Supplemental Information

Enhanced degradation of misfolded proteins promotes tumorigenesis

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and Xiaolu Yang

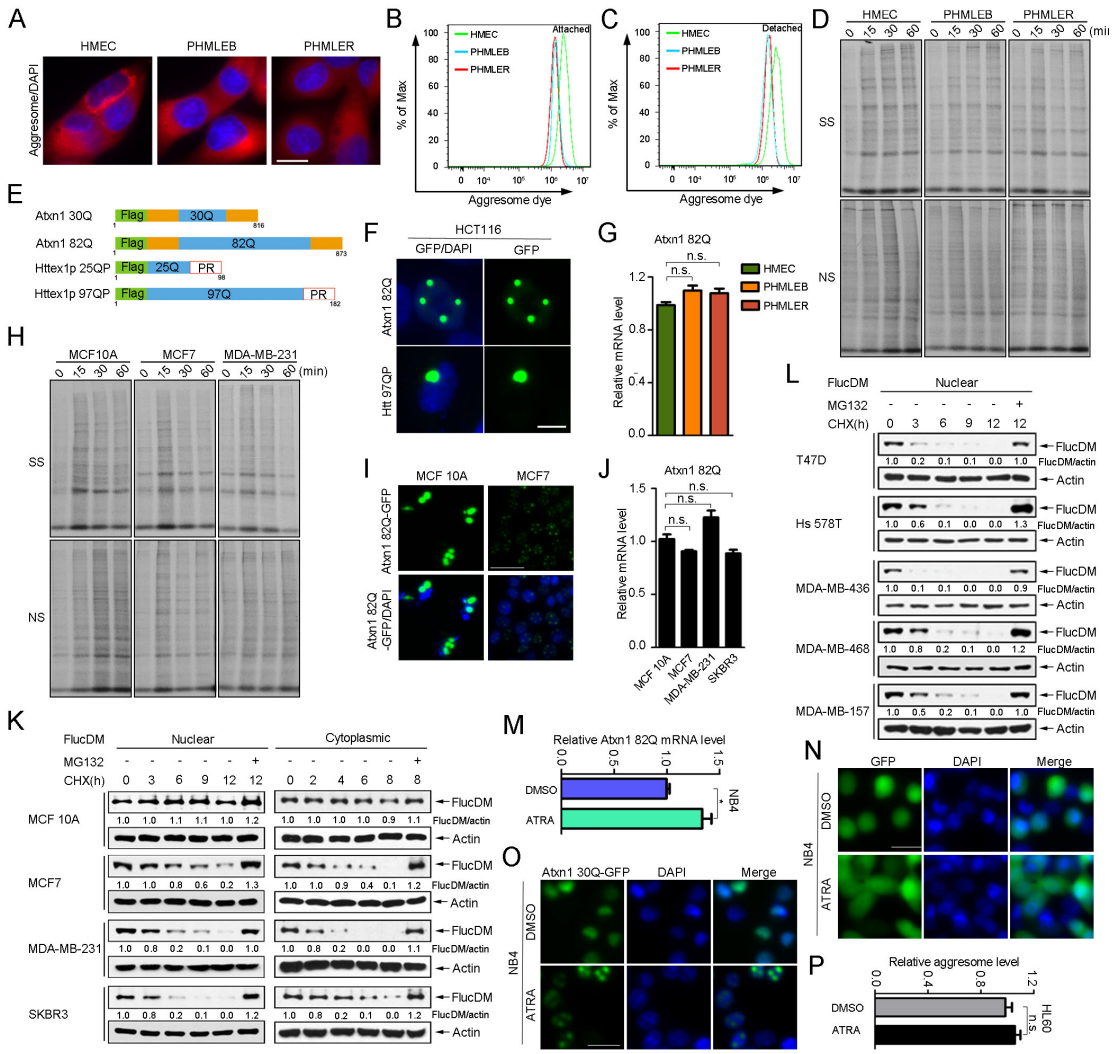

Figure S1. The capability to clear misfolded proteins is enhanced during oncogenic transformation of HMEC and in breast cancer cells, and is decreased upon the differentiation of leukemia cells (related to Figure 1 and Table S2)

(A) Representative images of aggresomes in HMEC, PHMLEB, and PHMLER cells.

(B and C) Levels of aggresome in HMEC, PHMLEB, and PHMLER cells grown under matrix-attached (on adherent plates) and matrix-detached (non-adherent, poly-HEMA-treated plates) conditions were analyzed by flow cytometry. Representative data are shown.

(D and H) Representative images of the autoradiograph gels quantified in Figure 1E (D) and Figure 1K (H). 50% of NS and 25% of SS were analyzed.

(E) Schematic diagram of polyQ constructs (not drawn to scale). Each construct was tagged with the Flag epitope or with both the Flag epitope and green fluorescence protein (GFP). The numbers of amino acids and polyQ regions are indicated. Httex1p is encoded by the first exon of the HD gene, containing the polyQ region and a proline-rich (PR) region. In all the other figures, Httex1p 25QP and Httex1p 97QP are labeled as Htt 25QP and Htt 97QP, respectively.

(F) HCT116 cells stably expressing Atxn1 82Q-GFP or Httex1p 97QP-GFP were stained with DAPI (blue). Scale bar, 10 μm. Note that Atxn1 82Q-GFP was localized in the nucleus, while Httex1p 97QP-GFP in the cytoplasm, sometime in the perinuclear region.

(G and J) Relative Atxn1 82Q mRNA levels in the indicated cells.

(I) Florescent images of MCF 10A and MCF7 cells infected with lentiviruses expressing Atxn1 82Q-GFP and stained with DAPI (blue). Scale bar, 50 μm.

(K and L) Stability of nuclear (K) and cytoplasmic (L) FlucDM proteins in MCF 10A and breast cancer cells. Cells were transfected with FlucDM plasmids and treated with 50 μg/ml cycloheximide (CHX) and 10 μM MG132 as indicated. Relative ratios of FlucDM versus

actin are indicated.
(M) Relative Atxn1 82Q mRNA levels in DMSO- and ATRA-treated NB4 cells.
(N and O) Images of DMSO- and ATRA-treated NB4 cells transfected with GFP (M) or Atxn1 30Q-GFP (O). Cells were stained with DAPI (blue). Scale bar, 50 μm.
(P) Relative aggresome levels in HL60 cells treated with DMSO or ATRA.
In (G), (J), (M), and (P), data are mean ± SEM (n = 3).

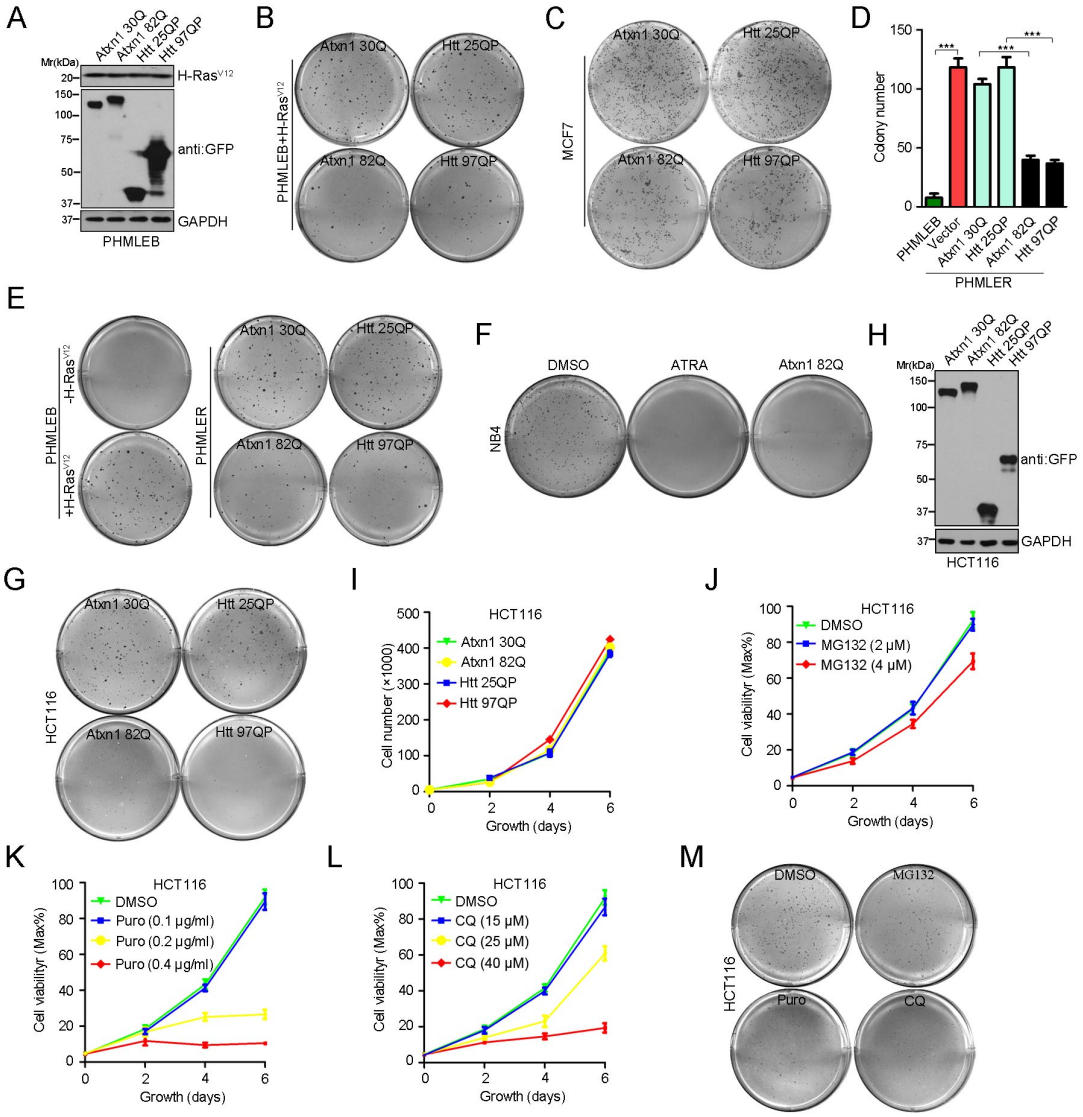

Figure S2. Exogenous and endogenous misfolded proteins inhibit the initiation and maintenance of malignant phenotypes (related to Figure 2)

(A and B) PHMLEB cells stably expressing polyQ proteins (via lentiviral infection) were infected with retrovirus expressing H-Ras^{V12}. Protein expression was analyzed by Western blot (A), and anchorage-independent growth by colony formation in soft agar (B).

(C) MCF7 cells stably expressing the indicated polyQ proteins were assayed for colony formation in soft-agar. Representative images are shown.

(D and E) PHMELB cells stably infected with and without the H-Ras^{V12} retroviruses, and PHMELR cells stably infected with lentiviruses expressing the indicated polyQ proteins, were grown in soft agar. Numbers of colonies (mean ± SEM, n = 3) and representative images of colony formation (E) are shown.

(F) Soft agar colony formation assay of NB4 cells treated without and with ARTA, as well as untreated NB4 cells expressing Atxn1 82Q.

(G to I) HCT116 cells were infected with lentiviruses expressing Atxn1 30Q, Atxn1 82Q, Httex1p 25QP, and Httex1p 97QP. Growth in soft agar (G), protein expression (H), and proliferation on adherent plates (I; n=3) are shown.

(J to L) Proliferation of HCT116 cells on adherent plates in the presence of different concentrations of MG132 (J), Puro (K), or CQ (L). Data represent means (n = 3).

(M) Soft agar colony formation assay of HCT116 in the presence of DMSO, MG132 (2 μM), Puro (0.1 μg/ml), or CQ (15 μM).

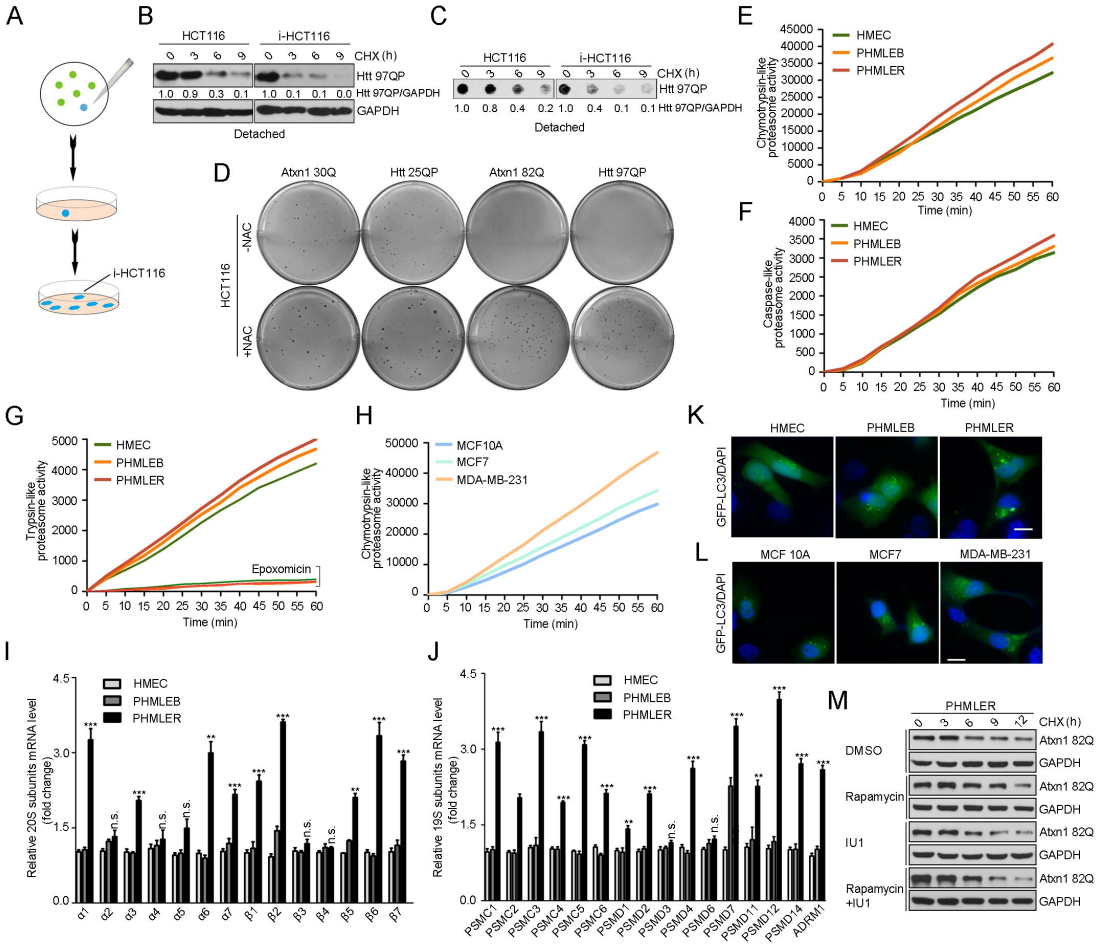

Figure S3. The role of ROS in anchorage-independent growth, and increase in proteasome activities and subunits during HMEC transformation (related to Figures 3, 4 and Table S2)

(A) Schematic diagram of isolating HCT116 cells grown in soft agar medium (i-HCT116).

(B and C) Stability of Httex1p 97QP in matrix-detached i-HCT116 and parental HCT116 cells was assayed by CHX chase. Total cell lysates (B) and the SDS-resistant fraction (SR; C) were analyzed by Western blot and filter retardation assay, respectively. Relative Htt 97QP/GAPDH ratios are shown.

(D) HCT116 cells stably expressing polyQ proteins were grown in soft agar in the presence or absence of NAC. Representative images of colonies are shown.

(E to G) Chymotrypsin-like (E), caspase-like (F) and trypsin-like (G) proteasome activities in HMEC, PHMLEB, and PHMLER cells were measured using fluorometric substrates Suc-LLVY-AMC, Ac-nLPnLD- AMC, and Ac-RLR-AMC, respectively. For (G), cell lysates were also treated with the proteasome inhibitor epoxomicin (2 μM) for 30 min before measurement.

(H) Chymotrypsin-like proteasome activity in MCF 10A, MCF7, and MDA-MB-231 cells was measured using the fluorometric substrate Suc-LLVY-AMC.

(I and J) Relative mRNA levels of all proteasome subunits in HMEC, PHMLEB, and PHMLER cells (mean ± SEM, n = 3).

(K and L) Representative images of the GFP-LC3 localization quantified in Figures 4E (K) and 4F (L).

(M) Representative Western blot of Atxn1 82Q stability quantified in Figure 4J.

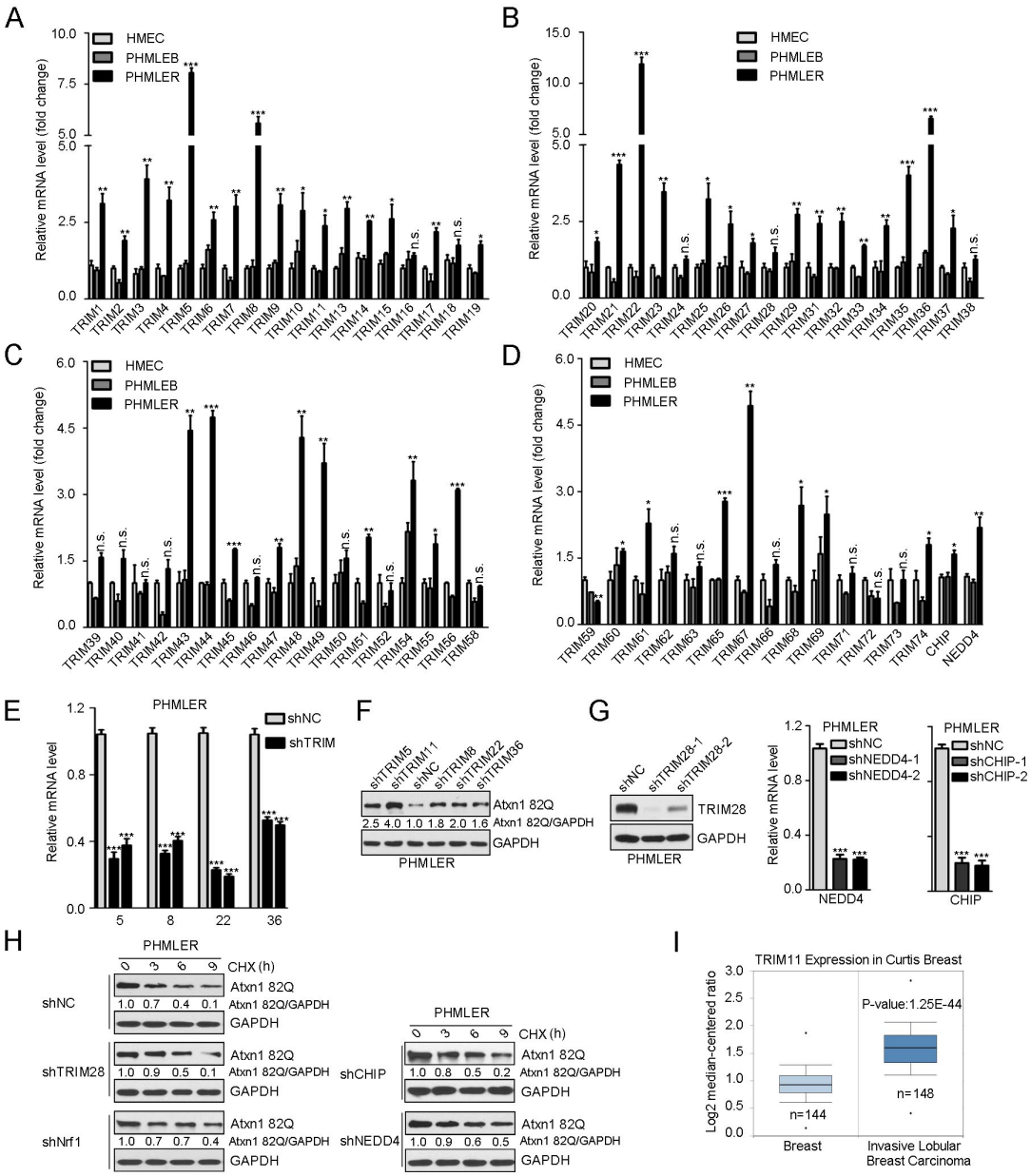

Figure S4. Expression of TRIM genes in human cancer (related to Figure 5, Table S1 and Table S2)

(A-D) Relative mRNA levels of all human TRIMs in HMEC, PHMLEB, and PHMLER cells. TRIM12, TRIM30 (both of murine origin), and TRIM57 (a pseudogene) were not tested. TRIM57 is the same as TRIM59.

(E) PHMLER cells were stably infected with lentiviruses expressing a control shRNA (shNC) or two independent shRNAs against TRIM5, 8, 22, and 36 each. Shown are relative mRNA levels.

(F) Steady-state levels of Flag-Atxn1 82Q in PHMLER cells stably transfected with shNC or shRNAs against TRIM5, 8, 11, 22, or 36. Relative Atxn1 82Q/GAPDH ratios are indicated.

(G) Western blot analysis of TRIM28 knockdown in PHMLER cells (left), and quantitative real-time PCR analysis of NEDD4 and CHIP knockdown in PHMLER cells. Two independent shRNAs were used for TRIM11, NEDD4, and CHIP each. shNC: control shRNA.

(H) PHMLER cells were stably infected with an shNC or an shRNAs against the indicated genes. Stability of the Atxn1 82Q protein was assayed, and relative Atxn1 82Q/GAPDH ratios are indicated.

(I) Box plot comparing TRIM11 mRNA levels in human breast cancers and normal tissues.

The data (https://www.oncomine.org/) (Curtis et al., 2012) are plotted on a log2 scale and centered on the median of its expression levels. The *P*-value was obtained using a two-sample t test, and "n" indicates the number of samples.
In (A-E) and (G), data represent mean ± SEM (n = 3 unless otherwise indicated).

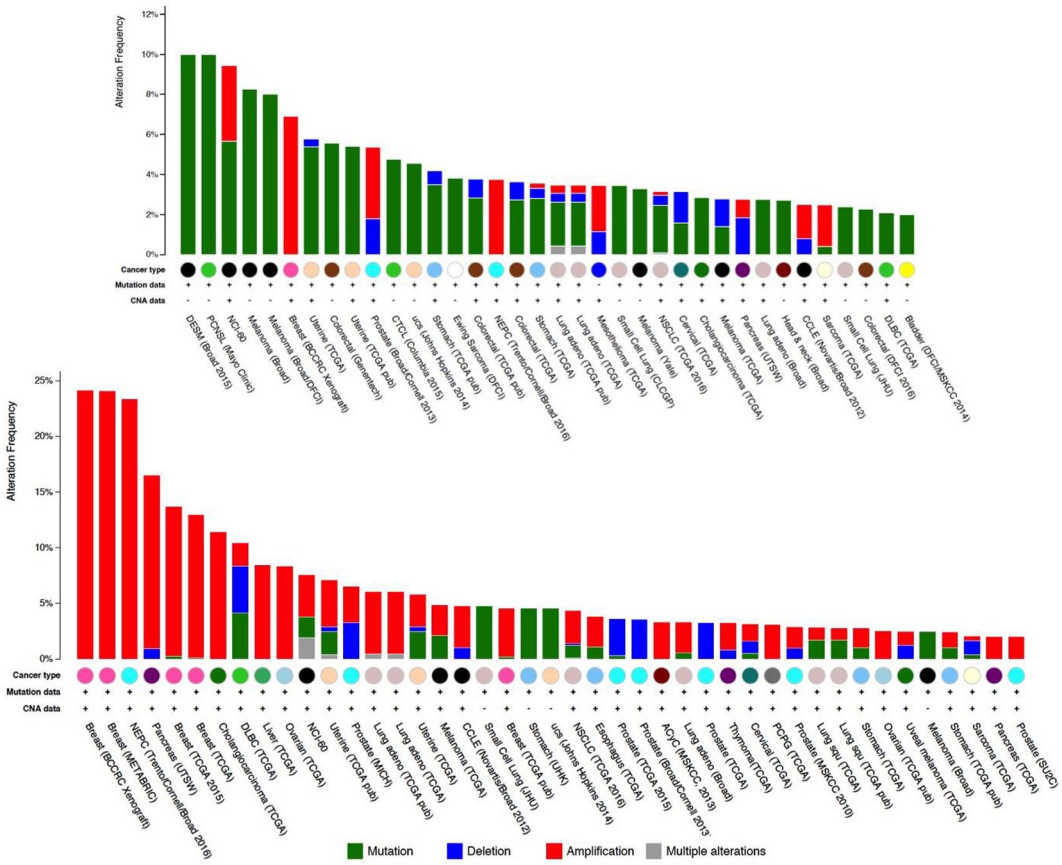

Figure S5. Alternations of NEDD4 (top) and TRIM11 (bottom) in human tumors (related to Figure 5)

Data were from BioPortal (http://www.cbioportal.org) (related to Figure 5)

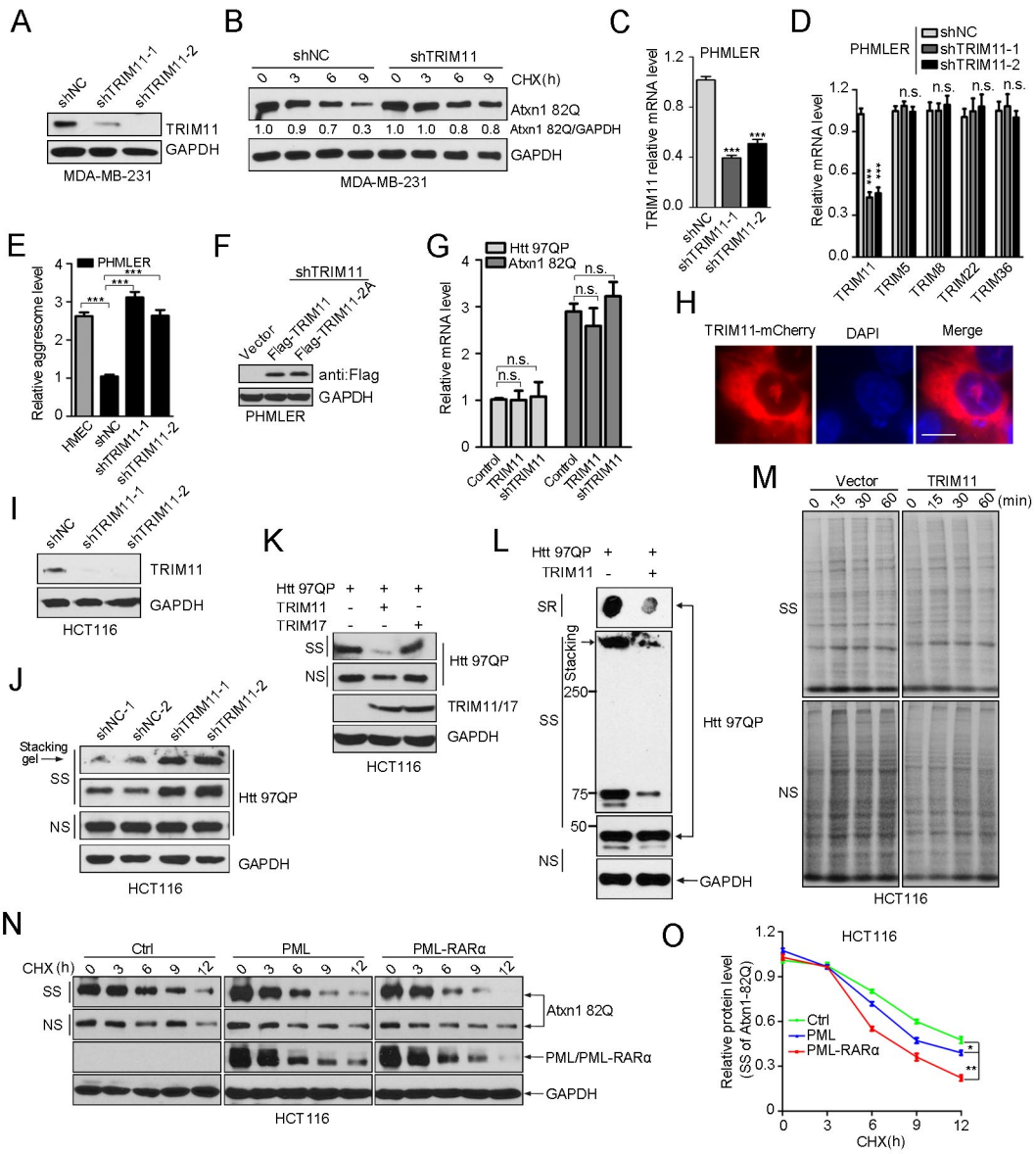

Figure S6. Role of TRIM11 and PML-RARα in the degradation of misfolded proteins (related to Figure 5 and Table S2)

(A and B) TRIM11 knockdown in MDA-MD-231 cells (A) and its effect on the stability of Atxn1 82Q (B). Relative Atxn1 82Q/ GAPDH ratios are indicated.

(C) TRIM11 mRNA levels in PHMLER cells stably expressing shNC and TRIM11 shRNAs (mean ± SEM, n =3).

(D) mRNA levels of TRIM5, 8, 22 and 36 in control and TRIM11-knockdown PHMLER cells (mean ± SEM, n =3).

(E) Relative levels of aggresomes in HMECs, as well as in PHMLER cells expressing shNC or TRIM11 shRNAs (mean ± SEM, n =3).

(F) Western blot analysis of TRIM11-knockdown PHMLER cells infected with lentiviruses expressing Flag-TRIM11 or Flag-TRIM11-2A.

(G) Relative Htt 97QP and Atxn1 82Q mRNA levels in control, TRIM11-overexpressing, and TRIM11-knockdown HCT116 cells (mean ± SEM, n =3).

(H) HCT116 cells were stably infected with lentiviruses expressing TRIM11-mCherry. Cells were stained with DAPI. Scale bar, 10 μm.

(I) shRNA-mediated TRIM11 knockdown in HCT116 cells.

(J) Levels of Httex1p 97QP in HCT116 cells stably expressing control or TRIM11 shRNAs. The species in stacking gel represented the SR form of Httex1p 97QP.

(K) Effect of TRIM11 and TRIM17 on Httex1p 97QP. HCT116 cells were transfected with Httex1p 97QP alone, or together with TRIM11 or TRIM17.
(L) HCT116 cells were transfected with Httex1p 97QP alone or together with TRIM11. The SR fraction of cell lysates was analyzed by filter retardation assay, and the NS and SS fractions by Western blot.
(M) Representative images of the autoradiograph gels quantified in Figure 5N. 50% of NS and 25% of SS were analyzed.
(N and O) HCT116 cells were transfected with Atxn1 82Q together with vector (ctrl), PML or PML-RARα, and treated with CHX for the indicated time. NS and SS fractions of the cell lysates were analyzed by Western blot analysis (N) and the relative SS Atxn1 82Q/GAPDH ratios were quantified (O). Data present mean (n =3).

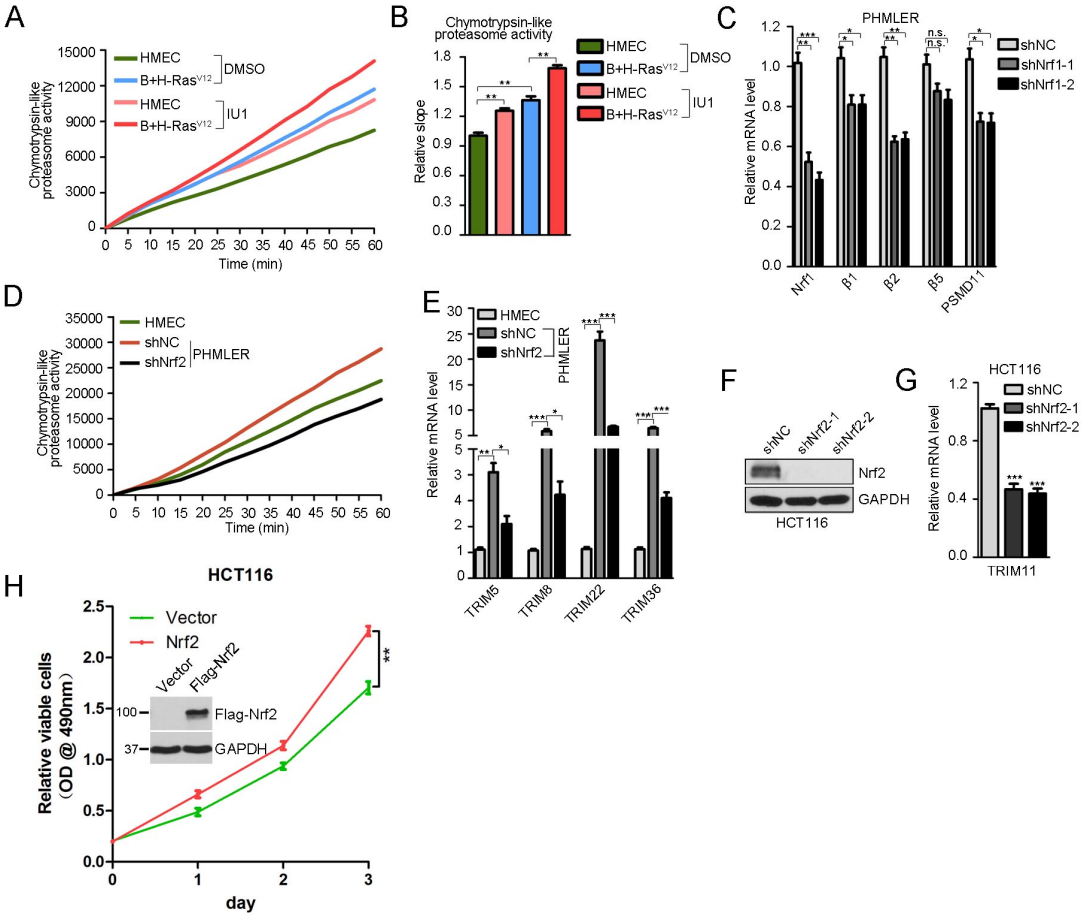

Figure S7. Regulation of TRIM proteins by Nrf1 and Nrf2, and the role of TRIM 11 in Nrf2-mediated PQC and tumor growth (related to Figures 6, 7 and Table S2)

(A and B) HMECs, as well as in PHMLEB cells infected with H-Ras^{V12}, were treated with DMSO or IU1 (50 μM). Relative chymotrypsin-like proteasome activity is shown (n = 6 for HMECs, and 7 for PHMLEB cells infected with H-Ras^{V12}).

(C) Relative mRNA levels of the proteasome subunits β1, β2, β5, and PSMD11 in PHMLER cells expressing shNC or Nrf1shRNAs.

(D) Chymotrypsin-like proteasome activity in HMECs, as well as in PHMLER cells expressing shNC and Nrf1shRNAs.

(E) Relative mRNA levels of TRIM5, 8, 22, and 36 in PHMECs, and in PHMLER cells expressing shNC and Nrf2 shRNAs.

(F and G) shRNAs-mediated knockdown of Nrf2 (F) and its effect on TRIM11 mRNA levels (G).

(H) Proliferation of control and Nrf2-overexpressing HCT116 cells.

In (B), (C), (E), (G), and (H), values represent mean or mean ± SEM (n = 3).

Table S1. Change of TRIM mRNA levels in PHMLER cells compared to HMECs (related to Figure S4). Statistical significance was assessed using unpaired two-tailed Student's *t*-tests. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Up-regulated		Down-regulated		No significant change
*	TRIM10, TRIM11, TRIM15, TRIM19, TRIM20, TRIM25, TRIM26, TRIM27, TRIM37, TRIM55, TRIM60, TRIM61, TRIM68, TRIM69, TRIM74	**	TRIM59	TRIM16, TRIM18, TRIM24, TRIM28, TRIM38, TRIM39, TRIM40, TRIM41, TRIM42, TRIM46, TRIM50, TRIM52, TRIM58, TRIM62, TRIM63, TRIM66, TRIM71, TRIM72, TRIM73
**	TRIM1, TRIM2, TRIM3, TRIM4, TRIM6, TRIM7, TRIM9, TRIM13, TRIM17, TRIM23, TRIM29, TRIM31, TRIM32, TRIM33, TRIM34, TRIM43, TRIM47, TRIM48, TRIM49, TRIM51, TRIM54, TRIM67			
** *	TRIM5, TRIM8, TRIM21, TRIM22, TRIM35, TRIM36, TRIM44, TRIM45, TRIM56, TRIM65			

Table S2. Primer sequences for quantitative real-time PCR (related to Figures S1, S3, S4, S6 and S7).

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Plasmids

PML-RARα (containing PML amino acids 1-394 and RARα amino acids 61-462) was cloned into pRK5 with an NH_2-terminal Flag tag. Flag-FlucDM-GFP (which expressed in cytoplasm a *firefly* luciferase mutant in which R188 and R261 were changed to Glu) was made in pRK5 with an NH_2-terminal Flag epitope and a COOH-terminal enhanced green fluorescence protein (GFP), based on a plasmid provided by Dr. S. Raychaudhuri (Gupta et al., 2011). The nucleus-localized variant of Flag-FlunDM-GFP was generated by fusing the SV40 nuclear localization signal (PKKKRKV) at the NH_2-terminus of FlucDM. The following plasmids have been described previously: Flag-PML/pRK5 (isoform IV), Flag-Atxn1 82Q/pRK5, and Flag-Httex1p 97QP/pRK5 (Guo et al., 2014); TRIM11 (COOH-terminally Flag-tagged) (Ishikawa et al., 2006); and TRIM17 (NH_2-terminally HA-tagged) (Versteeg et al., 2013). TRIM17 was also cloned into pRK5 vector with an NH_2-terminal Flag tag. pEGFP-LC3 (human) was a gift from Toren Finkel (Addgene plasmid # 24920) (Lee et al., 2008), and NC16 pCDNA3.1 Flag-NRF2 was a gift from Randall Moon (Addgene plasmid # 36971)(Camp et al., 2012).

Lentiviral vectors expressing polyQ proteins, TRIM11, and TRIM11 2A (Cys16/Cys19-to-Ala) were constructed in GFP-T2A-mCherry/pTRPE (provided by J. L. Riley). These proteins were fused NH_2-terminally with GFP (GFP-Atxn1 30Q, GFP-Atxn1 82Q, GFP-Httex1p 25QP, and GFP-Httex1p 97QP), NH_2-terminally with both GFP and a Flag tag (GFP-Flag-Atxn1 30Q, GFP-Flag-Atxn1 82Q, GFP-Flag-Httex1p 25QP, and GFP-Flag-Httex1p 97QP), or NH_2-terminally with Flag and COOH-terminally with mCherry (Flag-TRIM11-mCherry). The Atxn1 lentiviral plasmids were made based on the corresponding plasmids in pcDNA1Amp (Riley et al., 2005), and Httex1p and TRIM11 lentiviral plasmids based on the corresponding plasmids in pcDNA3.1 (Ishikawa et al., 2006; Steffan et al., 2004).

The following lentiviral plasmids expressing shRNAs were made in pLKO.1-puro and purchased from Sigma, with the clone numbers indicated in the parentheses: control, TRIM11 (TRCN0000033959 and TRCN0000033960), TRIM5 (TRCN0000005896), TRIM8 (TRCN0000033966), TRIM22 (TRCN0000005899), TRIM36 (TRCN0000040289), TRIM28 (TRCN0000017998 and TRCN0000017999), Nrf2 (TRCN0000273494 and TRCN0000284999), Nrf1 (TRCN000016903 and TRCN0000016904), NEDD4 (TRCN000007550 and TRCN0000007551), and CHIP (TRCN000007525 and TRCN0000007526).

The retroviral plasmid pBABE puro H-Ras^{V12} was a gift from William Hahn (Addgene plasmid # 9051) (Elenbaas et al., 2001).

Antibodies, Reagents, and siRNA

Primary antibodies against the following proteins/epitopes were purchased from the indicated sources: mCherry (ChromoTek); GAPDH (Novus Biologicals); SV40 large T-antigen (ab16879; Abcam); TRIM11 (EMD Millipore); 20S proteasome α/β subunits and proteasome activator 11S α/β/γ subunits (Enzo Life Sciences); ATG7 and K48-linked poly-ubiquitin (Cell Signaling Technology); FLAG and p62 (Sigma); GFP and LC3B (MBL); H-Ras (sc-896), Nrf2 (sc-13032), and ubiquitin (P4D1) (Santa Cruz Biotechnology); TRIM28, PSMD1, and PSMD11 (Bethyl Laboratories). Secondary antibodies were conjugated to HRP (Santa Cruz) or AlexaFluor 568 (Invitrogen).

Cycloheximide (CHX), the USP14 inhibitor IU1, and rapamycin was purchased from Calbiochem. The following reagents were from Sigma: epoxomicin, all-trans

retinoic acid (ATRA), benzonase, chloroquine, 2', 7'-dichlorodihydrofluorescein diacetate (H2-DCFDA), paraformaldehyde (PFA), polybrene, poly(2-hydroxyethyl methacrylate) (poly-HEMA), puromycin, N-acetylcysteine (NAC), Z-Leu-Leu-Leu-al (MG132), puromycin (Puro), and chloroquine (CQ).

Human ATG7 siRNAs were purchased from GE Dharmacon (siATG7-1, J-020112-05; siATG7-1, J-020112-06).

Cell culture

Cells were purchased from ATCC unless otherwise indicated. HEK293T, MCF7, MDA-MB-231, SK-BR-3, Hs 578T, MDA-MB-436, MDA-MB-468, and MDA-MB-157 cells were cultured in Dulbecco's Modified Eagle's medium; HCT116 cells (from B. Vogelstein) in McCoy's 5A medium; and NB4 (from M. Lanotte) (Lanotte et al., 1991), HL-60, and T47D cells in RPMI 1640 medium. All medium (Life Technologies) were supplemented with 10% fetal bovine serum (HyClone). HMEC, PHMLEB, and PHMLER cells (from R. Weinberg) (Elenbaas et al., 2001) were cultured in mammary epithelial cell growth medium (MEGM basal medium, Lonza). MCF 10A cells were cultured in DMEM/F12 supplemented with MEGM SingleQuots™ Kit (Lonza) containing human EGF (100 mg/ml), insulin (10 mg/ml) and hydrocortisone (1 mg/ml); 5% horse serum (Invitrogen); and cholera toxin (100 ng/mL, Sigma). Suspension cells were cultured in appropriate medium and grown in poly-HEMA-coated (12 mg/ml) plates. All cells were grown at 37°C with 5% CO_2.

Lentiviruses, retroviruses, and stable cell lines

For lentivirus production, HEK293T cells were transfected with each lentiviral vector together with the helper plasmids Gag, Rev and VSVG. For retrovirus production, Phoenix-E cells were transfected with each retroviral vector and the PCL-ampho helper plasmid. Forty-eight to 72 h after transfection, virus-containing media were collected by centrifugation at 100 g for 5 min and concentrated by centrifugation at 10,000 g for 18 h. Cells were infected with lentiviruses or retroviruses in the presence of 8 μg/ml polybrene and selected with appropriate antibiotics.

Transformation and differentiation

H-Ras^{V12}-expressing retroviruses were used to transforme PHMLEB cells as previously described (Elenbaas et al., 2001). Differentiation of NB4 cells was induced using 1 μM ATRA (Lanotte et al., 1991).

Telomeric repeat amplification protocol (TRAP) assay

Cells were lysed in CHAPS lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol, 5 mM b-ME, 1 mM NaF, 1 mM PMSF, and 1x Roche complete protease inhibitor cocktail). Equal amounts of proteins were mixed with 0.1 μg ACX primer (GCGCGGCTTACCCTTACCCTTACCCTAACC), 0.1 μg TS primer (AATCCGTCGAGCAGAGTT), 2.5 units Taq polymerase, and 200 nM dNTPs, in 1x TRAP buffer (20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 63 mM KCl, 0.05% Tween-20, 1 mM EGTA, and 0.5 mg BSA) and 50 μL total volume. Reaction was run on a thermocycler: Lid to 105°C; Step 1, 30 min @ 30°C; Step 2, 5 min @ 95°C; Step 3, 30 sec @ 95°C; Step 4, 30 sec @ 52°C; Step 5, 90 sec @ 72°C; Repeat steps 3-5 36x; Step 6, 5 min @ 72°C; Hold @ 4°C. Samples analyzed via SDS free PAGE with TAE running buffer, stained with EtBr. DNA fragments were visualized with UV.

Aggresome measurement

Cells were harvested, fixed with 4% PFA for 30 min at 37 °C, and permeabilized with 0.1% Triton X-100 for 15 min at room temperature (RT). Cells were incubated with ProteoStat® aggresome dye (Enzo Life Sciences) for 30 min at RT, and washed with PBS. Fluorescence signals were immediately measured by a BD Accuri™ C6 flow cytometer and analyzed by FlowJo software.

Cell lysate fractionation, Western blot, and filter retardation assay

Cell lysates fractionation and filter retardation assay were performed as previously described (Guo et al., 2014). Cells were lysed in NP-40-containing buffer for 30 min on ice and subsequently were centrifuged at 16,000 g for 15 min at 4 °C. The supernatant was designated as the NP-40-soluble (NS) fraction. The protein concentrations in the NS fraction were measured by Bradford assay (Bio-Rad Labs). The pellets were re-suspended in the pellet buffer and boiled in 2% SDS with 50 mM DTT (SDS buffer). SDS-soluble (SS) fraction of the pellet and the NS fraction were analyzed by Western blot. The SDS-insoluble (SS) fraction of the pellets, which could not enter the SDS-PAGE, was detected by filter retardation assay. After boiling in SDS buffer, the pellet fraction was applied to a membrane with a pore size of 0.2 μm, and the proteins retained on the membrane analyzed by immunoblot. In some experiments, the SS fraction was also detected as a band at the top of the stacking gel.

For detecting K48 polyUb-modified proteins, 25% NS and 50% SS fractions from matrix-attached cells, or 25% of NS and 20% of SS fractions from matrix-detached cells, were loaded onto SDS-PAGE. Relative ratios of SS versus total (NS+SS) fractions were determined based on the relative loading. To analyze protein half-life, Flag-Atxn1 82Q and Flag-Httex1p 97Q were used. 12 h after transfection or 48 h after infection, cells were treated with CHX (50 μg/ml) for different durations. Cells were fractionated and analyzed by Western blot or filter retardation assay using anti-Flag antibody.

Immunofluorescence and Immunoprecipitation

Cells were transfected or infected with GFP, GFP-Atxn1 82Q, and GFP-Httex1p 97QP. Transfected and untransfected cells cultured on coverslips were fixed with 4% PFA for 30 min and permeabilized with 0.15% Triton X-100 for 15 min. After being washed twice with PBS, cells were blocked with 3% BSA for 30 min, incubated with primary antibody overnight at 4 °C, and the secondary antibody for 1 h at RT. Cells were mounted using medium containing DAPI (Vector Labs) and observed under a fluorescence microscope (Olympus). For quantifying GFP-Atxn1 82Q, and GFP-Httex1p 97QP inclusions, 200 randomly selected cells were examined.
For analyzing Atxn1 82Q ubiquitination, GFP-Flag-Atxn1 82Q was expressed alone or together with HA-TRIM11 in HCT116 cells.

Cells were lysed in SDS-containing buffer, boiled, and centrifuged. 10% and 25% of lysates from vector- and HA-TRIM11-transfected cells, respectively, which contained similar levels of unmodified Flag-Atxn1 82Q, were used as input for immnoprecipitation. After being diluted with buffer containing no SDS, the lysates were incubated with anti-Flag antibody (M2)-conjugated beads at 4 °C overnight. The beads were sequentially washed in the NP-40-containing buffer with additional 0, 0.5, and 1 M KCl. The beads were boiled in loading buffer and were analyzed by Western blot. For testing TRIM11-Atxn1 82Q interaction, GFP-Atxn1 82Q was expressed alone or together with Flag-TRIM11 in HCT116 cells. Cells were lysed in NP-40-containing buffer, and the lysates were incubated with the M2 beads at 4 °C overnight. After washed 4 times with NP-40-containing buffer, the beads and cell lysates were boiled and analyzed by Western blot.

Pulse-chase assay

Analysis of DRiPs was performed as previously described (Schubert et al., 2000). Cells were cultured in Met-free DMEM medium for 30 min, and pulse labeled with [^{35}S]Met (100 μCi/ml) for 30 seconds. Afterwards, cells were washed twice with cold PBS and chased in complete medium for 0-60 min at 37 °C. Cell lysates were fractionated into the NS and SS fractions, which were subsequently analyzed by SDS-PAGE and autoradiography. The total signals of each lane, relative to time 0, were quantified by Image J. The fractions of DRiP were calculated by dividing the signals in the SS fraction with the total signal of NS and SS fractions, adjusted by the percentage of loading for each fraction.

Analysis of Atxn1 82Q stability was performed as previously described (Guo et al., 2014). Cells expressing GFP-Flag-Atxn1 82Q were cultured for 30 min in Met-free DMEM medium, and pulse labeled for 30 min with [^{35}S]Met (100 μCi/ml). Cells were chased in complete medium for 0-24 h and lysed in SDS-containing buffer. After boiled and diluted into buffer without SDS, the supernatants were incubated with anti-Flag M2 beads at 4 °C overnight. The beads were sequentially washed with additional 0, 0.5, and 1 M KCl in the NP-40-containing buffer, and samples were resolved by SDS-PAGE and detected by autoradiography.

Quantitative real-time PCR

mRNA levels were analyzed by quantitative real-time PCR. Total RNAs were extracted using TRIzol (Invitrogen). 2 μg RNA was reverse transcribed to cDNA using the First Strand cDNA Synthesis Kit (Marligen Biosciences). Quantitative real-time PCR was performed using the SYBR Green PCR Master Mix in the 7900HT Fast Real-Time PCR System (Applied Biosystems) as previously described (Jiang et al., 2013). The primers for reverse transcription are described in Table S2.

26S Proteasome Activity

The activities of the 26S proteasome were assayed as previously described (Kisselev and Goldberg, 2005). Cells were lysed in a cytosolic extract buffer (50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA, and 0.025% digitonin) on ice for 20 min. Cell lysates were centrifuged for 15 min at 10,000 g and 4 °C. The protein concentrations in the supernatant were measured by Bradford assay. Cell lysates containing 5-10 μg of total protein was diluted with 26S proteasome assay buffer (50 mM Tris-HCl, pH 7.5, 40 mM KCl, 5 mM $MgCl_2$, 0.5 mM ATP, 1 mM DTT, 0.5 mg/ml BSA) in a 96-well microtiter plate (BD Falcon), and incubated with fluorogenic substrates. Chymotrypsin-, caspase-, and trypsin-like proteasome activities were measured by using the substrate Suc-LLVY-AMC, Ac-nLPnLD, and Ac-RLR-AMC (Enzo), respectively. Fluorescence (380nm/460nm, excitation/emission) released by AMC fluorescence was monitored on a microplate fluorometer (Infinite M200, Tecan) every 5 min at 37 °C for 1 h.

Cell growth and MTT assay

Cells were seeded in triplicates in 96-well plates at 3,000 cells/well and cultured in medium containing 10% FBS. Cells were shifted to 6-well plates when reaching confluence. Relative viable cells were determined by counting cell number or measuring the OD at an absorbance wavelength of 490 nm of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Promega) at the indicated time points.

Soft agar colony formation assay
0.5-1 × 10^4 cells were suspended in 1.5 ml growth media that contained 0.35% of top agar (Lonza) and was pre-warmed to 40 °C. The mixed suspension was added to wells of six-well plates, which contained 1.5 ml pre-solidified media with 0.6% of bottom agar. After the top agar became solidified, 1 ml of growth medium was added to the wells. Medium was replaced with fresh one every 3 or 4 days. Two to four weeks later, colonies were stained with 1 ml 0.005% crystal violet (Invitrogen) in PFA, pictured, and scored using Image J.

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