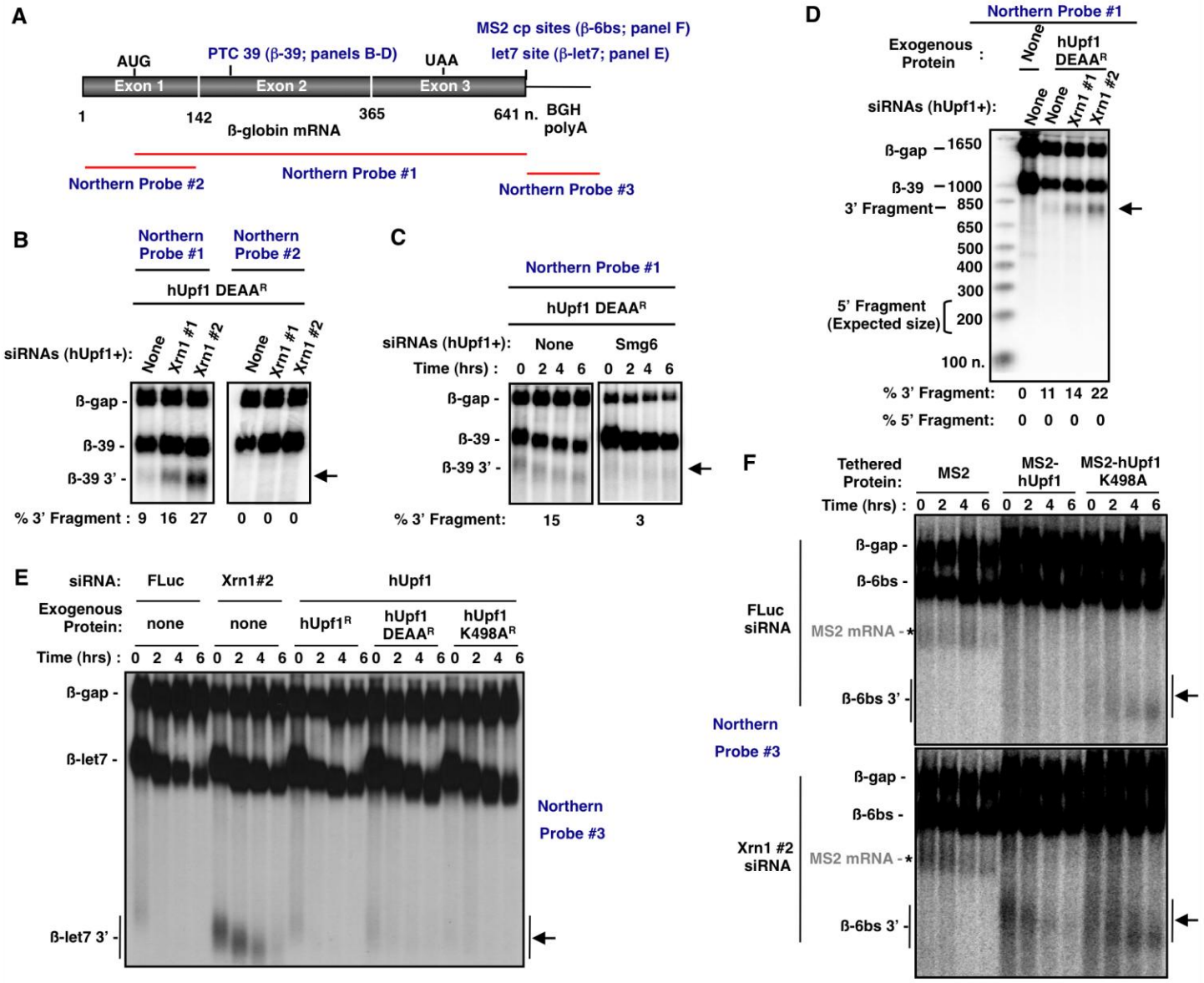


Figure S1, related to Figure 1. The hUpf1 ATPase mutant-dependent NMD intermediate is the 3' endonucleo-lytic fragment generated by Smg6, is specific to the NMD pathway and also occurs upon hUpf1 tethering.

(A) Schematic showing the used β-globin reporter mRNAs and Northern probes used to detect the corresponding full-length (Northern Probe #1), 5' ends (Northern Probe #2) or 3' ends (Northern Probe #3) of the reporters.

(B) Northern blots showing the detection of a faster migrating band upon hUpf1 ATPase mutant (hUpf1 DEAA) expression in the presence of no siRNA (None), Xrn1 #1 siRNA, or Xrn1 #2 siRNA with a probe against the full-length ß-39 mRNA (left panel) but not a probe against the 5' end (right panel) of the ß-39 mRNA. β-gap is an internal control mRNA. Quantifications of the amount of 3' fragment (in percent of total ß-39 mRNA) observed in each lane are shown below.

(C) Northern blot showing the disappearance of the faster migrating band in cells expressing hUpf1 DEAA when cells are treated with a Smg6 siRNA (right panel), but not when cells are treated with no siRNA (left panel). Note: The left panel is the same as shown in Figure 1A (top panel). The right panel was run on the same gel, but was not included in Figure 1A. Quantifications of the percent 3' fragment observed in the '0 hour' lanes are shown below.

(D) Northern blot showing no detectable accumulation of the 5' ß-39 mRNA fragment when no exogenous hUpf1 protein (None) or hUpf1 DEAA protein is expressed in the presence of no Xrn1 siRNA (None), Xrn1 #1 siRNA or Xrn1 #2 siRNA. Quantifications of the percent 3' and percent 5' fragment observed in each lane are shown below.

(E) Northern blots showing the detection of a 3' decay intermediate in pulse-chase mRNA decay assays in HeLa Tet-off cells expressing β-globin mRNA containing a sequence perfectly complementary to endogenous let-7 miRNA, upon Xrn1 knock-down, but not upon expression of hUpf1 proteins.

(F) Northern blots showing the detection of a 3' decay intermediate in pulse-chase mRNA decay assays in HeLa Tet-off cells of β-globin mRNA tethered, via MS2 coat protein, to hUpf1 K498A (right lanes). Tethered wild-type hUpf1 only generates the 3' fragment upon Xrn1 knockdown (middle panel). *: the mRNA encoding unfused MS2 coat protein hybridizes with the Northern probe.

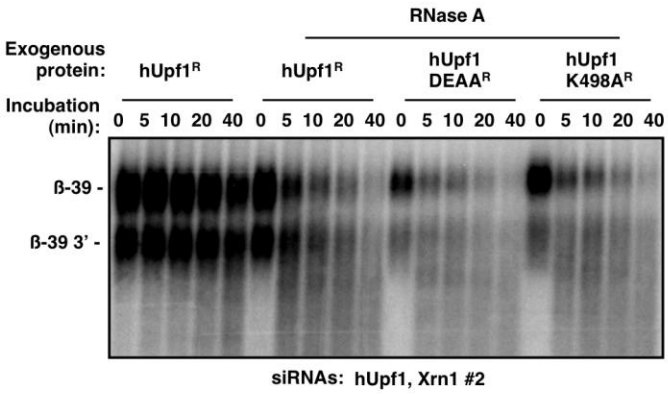


Figure S2, related to Figure 2.
Northern blot monitoring the degradation in the absence or presence of RNase A (as indicated) of β-39 mRNA and β-39 mRNA 3' fragment in extracts from HeLa Tet-off cells treated with siRNAs against hUpf1 and Xrn1 and expressing exogenous wild-type or mutant hUpf1 proteins from siRNA resistant mRNAs, as indicated.

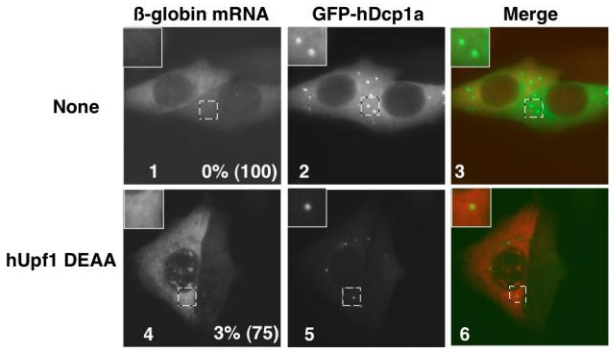


Figure S3, related to Figure 3. Wild-type ß-globin mRNA accumulates at low levels in PBs upon hUpf1 DEAA expression.

FISH assays showing the localization of wild-type ß-globin mRNA in the presence of no exogenous hUpf1 protein (panel 1-3) or hUpf1 DEAA (panel 4-6). GFP-hDcp1a was used as a processing body marker (panels 2 and 5). Merged images are shown in panes 3 and 6 (β-globin mRNA, red; GFP-hDcp1a, green). The percentage of cells which contained detectable quantities of ß-globin mRNA in PBs is shown with the number of cells quantified in parenthesis. An enlarged image corresponding to the dotted box is displayed in the upper left corner of each image.

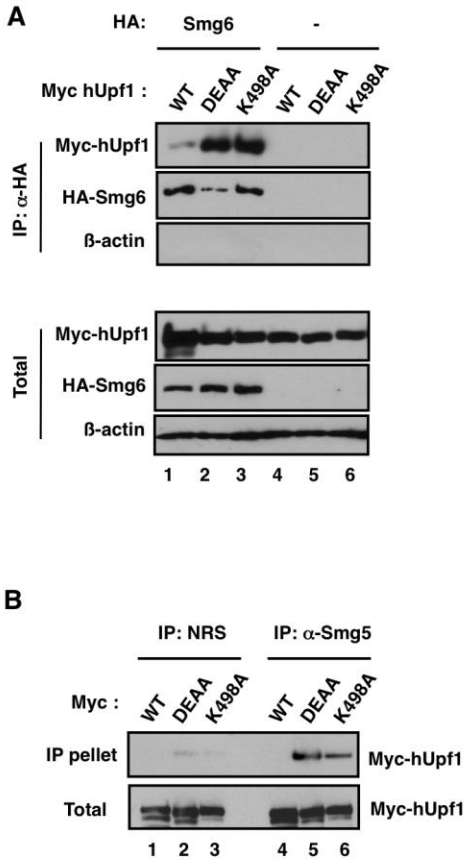


Figure S4, related to Figure 6. ATPase-deficient mutant hUpf1 proteins co-purify more efficiently than wild-type hUpf1 with Smg5 and Smg6.

(A) Western blots of anti-HA immunoprecipitates (top panels) from extracts of HEK 293T cells co-expressing Myc-tagged wild-type (WT) or ATPase mutant (DEAA, K498A) hUpf1 proteins with HA-tagged Smg6 or no HA tagged protein as indicated. Extracts were not treated with RNase. 5% of total extracts are shown in the bottom panels.

(B) Western blots of anti-Smg5 immunoprecipitates (panels 5-8) or immunoprecipitates using normal rabbit serum (NRS; panels 1-4) from extracts of HEK 293T cells expressing Myc-tagged wild-type (WT) or ATPase mutant (DEAA, K498A) hUpf1, or TTP as indicated. The bottom panel shows 5% of the total input extract.

Supplemental Experimental Procedures

Plasmid Constructs

Plasmids encoding the ß-39 (pPC-ß39), ß-ARE (pPC-ßwtATGMCSF) and GPx1-46 (pPC-GPx1-46) reporter mRNAs and the control mRNA ß-Gap (pcß-Gap) have been described previously (Singh et al., 2007). A plasmid encoding a tetracycline-responsive activator protein was used to activate transcription of reporter mRNAs (pTet-TTA; Clontech). Myc-tagged hUpf1 constructs have been described previously. Constructs expressing hUpf1 DEAA and K498A mutant proteins and hUpf1 mRNAs resistant to the hUpf1 siRNA (for the latter, silent mutations changed the DNA sequence encoding amino acids 631-633 from cgctccatt to agatcgata) were created using the quick-change site directed mutagenesis method (Stratagene). The plasmid pcNEGFP-hDcp1a, which was used as a PB marker, has two tandem copies of the enhanced Green-Fluorescent Protein (EGFP) inserted into the *Hin*dIII site of pcDNA3 (Invitrogen) and the open reading frame of hDcp1a inserted between *Eco*RI and *Not*I sites. The plasmid used to knock down hUpf1 expression (pSHAG-hUpf1; Figure 3) was described previously (Singh et al., 2007). The plasmid encoding HA-tagged Smg6 was a generous gift from Dr. Oliver Mühlemann (Eberle et al., 2009). Plasmid sequences are available upon request.

Antibodies

Rabbit polyclonal antibodies were raised (Cocalico Biologicals Inc, Reamstown, PA) against bacterially produced fragments of human Smg1 (amino acids 1-188), Smg5 (amino acids 405-511), Smg6 (amino acids 80-240) and Smg7 (amino acids 521-645) with N-terminal Glutathione-S-Transferase (GST) tags. Rabbit polyclonal antibodies against hUpf1, hUpf2, hUpf3b (Lykke-Andersen et al., 2000), eIF4A3, Y14, Xrn1 (Lykke-Andersen and Wagner, 2005; Singh et al., 2007) and mouse monoclonal anti-HuR 3A2 (Gallouzi et al., 2000) were described earlier. Human IC-6 serum was a generous gift from Drs. Marv Fritzler, Ed Chan and Donald

Bloch. The rabbit polyclonal antibodies used to detect ß-actin and PABPC1 were obtained from Cell Signaling Technologies and AbCam, respectively. Antibodies against Smg5, Smg7, hUpf2, hUpf3b and Y14 were affinity purified using their His_6-tagged antigens.

mRNA decay and RNA-immunoprecipitation assays

HeLa tet-off cells were seeded onto 2.5-cm wells (Figure 1) or 10 cm plates (Figure 2A and Figure 6B) at ~ 25% confluency 20 hours prior to siRNA transfection. On the following day, cells were transfected with Silentfect transfection reagent (Biorad) and Xrn1 and/or hUpf1 siRNAs (see sequences in the Supplemental Experimental Procedures) at a final concentration of 20 nM. On the following day, cells were placed in fresh media and transfected with 400 ng reporter mRNA plasmid (4 μg for Figures 2A,B and 6B), 40 ng pcß-gap (Figure 1 only), 100 ng of myc-hUpf1 plasmid (1 μg for Figures 2A, B) or mutants thereof and pcDNA3 plasmid to 1 μg total DNA (10 μg for Figures 2A, B and 6B) using TransIT HeLa reagent (Mirus). In Figure 6B, plasmids expressing the indicated tagged NMD factors were co-transfected. Expression of mRNA reporters was induced for 6 hours by the removal of tetracycline 40 hours after the plasmid transfection. For mRNA decay assays (Figure 1), total RNA was prepared using Trizol reagent (Invitrogen), 0, 2, 4, or 6 hours after addition of 1μg/ml tetracycline. For RNA-immunoprecipitation assays (Figures 2A,B and 6B), cells were washed with 10 ml PBS and lysed with 1.0 ml of isotonic gentle lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl pH 7.5, 200 mM NaCl, 0.5 mM PMSF, 2 μg/ml aprotinin and leupeptin) containing yeast RNA at a final concentration of 100 ng/ml. Cell lysates were incubated with mouse monoclonal anti-myc, or rabbit anti-hUpf2 antibodies conjugated to protein A sepharose, or preconjugated anti-FLAG or anti-HA beads (Sigma), at 4ºC for 1 hour. Beads were washed 8 times with NET-2 buffer. To elute myc-hUpf1 complexes, beads were lightly shaken with ~ μl of NET-2 buffer containing 1 μg/ml myc peptide and 40 units RNaseOut (Invitrogen) for 2 hours. Other complexes were

eluted directly into Trizol. Elutions were transferred to 1 ml Trizol (Invitrogen). Total RNA was prepared and subjected to Northern blotting as described previously (Singh et al., 2007). The efficiency of siRNA knockdowns was monitored by Western blotting.

In vitro 5'-to-3' mRNA decay assays

Hela Tet-off cells in 10-cm plates were transfected with siRNAs against hUpf1 (see and siRNA-resistant hUpf1, hUpf1 DEAA, or hUpf1 K498A protein expression vectors as described above. 3 days after siRNA transfection, cells were lysed in 1 ml of hypotonic gentle lysis buffer (0.1% Triton X-100, 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 0.5 mM PMSF, 2 μg/ml aprotinin and leupeptin). For decay assays conducted directly in the lysate, 250 µl of each sample was split off and added to 25 µl of 10x Terminator buffer (Epicentre Biotechnologies) in the presence of 1.25 units of Terminator enzyme (Epicentre). In the experiments using RNase A, 10 ng/ml of RNase A was added instead of Terminator. After mixing, five 50 µl aliquots were split into separate tubes and samples were incubated at 30ºC (37°C for RNase A assay) for the indicated time and subjected to phenol-chloroform extraction followed by ethanol precipitation. For decay assays conducted after phenol extraction (Figure 2C), 250 µl of lysate was split off from each sample and subjected to phenol-chloroform extraction and ethanol precipitation. Pellets were dissolved in 250 µl of RNase-free water and incubated in the presence of 25 µl of Terminator buffer and 1.25 units of Terminator enzyme. Decay assays were conducted the same as described above. mRNA levels were visualized by northern blotting for ß-globin mRNA.

Indirect Immunofluorescence Assays

HeLa cells in DMEM/10% fetal bovine serum (FBS) at ~30% confluency in 2.5 cm wells were transfected using TransIT HeLaMonster reagent according to manufacturer's protocols (Mirus),

with a total of 1 μg of plasmid. Cells were split to chamber slides twenty-four hours later. For indirect immunofluorescence experiments, cells were transfected with 100 ng of pcDNA3-Myc-hUpf1 or derivative thereof, and 0.9 μg of empty pcDNA3 vector (for details on plasmids, see the Supplemental Experimental Procedures). Forty-eight hours after transfection, cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) for 15 mins, and permeabilized and blocked with PBS/1% goat serum/0.1% Triton X-100 for 30 mins. Cells were then incubated with human IC-6 serum (1: 8,000 dilution) (Figure 5A-B) and either mouse anti-myc (9B11, Cell Signaling) antibodies (1:200 dilution) (Figure 5A) or a rabbit antibody against an endogenous NMD factor (1:100-1:1,000 dilution) (Figure 5B) for 2 hours (for details on antibodies, see the Supplemental Experimental Procedures). Following removal of the primary antibody, cells were incubated for 1 hour with 4 μg/ml of secondary anti-human antibodies labeled with Texas Red fluorophore (Molecular Probes) and anti-mouse or anti-rabbit antibodies labeled with Alexa-488 fluorophore (Molecular Probes). In the experiments in Figure 5B, 50 ng plasmid expressing DsRed with a nuclear localization signal (NLS-DsRed) was co-transfected to label transfected cells.

Flourescence In Situ Hybridization (FISH) Assays

For FISH experiments, cells were transfected in the presence of 50 ng/ml tetracycline with 400 ng of reporter mRNA expression plasmid, 400 ng pTet-TTA, 250 ng pSHAG hUpf1, 75 ng pcNEGFP-hDcp1a and 100 ng of myc-hUpf1 plasmid or derivatives thereof (Figure 3). 60 hours after transfection, transcription of reporter mRNAs was initiated by washing cells in PBS and placing them in DMEM/10% FBS containing no tetracycline. After a transcriptional pulse of 10 hours (see section on pulsed expression experiments), cells were fixed in 4% paraformaldehyde in PBS for 15 minutes and permeabilized overnight in 70% ethanol at 4ºC. Cells were then rehydrated for 10 minutes in 50% formamide, 2X SSC (300 mM NaCl, 30 mM sodium citrate,

PH 7.0). Next, cells were incubated overnight at 37°C in a solution containing 50% formamide, 2X SSC, 0.02% Bovine Serum Albumin (BSA), 2 mM vanadyl-ribonucleoside complexes, 1 μg/ml total yeast RNA, and 0.1 mg/ml dextran sulfate. In order to detect the localization of full-length ß-globin mRNA, four Texas-Red labeled 50-nucleotide DNA oligo probes complementary to sequences in exons 1, 2, and 3 were added (Figure 3A) at a concentration of 20 ng/ml each (sequences given in Supplemental Experimental Procedures). To detect GPx-1 mRNA two oligos were used to detect exon 1 and 2. In the experiments in Figure 3B and 3C, single probes were used at a concentration of 20 ng/ml. 24 hours later, cells were washed twice for 30 minutes at 37°C in 50% formamide, 2X SSC prior to visualization. To quantify the number of cells with reporter mRNAs concentrated in PBs, transfected cells expressing detectable quantities of reporter mRNA were scored for reporter mRNA co-localization with GFP-hDcp1a. The cell counts from at least three experiments were averaged to produce a final percentage.

Co-Immunoprecipitation Assays

Human embryonic kidney 293T cells in 3.5-cm wells were transfected with 200 ng of Myc-tagged hUpf1 or mutants thereof and 1.8 μg of pcDNA3 vector. 48 hours after transfection, cells were lysed in hypotonic gentle lysis buffer in the presence or absence of RNase A (100 ng/mL). Lysates were incubated for 4 hours with mouse monoclonal anti-myc antibodies (9B11, Cell Signaling) conjugated to protein A sepharose. Beads were washed 8 times with NET-2 buffer (0.05% Triton X-100, 50 mM Tris-HCl pH 7.5, 150 mM NaCl) and placed in 20 μl of SDS load buffer (100 mM Tris-HCl (ph 6.8), 4 % SDS, 0.2% bromophenol blue, 20% glycerol). Samples were analyzed by SDS-PAGE and Western blot.

siRNA target sequences (Dharmacon)

Xrn1 #1: 5’ GUCAUGGCAAGGAGUUACCAU 3’ + 5’ GGUAACUCCUUGCCAUGACUU 3’

Xrn1 #2: 5’ AGAUGAACUUACCGUAGAAUU 3’ + 5’ UUCUACGGUAAGUUCAUCUUU 3’

Upf1: 5’ CCAAGAUGCAGUUCCGCUCCAUU 3’ + 5’ UGGAGCGGAACUGCAUCUUGGUU 3’

Smg6: 5’ AGGAGGAAGUUGCGAAUAAUU 3’ + 5’ UUAUUCGCAACUUCCUCCUUU 3’

FISH Probes (5’ end labeled with Texas-red; Invitrogen)

ß-globin

ßgl Ex 1-1: 5’ TAACGGCAGACTTCTCCTCAGGAGTCAGGTGCACCATGGTGTCTGTTTGA 3’

ßgl Ex 1-2: 5’ TGCCCAGGGCCTCACCACCAACTTCATCCACGTTCACCTTGCCCCACAGG 3’

ßgl Ex 2-1: 5’ CATAACAGCATCAGGAGTGGACAGATCCCCAAAGGACTCAAAGAACCTCT 3’

ßgl Ex 2-2: 5’ GAAGTTCTCAGGATCCACGTGCAGCTTGTCACAGTGCAGCTCACTCAGTG 3’

ßgl Ex 3: 5’ TAGTTGGACTTAGGGAACAAAGGAACCTTTAATAGAAATTGGACAGCAAG 3’

GPx-1

GPx1 Ex1:

5’ GCGCGCGGAGAAGGCATACACGGTGGACTGTGCCACCGCGGAGAGCCGAG 3’

GPx1 Ex 2:

5’ ACAGCAGGGCTTCTATATCGGGTTCGATGTCGATGGTGCGAAAGCGCCTG 3’

Supplemental Figure 1

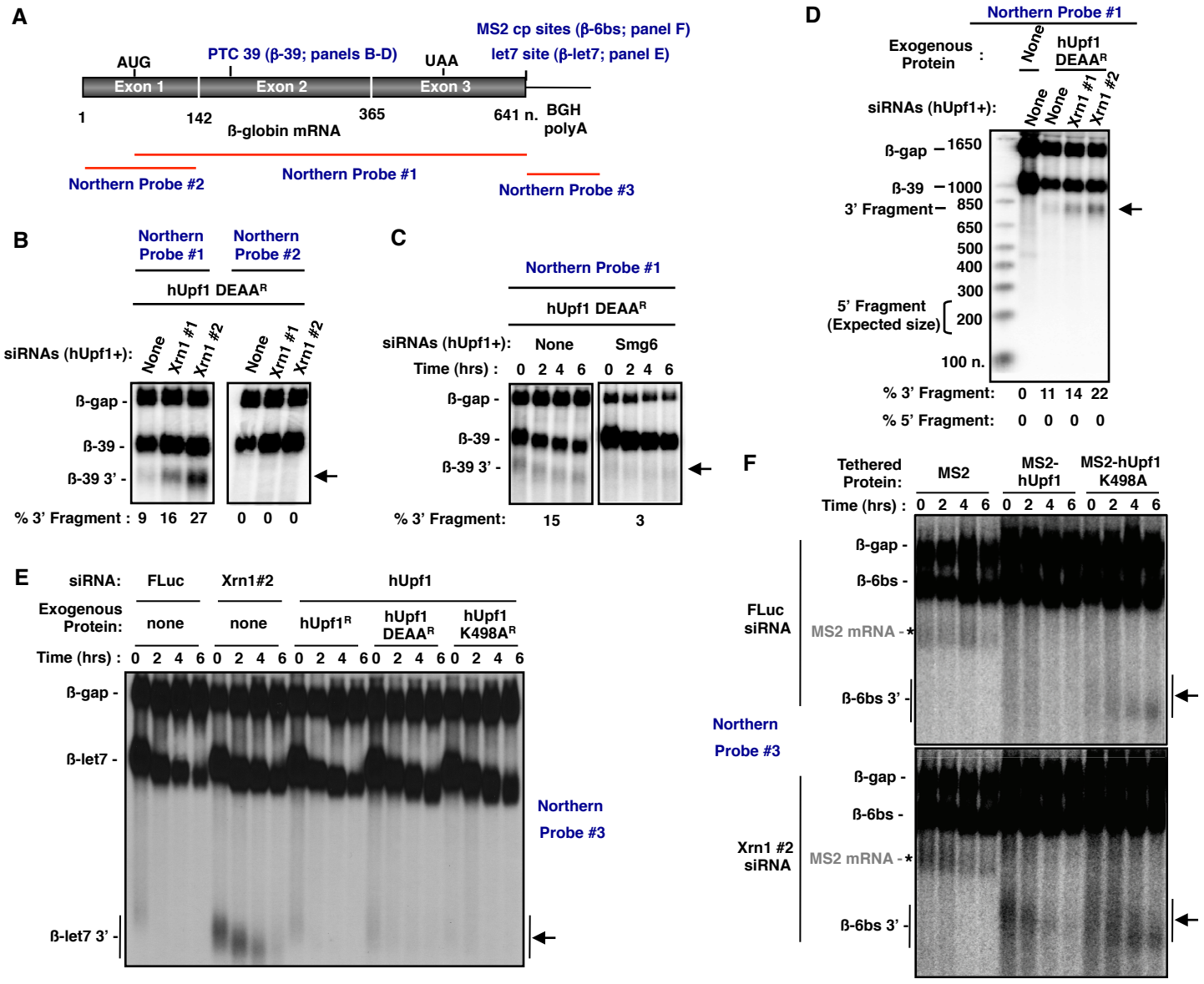


Figure S1, related to Figure 1. The hUpf1 ATPase mutant-dependent NMD intermediate is the 3' endonucleo-lytic fragment generated by Smg6, is specific to the NMD pathway and also occurs upon hUpf1 tethering.

(A) Schematic showing the used β-globin reporter mRNAs and Northern probes used to detect the corresponding full-length (Northern Probe #1), 5' ends (Northern Probe #2) or 3' ends (Northern Probe #3) of the reporters.

(B) Northern blots showing the detection of a faster migrating band upon hUpf1 ATPase mutant (hUpf1 DEAA) expression in the presence of no siRNA (None), Xrn1 #1 siRNA, or Xrn1 #2 siRNA with a probe against the full-length ß-39 mRNA (left panel) but not a probe against the 5' end (right panel) of the ß-39 mRNA. β-gap is an internal control mRNA. Quantifications of the amount of 3' fragment (in percent of total ß-39 mRNA) observed in each lane are shown below.

(C) Northern blot showing the disappearance of the faster migrating band in cells expressing hUpf1 DEAA when cells are treated with a Smg6 siRNA (right panel), but not when cells are treated with no siRNA (left panel). Note: The left panel is the same as shown in Figure 1A (top panel). The right panel was run on the same gel, but was not included in Figure 1A. Quantifications of the percent 3' fragment observed in the '0 hour' lanes are shown below.

(D) Northern blot showing no detectable accumulation of the 5' ß-39 mRNA fragment when no exogenous hUpf1 protein (None) or hUpf1 DEAA protein is expressed in the presence of no Xrn1 siRNA (None), Xrn1 #1 siRNA or Xrn1 #2 siRNA. Quantifications of the percent 3' and percent 5' fragment observed in each lane are shown below.

(E) Northern blots showing the detection of a 3' decay intermediate in pulse-chase mRNA decay assays in HeLa Tet-off cells expressing β-globin mRNA containing a sequence perfectly complementary to endogenous let-7 miRNA, upon Xrn1 knock-down, but not upon expression of hUpf1 proteins.

(F) Northern blots showing the detection of a 3' decay intermediate in pulse-chase mRNA decay assays in HeLa Tet-off cells of β-globin mRNA tethered, via MS2 coat protein, to hUpf1 K498A (right lanes). Tethered wild-type hUpf1 only generates the 3' fragment upon Xrn1 knockdown (middle panel). *: the mRNA encoding unfused MS2 coat protein hybridizes with the Northern probe.

Supplemental Figure 2

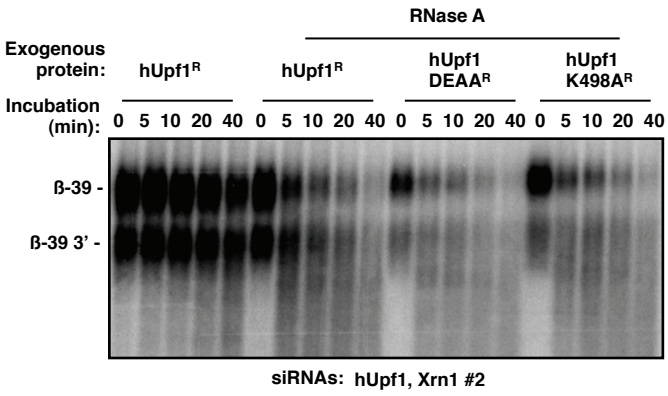


Figure S2, related to Figure 2.
Northern blot monitoring the degradation in the absence or presence of RNase A (as indicated) of β-39 mRNA and β-39 mRNA 3' fragment in extracts from HeLa Tet-off cells treated with siRNAs against hUpf1 and Xrn1 and expressing exogenous wild-type or mutant hUpf1 proteins from siRNA resistant mRNAs, as indicated.

Supplemental Figure 3

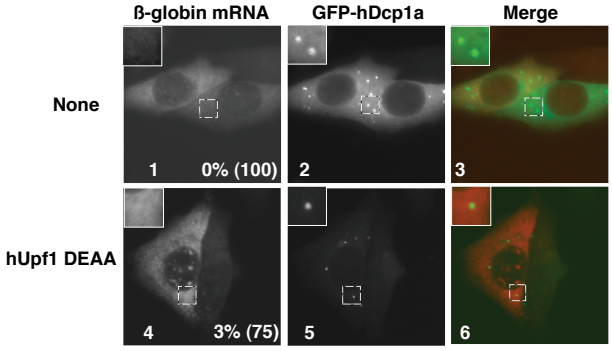
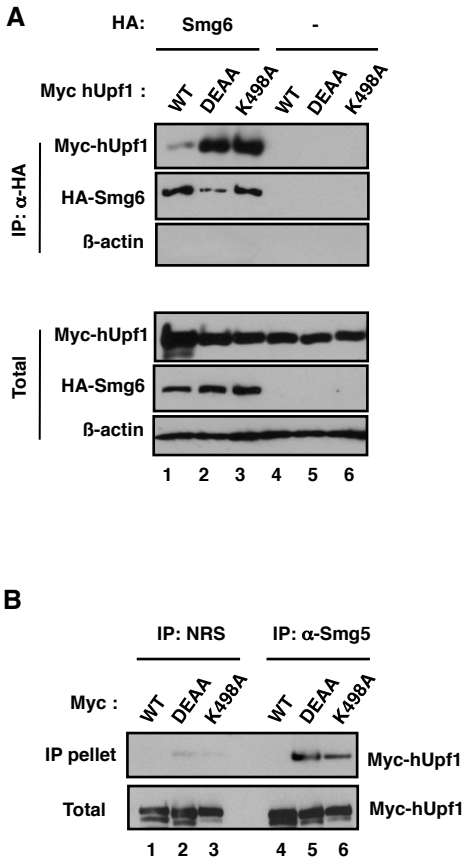


Figure S3, related to Figure 3. Wild-type ß-globin mRNA accumulates at low levels in PBs upon hUpf1 DEAA expression.

FISH assays showing the localization of wild-type ß-globin mRNA in the presence of no exogenous hUpf1 protein (panel 1-3) or hUpf1 DEAA (panel 4-6). GFP-hDcp1a was used as a processing body marker (panels 2 and 5). Merged images are shown in panes 3 and 6 (β-globin mRNA, red; GFP-hDcp1a, green). The percentage of cells which contained detectable quantities of ß-globin mRNA in PBs is shown with the number of cells quantified in parenthesis. An enlarged image corresponding to the dotted box is displayed in the upper left corner of each image.

Supplemental Figure 4



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(A) Western blots of anti-HA immunoprecipitates (top panels) from extracts of HEK 293T cells co-expressing Myc-tagged wild-type (WT) or ATPase mutant (DEAA, K498A) hUpf1 proteins with HA-tagged Smg6 or no HA tagged protein as indicated. Extracts were not treated with RNase. 5% of total extracts are shown in the bottom panels.

(B) Western blots of anti-Smg5 immunoprecipitates (panels 5-8) or immunoprecipitates using normal rabbit serum (NRS; panels 1-4) from extracts of HEK 293T cells expressing Myc-tagged wild-type (WT) or ATPase mutant (DEAA, K498A) hUpf1, or TTP as indicated. The bottom panel shows 5% of the total input extract.