*c*) TAAGACATCTGGCATCATT, and (*d*) CATCAAAGTGGACAAATTT. Other siRNA pools were Uch37 (M-006060-00-50), Usp14 (M-006065-00-50), and the nontargeting control GL2 (D-001100-01-20).

Cell Culture

HeLa Tet-On cells (C3000-1, Becton Dickinson, Franklin Lakes, NJ) were maintained in 90% DMEM (Invitrogen), 10% tetracycline-free fetal bovine serum (Becton Dickinson), 1% L-glutamine (Invitrogen), and 100 μg/mL geneticin (Invitrogen). All cells were grown at 37°C in a humidified atmosphere with 5% $C0_2$.

Reverse Transcription and Quantitative PCR

Total RNA was isolated using RNeasy technology and DNase treatment according to the manufacturer's protocol (Qiagen, Valencia, CA). First-strand cDNA synthesis was done with a reverse transcription system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol, except that both oligo(dT) and random hexamers were used for priming. Primers and MGB Eclipse probes (Supplementary Table S1) for real-time PCR were designed and validated by Nanogen (San Diego, CA). mRNA transcripts were assayed in a multiplexed format, using human β2-microglobulin RNA as an internal control. Transcripts were amplified using Taq polymerase (Sigma, St. Louis, MO) and cycling parameters of an initial 95°C for 2 min and then 40 cycles of 95°C for 20 s, 58°C for 20 s, and 76°C for 20 s. Data were analyzed using SDS 1.7 (Applied Biosystems) software, using the comparative C_t method and normalization to β2-microglobulin transcripts.

Expression Vectors and Cell Lines

A full-length human Poh1 cDNA clone was isolated from an adrenal gland cDNA library generated at Millennium Pharmaceuticals (Cambridge, MA) and used as template for the generation of the H113A/H115A double mutant by PCR. The sequences of the mutagenic primers were as follows: 5′-**GTAT*GCA*AGT*GCA*CCTGGCTTTGGTTG**-3′ (forward) and 5′-**CCAGG*TGC*ACT*TGC*ATACCAACCAAC**-3′ (reverse; mutations are in italics). Both the wild-type (WT) and the double mutant open reading frames were PCR amplified with gene-specific primers and subcloned into the *Kpn*I and *Xba*I sites of the mammalian expression vector pTracer-EF/V5-His (Invitrogen TM, Life Technologies, Inc., Carlsbad, CA) to incorporate the V5-His tag of the vector at the 3′ end of the gene. The sequences of the PCR primers used for subcloning were as follows: 5′-gg*GGTACCAGATCT*ACC**ATGGACAGACTTCTTAGACTTGGAGGAG**-3′ (forward) and 5′-gc*TCTAGA***TTTAAATACGACAGTATCCAACAATAGCTGCTAAAC**-3′ (reverse). Gene-specific sequences are in bold font, and restriction enzyme sequences are in italics. The PCR product was ligated into pTracer-EF/V5-His as described previously (25). To generate inducible retroviral expression vectors, the WT and double mutant cDNAs with 3′-V5-His tags were excised from pTracer-EF/V5-His with *Bgl*II and *Pme*I and ligated to *Bam*HI-*Hpa*I digested pRevTRE (Clontech/BD Biosciences, Mountain View, CA). DH5α cells were used for all transformations.

HeLa Tet-On cells were transfected with the vectors above and maintained in 250 μg/mL hygromycin B (Invitrogen) to select for cells with doxycycline-inducible expression of WT Poh1 (V5-$Poh1^{WT}$) or mutant Poh1 (V5-$Poh1^{AXA}$). Clones of the HeLa cell pools were isolated for Western and protease assay work, based on improved regulation of the Tet-responsive V5-Poh1 protein and better knockdown of endogenous Poh1 (data not shown). The cell clones performed comparably to the cell pools in viability RNA interference (RNAi) complementation assays (Supplementary Fig. S1), albeit with partial rescue by V5-$Poh1^{WT}$.

Viability Assay with RNAi or RNAi Complementation

Cells were seeded 24 h before transfection into 96-well or six-well plates at a density of 1×10^3 or 2.5×10^4 to 4.5×10^4 per well, respectively. Transfections were done using 10 nmol/L siRNA duplexes and the LipofectAMINE 2000 reagent kit (Invitrogen). Cell viability was measured at 48 to 120 h posttransfection using the WST-1 assay (Roche Diagnostics, Palo Alto, CA) and an absorbance plate reader (Molecular Devices, Sunnyvale, CA) at 450 to 490 nm. Background absorbance was subtracted from all values before normalizing data. In Figure 1, error is represented as the SD of triplicate data. In Figure 3, error is represented as the SE for 12 replicates from six independent experiments. Heteroscedascity was assessed using an *f* test, and statistical significance was assessed using a two-tailed *t* test.

Western Blot

For denaturing polyacrylamide gels, cell pellets were lysed in buffer containing 10 mmol/L Tris (pH 7.5), 1% Triton X-100, and protease inhibitors (complete mini tablets, Roche). The protein concentrations of cleared lysates were determined using a Bradford assay, and equal amounts of total protein (25 μg) were resolved in 4% to 20% Tris-glycine SDS-PAGE gels (Invitrogen). Proteins were transferred to nitrocellulose membranes (Invitrogen), using Tris-glycine transfer buffer (Invitrogen) and an XCell Blot Module for 1.5 h at 30 V.

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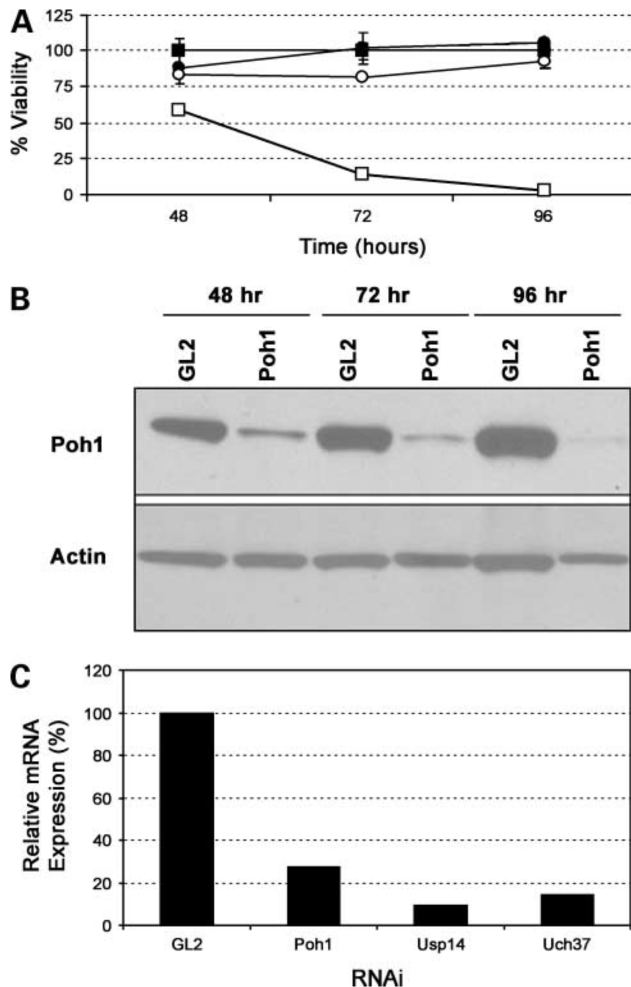

Figure 1. Poh1 RNAi reduces cell viability and Poh1 protein. HeLa cells were transfected with siRNA pools targeting deubiquitinases Poh1 (□), Usp14 (●), or Uch37 (○). The control siRNA was GL2 (■). **A,** cell viability was assessed at the indicated times posttransfection. **B,** expression of Poh1 protein was assessed in HeLa cells transfected with Poh1 or GL2 siRNAs. **C,** expression levels of Poh1, Usp14, and Uch37 mRNA were assessed at 48 h posttransfection.

Native gels were prepared and run as described previously (26). For native gels, cell pellets were lysed in a hypotonic buffer of 5 mmol/L EDTA (pH 8.0) for 30 min followed by two rapid freeze-thaws, using dry ice. Lysates were stored at −80°C overnight, thawed, and cleared by centrifugation. The protein concentrations of cleared lysates were determined using a Bradford assay, and equal amounts of total protein (25 μg) were resolved in 3.5% nondenaturing polyacrylamide gels, using 5× native sample buffer [250 mmol/L Tris-HCL (pH 7.4), 50% glycerol, 0.007% xylene cyanol]. Human 26S proteasomes (Immatics, Tuebingen, Germany) and human 20S proteasomes (Boston Biochem, Cambridge, MA) were run as size controls. Gels were soaked for 15 min in transfer buffer containing 0.1% SDS, before transfer onto polyvinylidene fluoride membranes (Immobilon transfer membrane, Millipore, MA) pretreated by soaking in methanol for 30 s, rinsing with distilled water, and soaking in transfer buffer for 1 min. Following transfer, membranes were soaked in 5% acetic acid for 15 min, rinsed with distilled water, and dried for 10 min at room temperature.

Membranes were incubated with primary antibodies at the following dilutions: anti-Poh1 at 1:500 and anti-actin, anti-V5, anti-ubiquitin, and anti-subunit α6 at 1:1,000. Detection was via enhanced chemiluminescence (Amersham, GE Healthcare, Piscataway, NJ).

Proteasome Activity Assays

26S and 20S proteasome peptidase activities were evaluated using an *in vitro* peptide hydrolysis assay. Proteasome activity was quantitatively assessed in cell extracts using the β5-selective fluorogenic substrate succinyl-leucine-leucine-valine-tyrosine-4-methyl-7-courmarylamide (Suc-LLVY-AMC) as described previously (27). Parallel reactions were done in either the absence or presence of recombinant proteasomal activator, PA28α (Boston Biochem), to determine the activity of the 26S and 20S proteasome, respectively. Briefly, extracts were diluted to 200 μg/mL in 5 mmol/L EDTA (pH 8.0) and dispensed into a 96-well black opaque plate to give 10 μg protein per reaction. Reactions were initiated by addition of 150 μL of 20 mmol/L HEPES (pH 7.4), containing 0.5 mmol/L EDTA, and 133 μmol/L Suc-LLVY-AMC, with or without 16 nmol/L PA28α. Peptidase activity was measured at 37°C by monitoring AMC production with a Polarstar Galaxy fluorometer (BMG Labtechnologies, Offenburg, Germany) and quantified with reference to an AMC calibration curve. Suc-LLVY-AMC was obtained from AnaSpec, Inc. (San Jose, CA).

Results

Poh1 Is Required For Cell Viability

RNAi was used to determine the essentiality of three proteasome-associated deubiquitinases, Poh1, Usp14, and Uch37, to HeLa cell viability. Knockdown of Poh1 severely reduced HeLa cell viability to 3% of the control siRNA–treated cells at 96 h (Fig. 1A). Knockdown of Usp14 or Uch37 had no negative effect on HeLa cell viability. Similar results were also observed in the HCT-116 colon cancer cell line (Supplementary Table S2). The amount of Poh1 protein was effectively reduced at 48 h posttransfection and became undetectable by 96 h (Fig. 1B). In lieu of having good antibody reagents for evaluating protein knockdown of Usp14 and Uch37, RNA levels were quantitated as a measure of siRNA efficacy. At 48 h posttransfection, Poh1, Usp14, and Uch37 mRNAs were reduced 4-, 11-, and 7-fold, respectively (Fig. 1C).

To determine if RNAi of these deubiquitinases affected the ubiquitin proteasome pathway, we evaluated levels of total ubiquitinated proteins. Western blotting for ubiquitin in lysates from siRNA-transfected cells showed that Poh1 knockdown resulted in a dramatic increase in ubiquitinated protein levels at 48 h posttransfection (Fig. 2). Poh1 protein was not reduced with RNAi of Usp14 or Uch37, and no

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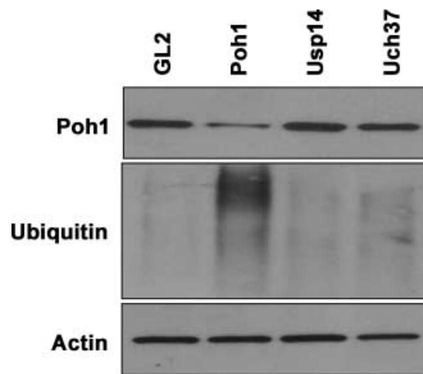

Figure 2. Knockdown of Poh1 protein increases cellular levels of ubiquitinated proteins. HeLa cells were transfected with siRNA pools targeting deubiquitinases Poh1, Usp14, or Uch37. The control siRNA was GL2. The expression levels of Poh1 protein and total ubiquitinated proteins were assessed at 48 h posttransfection.

increases in ubiquitinated protein levels were observed. The increase in total ubiquitinated proteins was maintained out to 72 h with Poh1 RNAi (Supplementary Fig. S2). The reduction in cell viability and increase in total ubiquitinated protein levels support our conclusion that the ubiquitin proteasome pathway has been disrupted by knockdown of Poh1 protein.

The JAMM Motif of Poh1 Is Required for Cell Viability

To test the importance of the Poh1 JAMM motif for cell viability, we engineered a RNAi complementation approach. HeLa cells were generated expressing inducible V5-tagged versions of Poh1, which were WT (V5-Poh1^{WT}) or mutant (V5-Poh1^{AXA}). The mutant Poh1 protein had alanines in place of two conserved histidines of the putative active site zinc-coordinating center. HeLa cells expressing inducible V5-Poh1^{AXA} were compared with HeLa cells expressing inducible V5-Poh1^{WT} in viability assays, combining knockdown of endogenous Poh1 with induction of exogenous V5-Poh1 (Fig. 3). siRNAs targeting endogenous Poh1 were specific to UTRs of the Poh1 mRNA that were not included in the induced V5-Poh1 transcripts.

The V5-Poh1^{WT} HeLa cell pool was used first to determine if we could achieve rescue by sufficiently coordinating the timing and levels of endogenous Poh1 protein knockdown, exogenous V5-Poh1 protein induction, and incorporation of exogenous V5-Poh1 protein into 26S proteasomes (Fig. 3, *columns 1–4*). In the uninduced V5-Poh1^{WT} cells, Poh1 UTR siRNA reduced viability to 9% at 96 h posttransfection (Fig. 3, *column 2* versus *column 1*). Induction of V5-Poh1^{WT} in cells with RNAi of endogenous Poh1 significantly rescued cell viability to 77% the level of control siRNA–transfected cells (Fig. 3, *column 4* versus *column 3*), with an 8-fold increase in cell viability versus noninduced cells (Fig. 3, *column 4* versus *column 2*; $P = 4.1 \times 10^{-5}$, Welch's two-tailed t test). The ability of induced V5-Poh1^{WT} to rescue the reduced viability observed with Poh1 RNAi proved that the Poh1 RNAi effect was specific to targeting of the Poh1 protein and not the result of off-target siRNA activity. This result validated the RNAi complementation system as a means by which to evaluate the Poh1 JAMM motif residues proposed to be required for catalytic function.

The effect of a mutant Poh1 JAMM motif was tested in a RNAi complementation experiment using the V5-Poh1^{AXA} HeLa cell pool (Fig. 3, *columns 5–8*). In the uninduced V5-Poh1^{AXA} cells, Poh1 UTR siRNA reduced viability to 7% at 96 h posttransfection (Fig. 3, *column 6* versus *column 5*). Induction of V5-Poh1^{AXA} in cells with RNAi of endogenous Poh1 did not rescue cell viability (Fig. 3, *column 8* versus *column 7*), with only 12% of cells remaining viable. The viability of V5-Poh1^{AXA} cells with Poh1 RNAi in the presence of induction was not significantly different from that in the absence of induction ($P = 0.09$, two-tailed t test). The inability of induced V5-Poh1^{AXA} to rescue the reduced viability observed with Poh1 RNAi suggests that the JAMM motif of Poh1 is required for cell viability. No dominant-negative effect was observed with expression of V5-Poh1^{AXA} in the absence of Poh1 RNAi (Fig. 3, *column 7*).

Endogenous Poh1 and tagged Poh1 protein levels were assessed in a RNAi complementation experiment by Western blotting at 96 h (Fig. 4). Endogenous Poh1 was reduced with Poh1 RNAi in both the V5-Poh1^{WT} and the V5-Poh1^{AXA} cells. The V5 tag of the induced Poh1 proteins provided a means to distinguish induced V5-Poh1 from endogenous Poh1. Western blotting with a V5 antibody confirmed an increase of both the V5-Poh1^{WT} and the V5-Poh1^{AXA} proteins on induction. Therefore, the inability of induced V5-Poh1^{AXA} to rescue the reduced viability observed with Poh1 RNAi could not be attributed to a lack of protein expression. The induced V5-Poh1 proteins were also detected with the anti-Poh1 antibody, although reactivity was weak. The weak reactivity may be attributed to levels below the detection limit of the anti-Poh1 antibody and/or weaker reactivity of the COOH terminal specific anti-Poh1 antibody to COOH-terminally tagged V5-Poh1

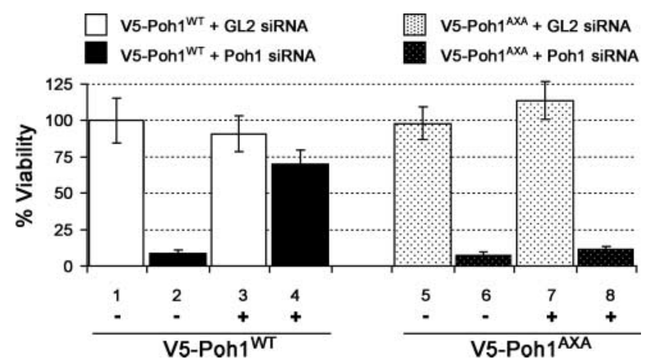

Figure 3. WT Poh1, but not a JAMM motif mutant, can rescue the loss of cell viability caused by endogenous Poh1 RNAi. HeLa cells with inducible expression of WT Poh1 (V5-Poh1^{WT}; *nonstippled columns*) or mutant Poh1 (V5-Poh1^{AXA}; *stippled columns*) were transfected with a control GL2 siRNA (*white columns*) or a siRNA targeting endogenous Poh1 (*black columns*) in the absence (−) or presence (+) of induction. Cell viability was assessed at 96 h posttransfection, and viabilities were normalized to the uninduced V5-Poh1^{WT} cells transfected with control GL2 siRNA.

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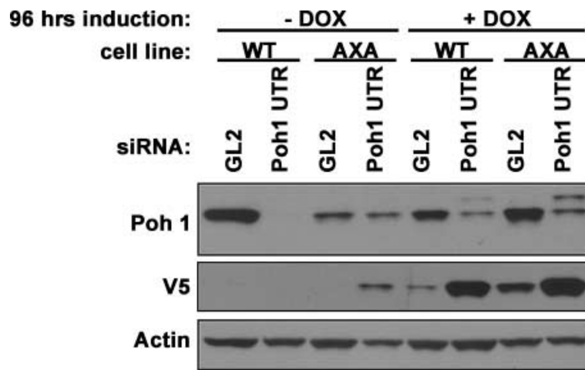

Figure 4. Poh1 protein knockdown and induction in a RNAi complementation experiment. HeLa cell clones with inducible expression of WT Poh1 (*WT*) or mutant Poh1 (*AXA*) were transfected with a control siRNA (*GL2*) or a siRNA targeting endogenous Poh1 (*Poh1 UTR*) in the absence (*−DOX*) or presence (*+DOX*) of induced V5-Poh1^{WT} or V5-Poh1^{AXA} proteins. Expression levels of endogenous Poh1 and induced Poh1 were assessed by immunoblotting of cell lysates with anti-Poh1 and anti-V5 antibodies, respectively.

versus endogenous Poh1. The observed increase in V5-Poh1 protein levels in induced Poh1 RNAi cells versus induced control siRNA–transfected cells may be a result of stabilization by incorporation into proteasomes. Western data at 120 h parallels the presented data (Supplementary Fig. S3).

Exogenous Poh1 Is Incorporated into Assembled 26S Proteasomes

The ability of exogenous V5-Poh1^{WT} to restore viability to cells with RNAi of endogenous Poh1 suggests that the tagged Poh1 protein is competent for incorporation into functional proteasomes. The Poh1 orthologue, Rpn11, is structurally required for yeast proteasome stability and function (10). To interpret the lack of complementation with mutant Poh1 protein, it was important to determine if the V5-Poh1^{AXA} protein was incorporating into proteasomes. Incorporation of the tagged Poh1 proteins into proteasomes was determined by testing for V5 expression in large macromolecular complexes on native polyacrylamide gels. Native gels were run with lysates from cells used in the complementation experiments, and purified human 26S and 20S proteasomes were run as size standards. Western blotting for the 20S core subunit α6 revealed both 26S and 20S complexes in cell lysates (Fig. 5). When the same membrane was blotted for V5-Poh1 proteins, predominant bands corresponding to the 26S complex were detected in lysates from both induced V5-Poh1^{WT} cells and induced V5-Poh1^{AXA} cells. Incorporation of the V5-Poh1 proteins was also observed at 120 h (Supplementary Fig. S4). Our ability to detect the V5-Poh1^{AXA} mutant in a complex that comigrated with native 26S proteasomes suggests that the lack of viability rescue in these cells was not due to a lack of incorporation into assembled 26S proteasomes. Interestingly, some increase in both V5-Poh1^{WT} and V5-Poh1^{AXA} proteins is observed in uninduced cells with Poh1 RNAi. We hypothesize that when endogenous Poh1 levels are reduced, low levels of the V5-Poh1 proteins may be stabilized by incorporation into 26S proteasomes.

26S Proteasome Peptidase Activity Is Restored by Incorporation of Exogenous WT Poh1 but not Mutant Poh1

To assess the proteolytic capacity of 26S proteasomes from cells used in the complementation experiments, an *in vitro* LLVY-AMC hydrolysis assay was run on total cell lysates (Fig. 6). Assays were run in the absence and presence of a 20S activator, PA28, to determine the contribution of proteolytic activity from 26S proteasomes alone versus the combined activity of 26S and 20S proteasomes. 26S activities are reported as percentages of total proteasome activities normalized to the control siRNA–transfected cells for each uninduced cell line. In the absence of induction, Poh1 RNAi reduced 26S activity by 30% to 36% in both V5-Poh1^{WT} cells and V5-Poh1^{AXA} cells at 96 h. In control siRNA–transfected cells, there was no difference in proteasome activity on induction, showing that doxycycline alone did not affect proteasome activity. With Poh1 RNAi, proteasome activity differed upon induction of V5-Poh1^{WT} versus V5-Poh1^{AXA} protein. Induction of V5-Poh1^{WT}, combined with Poh1 RNAi, restored 26S proteasome activity to control levels, whereas induction of V5-Poh1^{AXA}, combined with Poh1 RNAi, did not. Similar results were observed at 120 h (Supplementary Fig. S5). This result parallels the viability effects we observed, supporting our conclusion that the Poh1 JAMM motif is necessary to support full 26S proteasome activity and, consequently, cell viability.

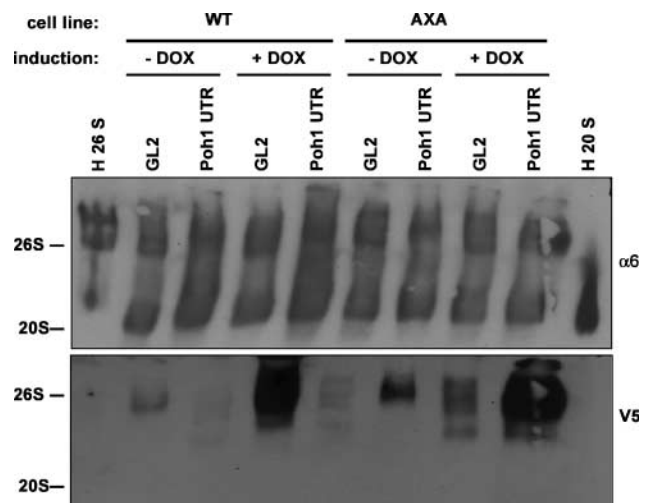

Figure 5. WT Poh1 and JAMM motif mutant Poh1 are incorporated into assembled 26S proteasomes. HeLa cell clones with inducible expression of WT Poh1 (*WT*) or mutant Poh1 (*AXA*) were transfected with a control siRNA (*GL2*) or a siRNA targeting endogenous Poh1 (*Poh1 UTR*) in the absence (*−DOX*) or presence (*+DOX*) of induced V5-Poh1^{WT} or V5-Poh1^{AXA} proteins. At 96 h posttransfection, cells were lysed hypotonically and whole-cell lysates were run on nondenaturing polyacrylamide gels to detect assembled 26S proteasomes and induced V5-Poh1^{WT} and V5-Poh1^{AXA} proteins by immunoblotting with anti-α6 and anti-V5 antibodies, respectively.

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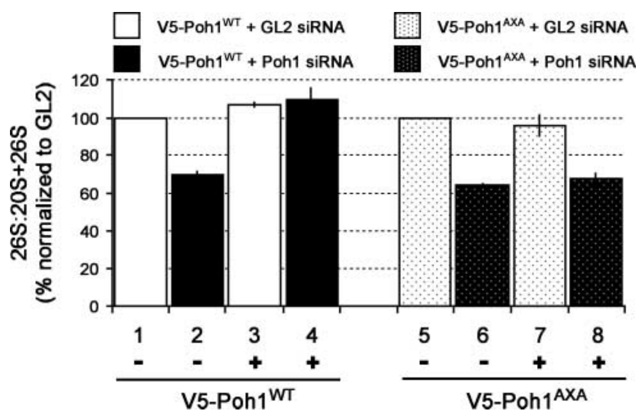

Figure 6. The proteolytic capacity of 26S proteasomes in whole-cell lysates differs between cells expressing WT Poh1 or JAMM motif mutant Poh1. HeLa cell clones with inducible expression of WT Poh1 (*WT*) or mutant Poh1 (*AXA*) were transfected with a control siRNA (*GL2*) or a siRNA targeting endogenous Poh1 (*Poh1 UTR*) in the absence (*−DOX*) or presence (*+DOX*) of induced V5-Poh1^{WT} or V5-Poh1^{AXA} proteins. Cells were lysed hypotonically and whole-cell lysates were used for *in vitro* peptidase assays at 96 h posttransfection. Peptidase activity of 26S proteasomes is presented as values normalized to total 20S + 26S proteasome activities for each uninduced cell line transfected with control siRNA.

Discussion

Deubiquitinase activities intrinsic to, or associated with, mammalian proteasomes include those of Poh1, Usp14, and Uch37. We have shown a requirement for the deubiquitinase Poh1 in maintaining mammalian cell viability. Poh1 RNAi resulted in a dramatic reduction in cell viability, whereas RNAi of Usp14 or Uch37 had no detrimental effect on cell viability. Similarly, the *S. cerevisiae* orthologue of Poh1, Rpn11, is essential for viability, whereas the orthologue of Usp14, Ubp6, is nonessential for viability (12). An orthologue of human Uch37 has not been defined in *S. cerevisiae*; however, the orthologue in *S. pombe*, Uch2, is nonessential for viability (15). The proposed functions for Uch37 and Usp14 are polyubiquitin chain shortening of proteasome-associated substrates and removal of full chains, respectively (5). The roles of Uch37 and Usp14 must be dispensable, compensated for, or sufficiently functional with any residual protein in our RNAi experiments. Better antibodies to Uch37 and Usp14 will be required to evaluate their knockdown in our experiments. Of the three deubiquitinases, only Poh1 knockdown globally affected the ubiquitin proteasome pathway, as evidenced by increased ubiquitinated protein levels.

As a component of the multienzymatic 26S proteasome complex, the reduced cell viability observed with Poh1 RNAi may be attributed to multiple factors, including a requirement for the enzymatic deubiquitinase activity of Poh1 and/or a structural requirement for Poh1 in assembled, functional 26S proteasomes. To address these possibilities, we used a RNAi complementation approach whereby endogenous Poh1 protein could be replaced with either WT Poh1 or a mutant form of Poh1 with alanines replacing two conserved histidines of the proposed catalytic motif. V5-Poh1^{WT} protein, but not V5-Poh1^{AXA} protein, rescued cell viability and 26S proteasome peptidase activity in whole-cell lysates, with both the V5-Poh1^{WT} and the V5-Poh1^{AXA} proteins being properly induced and incorporated into assembled proteasomes. The inability of V5-Poh1^{AXA} to restore 26S proteasome activity is consistent with the inability to restore viability, showing that the Poh1 JAMM motif is essential for 26 proteasome activity and, subsequently, cell viability.

Previous studies have provided evidence that Rpn11-mediated deubiquitination may be rate limiting for proteasomal degradation of ubiquitinated substrates (11). In whole-cell lysates, the inhibition of peptide hydrolysis by Poh1 RNAi was rescued by expression of WT, but not mutant, Poh1. Intriguingly, in preliminary fluorogenic peptide overlay assays of native gels, where proteasomes are partially purified, both WT and mutant Poh1 seemed to restore the decreased peptide hydrolysis caused by Poh1 RNAi (data not shown). Although peptide hydrolysis, as opposed to degradation of polyubiquitinated proteins, does not require the proteasome lid (4), cellular accumulation of polyubiquitinated substrates may prohibit access of peptide substrates to the catalytic chamber of 26S proteasomes containing mutant Poh1. Ongoing studies with affinity-purified mammalian 26S proteasomes will allow us to evaluate deubiquitinating and degradative activities of V5-Poh1^{AXA} containing proteasomes. Last, although we have focused on the proteasomal role of Poh1, additional roles for Poh1/Rpn11 have been reported in multidrug resistance (28, 29) and in maintaining mitochondrial morphology and function (18, 30, 31). It is unclear if all of these functions are connected to the deubiquitinating properties of Poh1/Rpn11.

In summary, our data provide support that human Poh1 is a required structural component of 26S proteasomes and that an intact JAMM motif is essential for cell viability through proper processing of ubiquitinated substrates. Preclinical and clinical activity of the proteasome inhibitor Velcade in multiple myeloma and other malignancies has validated the proteasome as a target in the treatment of cancer (32–36). Because deubiquitination is required for entry of proteasome substrates into the 20S core particle, by removing bulky, sterically hindering, polyubiquitin chains, a Poh1 inhibitor would be expected to block complete entry of proteins into the core particle, thereby blocking proteolysis. Targeting proteasome subunits other than the $\beta5$ protease of the 20S core particle may provide opportunities to modulate drug properties and/or proteasome function in ways that mimic or differ from those of Velcade. Exciting opportunities certainly exist for drug discovery exploring the regulation of protein homeostasis by targeting novel enzymatic activities either associated with the 26S proteasome or lying upstream in the ubiquitin proteasome pathway (37, 38).

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References

1. Glickman MH, Ciechanover A. The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev 2002;82: 373 – 428.

2. Ciechanover A. Proteolysis: from the lysosome to ubiquitin and the proteasome. Nat Rev Mol Cell Biol 2005;6:79 – 87.

3. Pickart CM, Cohen RE. Proteasomes and their kin: proteases in the machine age. Nat Rev Mol Cell Biol 2004;5:177 – 87.

4. Glickman MH, Rubin DM, Coux O, et al. A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3. Cell 1998;94: 615 – 23.

5. Guterman A, Glickman MH. Deubiquitinating enzymes are IN/(trinsic to proteasome function). Curr Protein Pept Sci 2004;5:201 – 11.

6. Glickman MH, Adir N. The proteasome and the delicate balance between destruction and rescue. PLoS Biol 2004;2:E13.

7. D'Andrea A, Pellman D. Deubiquitinating enzymes: a new class of biological regulators. Crit Rev Biochem Mol Biol 1998;33:337 – 52.

8. Wilkinson KD. Regulation of ubiquitin-dependent processes by deubiquitinating enzymes. FASEB J 1997;11:1245 – 56.

9. Lam YA, Xu W, DeMartino GN, Cohen RE. Editing of ubiquitin conjugates by an isopeptidase in the 26S proteasome. Nature 1997; 385:737 – 40.

10. Verma R, Aravind L, Oania R, et al. Role of Rpn11 metalloprotease in deubiquitination and degradation by the 26S proteasome. Science 2002; 298:611 – 15.

11. Yao T, Cohen RE. A cryptic protease couples deubiquitination and degradation by the proteasome. Nature 2002;419:403 – 7.

12. Guterman A, Glickman MH. Complementary roles for Rpn11 and Ubp6 in deubiquitination and proteolysis by the proteasome. J Biol Chem 2004;279:1729 – 38.

13. Borodovsky A, Kessler BM, Casagrande R, Overkleeft HS, Wilkinson KD, Ploegh HL. A novel active site-directed probe specific for deubiquitylating enzymes reveals proteasome association of USP14. EMBO J 2001;20:5187 – 96.

14. Leggett DS, Hanna J, Borodovsky A, et al. Multiple associated proteins regulate proteasome structure and function. Mol Cell 2002;10: 495 – 507.

15. Li T, Naqvi NI, Yang H, Teo TS. Identification of a 26S proteasome-associated UCH in fission yeast. Biochem Biophys Res Commun 2000; 272:270 – 5.

16. Holzl H, Kapelari B, Kellermann J, et al. The regulatory complex of *Drosophila* melanogaster 26S proteasomes. Subunit composition and localization of a deubiquitylating enzyme. J Cell Biol 2000;150: 119 – 30.

17. Wilkinson KD. Cell biology: unchaining the condemned. Nature 2002; 419:351 – 3.

18. Rinaldi T, Ricci C, Porro D, Bolotin-Fukuhara M, Frontali L. A mutation in a novel yeast proteasomal gene, RPN11/MPR1, produces a cell cycle arrest, overreplication of nuclear and mitochondrial DNA, and an altered mitochondrial morphology. Mol Biol Cell 1998;9:2917 – 31.

19. Maytal-Kivity V, Reis N, Hofmann K, Glickman MH. MPN$^+$, a putative catalytic motif found in a subset of MPN domain proteins from eukaryotes and prokaryotes, is critical for Rpn11 function. BMC Biochem 2002;3:28.

20. Tran HJ, Allen MD, Lowe J, Bycroft M. Structure of the Jab1/MPN domain and its implications for proteasome function. Biochemistry 2003; 42:11460 – 5.

21. Ambroggio XI, Rees DC, Deshaies RJ. JAMM: a metalloprotease-like zinc site in the proteasome and signalosome. PLoS Biol 2004;2:E2.

22. Ambroggio XI, Rees DC, Deshaies RJ. Structure and implications of JAMM, a novel metalloprotease. PLoS Biol 2004;2:E17.

23. Hochstrasser M. Molecular biology. New proteases in a ubiquitin stew. Science 2002;298:549 – 52.

24. Lundgren J, Masson P, Realini CA, Young P. Use of RNA interference and complementation to study the function of the *Drosophila* and human 26S proteasome subunit S13. Mol Cell Biol 2003;23:5320 – 30.

25. Pulido JC, Duyk GM. Construction of small-insert libraries enriched for short-tandem-repeat sequences by marker selection. In: Current protocols in human genetics, Vol. Unit 2.2. New York: Greene Publishing Associates and John Wiley and Sons, Inc.; 1994.

26. Elsasser S, Schmidt M, Finley D. Characterization of the proteasome using native gel electrophoresis. Methods Enzymol 2005;398:353 – 63.

27. Lightcap ES, McCormack TA, Pien CS, Chau V, Adams J, Elliott PJ. Proteasome inhibition measurements: clinical application. Clin Chem 2000;46:673 – 83.

28. Spataro V, Toda T, Craig R, et al. Resistance to diverse drugs and ultraviolet light conferred by overexpression of a novel human 26 S proteasome subunit. J Biol Chem 1997;272:30470 – 5.

29. Spataro V, Simmen K, Realini CA. The essential 26S proteasome subunit Rpn11 confers multidrug resistance to mammalian cells. Anticancer Res 2002;22:3905 – 9.

30. Rinaldi T, Ricordy R, Bolotin-Fukuhara M, Frontali L. Mitochondrial effects of the pleiotropic proteasomal mutation mpr1/rpn11: uncoupling from cell cycle defects in extragenic revertants. Gene 2002;286:43 – 51.

31. Rinaldi T, Pick E, Gambadoro A, et al. Participation of the proteasomal lid subunit Rpn11 in mitochondrial morphology and function is mapped to a distinct C-terminal domain. Biochem J 2004;381:275 – 85.

32. Adams J, Kauffman M. Development of the proteasome inhibitor Velcade (bortezomib). Cancer Invest 2004;22:304 – 11.

33. Adams J. Potential for proteasome inhibition in the treatment of cancer. Drug Discov Today 2003;8:307 – 15.

34. Adams J, Palombella VJ, Sausville EA, et al. Proteasome inhibitors: a novel class of potent and effective antitumor agents. Cancer Res 1999; 59:2615 – 22.

35. Rajkumar SV, Richardson PG, Hideshima T, Anderson KC. Proteasome inhibition as a novel therapeutic target in human cancer. J Clin Oncol 2005;23:630 – 9.

36. Richardson PG, Mitsiades C, Hideshima T, Anderson KC. Proteasome inhibition in the treatment of cancer. Cell Cycle 2005;4:290 – 6.

37. Wong BR, Parlati F, Qu K, et al. Drug discovery in the ubiquitin regulatory pathway. Drug Discov Today 2003;8:746 – 54.

38. Swinney DC. Targeting protein ubiquitination for drug discovery. What is in the drug discovery toolbox? Drug Discov Today 2001;6:244 – 50.

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