A post-translational regulatory switch on UPF1 controls targeted mRNA degradation

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Nonsense-mediated mRNA decay (NMD) controls the quality of eukaryotic gene expression and also degrades physiologic mRNAs. How NMD targets are identified is incompletely understood. A central NMD factor is the ATP-dependent RNA helicase upframeshift 1 (UPF1). Neither the distance in space between the termination codon and the poly(A) tail nor the binding of steady-state, largely hypophosphorylated UPF1 is a discriminating marker of cellular NMD targets, unlike for premature termination codon (PTC)-containing reporter mRNAs when compared with their PTC-free counterparts. Here, we map phosphorylated UPF1 (p-UPF1)-binding sites using transcriptome-wide footprinting or DNA oligonucleotide-directed mRNA cleavage to report that p-UPF1 provides the first reliable cellular NMD target marker. p-UPF1 is enriched on NMD target 3′ untranslated regions (UTRs) along with suppressor with morphogenic effect on genitalia 5 (SMG5) and SMG7 but not SMG1 or SMG6. Immunoprecipitations of UPF1 variants deficient in various aspects of the NMD process in parallel with Förster resonance energy transfer (FRET) experiments reveal that ATPase/helicase-deficient UPF1 manifests high levels of RNA binding and disregulated hyperphosphorylation, whereas wild-type UPF1 releases from nonspecific RNA interactions in an ATP hydrolysis-dependent mechanism until an NMD target is identified. 3′ UTR-associated UPF1 undergoes regulated phosphorylation on NMD targets, providing a binding platform for mRNA degradative activities. p-UPF1 binding to NMD target 3′ UTRs is stabilized by SMG5 and SMG7. Our results help to explain why steady-state UPF1 binding is not a marker for cellular NMD substrates and how this binding is transformed to induce mRNA decay.

[Keywords: UPF1; ATP-dependent RNA helicase; nonsense-mediated mRNA decay; phosphorylation; RIP-seq of transcriptome-binding sites for phospho-UPF1; mRNP composition]

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Nonsense-mediated mRNA decay (NMD) in human cells degrades newly synthesized mRNAs that are aberrant because they contain a premature termination codon (PTC) and thereby have the potential to encode a toxic truncated protein [Karam et al. 2013; Popp and Maquat 2013, 2014; Yamashita 2013]. NMD also degrades ~5%–10% of naturally occurring mRNAs, often as a means of maintaining cellular homeostasis [Huang and Wilkinson 2012; Schweingruber et al. 2013] or regulating developmental processes that include axon guidance, synaptic strength, and neuronal expression [Giorgi et al. 2007; Colak et al. 2013]. These functions explain at least in part the intellectual disabilities that typify NMD factor deficiencies [Nguyen et al. 2012, 2013].

In mammals, NMD largely targets newly synthesized mRNAs during a pioneer round of translation [Maquat et al. 2010; Trcek et al. 2013]. Depending on the rates at which newly synthesized transcripts are translated and maintain features that are recognized by the NMD machinery, NMD can degrade mRNAs bound at their caps by the cap-binding protein [CBP] heterodimer CBP80 and CBP20 or eukaryotic translation initiation factor 4E [eIF4E] [Ishigaki et al. 2001; Hosoda et al. 2005; Matsuda et al. 2007; Woeller et al. 2008; Sato and Maquat 2009; Durand and Lykke-Andersen 2013; Rufener and Mühlemann

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2013]. CBP80 promotes NMD [Hosoda et al. 2005; Hwang et al. 2010], as do exon junction complexes [EJC], which reside ~20–24 nucleotides [nt] upstream of ~80% of exon–exon junctions [Le Hir et al. 2000; Saulière et al. 2012; Singh et al. 2012]. EJCs often consist of the NMD factors upframeshifit 2 [UPF2] and UPF3 or UPF3X [also called UPF3a or UPF3b, respectively] as well as additional proteins [Popp and Maquat 2013, 2014; Yamashita 2013]. The essential splicing factor complexed with Cef1 protein phosphatase 2A (PP2A) [Anders et al. 2003; Chiu et al. 2003; Hogg and Goff 2010; Hurt et al. 2013; Kurosaki and Maquat 2013; Zünd et al. 2013; Gregersen et al. 2014]. Furthermore, while data indicate that UPF1 helicase activity remodels NMD targets during the decay process [Franks et al. 2010], assays of steady-state UPF1 binding to cellular RNA cannot differentiate NMD targets from mRNAs that are not NMD targets [Zünd et al. 2013; Gregersen et al. 2014].

To address these issues, we demonstrate using Förster resonance energy transfer [FRET] experiments and also immunoprecipitations of UPF1 from cellular lysates that UPF1 uses ATP hydrolysis as a means to dissociate from cellular RNAs. UPF1 variants [C126S or R843C] lacking RNA-binding ability are hypophosphorylated. However, a UPF1 variant [G495R/G497E] that lacks ATPase and helicase activities but retains the ability to bind RNA accumulates on RNA and becomes hyperphosphorylated, presumably as a consequence of its failure to dissociate. We conclude that ATP binding and hydrolysis generally modulate UPF1–RNA associations, which in turn regulate cellular UPF1 phosphorylation and NMD.

In addition to its quality control function, NMD also regulates many cellular processes, presumably because it controls the levels of many nonmutated cellular transcripts [Mendell et al. 2004; Wittmann et al. 2006; Viegas et al. 2007; Yepiskoposyan et al. 2011; Tani et al. 2012]. Genetic depletion studies by design identify both direct and indirect NMD targets, complicating our understanding of how NMD controls these processes. Alternative large-scale efforts to map steady-state UPF1-binding sites to the transcriptome have failed to identify endogenous NMD substrates. Here we map physiologic p-UPF1-binding sites on the cellular transcriptome using deep sequencing [RNA immunoprecipitation [RIP] plus sequencing [RIP-seq]] and show that, unlike UPF1, p-UPF1 binding is a reliable marker of NMD targets. We demonstrate that p-UPF1 binding is accompanied by SMG5 and SMG7 binding, which stabilize p-UPF1 on the 3’ UTRs of NMD targets. While PP2A phosphatase indeed reduces the degree of UPF1 phosphorylation, we unexpectedly found that PP2A is not enriched on NMD targets and does not detectably influence the efficiency of NMD. While other interpretations are possible, it may be that p-UPF1 is dephosphorylated after mRNA decay is initiated. Taken together, our data indicate that p-UPF1 provides the first discriminating mark of NMD targets whose generation is tightly controlled by an ordered series of events that constitute the NMD pathway.

Results

p-UPF1, unlike UPF1, marks cellular NMD targets

Steady-state—i.e., largely hypophosphorylated—UPF1 binding to cellular transcripts cannot be used as an identifier of NMD targets [Zünd et al. 2013; Gregersen et al. 2014; see below]. Reasons for this are likely to be multifaceted. Steady-state UPF1 may manifest a sufficient degree of nonspecific binding to RNA to partially
mask regulated UPF1 binding to NMD targets [Hurt et al.
2013; Zünd et al. 2013; Gregersen et al. 2014; see below].
Additionally, the ability of ribosomes to remove UPF1
from mRNA 5’ UTRs and coding regions leaves 3’ UTRs
as the primary sites of UPF1 binding [Hurt et al. 2013;
Zünd et al. 2013; Gregersen et al. 2014]; however, 3’ UTR
length cannot be reliably used to identify NMD targets
[Tani et al. 2012; Hurt et al. 2013] because other hall-
marks, such as the presence of a 3’ UTR EJC [Hurt et al.
2013; Kurosaki and Maquat 2013], and/or variables, such
as how a 3’ UTR folds [Eberle et al. 2008], must be
considered.

Since degradation of NMD targets involves UPF1 phos-
phorylation [Kashima et al. 2006; Isken et al. 2008], we
asked whether assays of p-UPF1 could identify cellular
NMD targets by mapping transcriptome-wide p-UPF1-
binding sites in vivo. We used an antigen-purified anti-p-
UPF1(S1116) antibody to isolate transcripts associated
with p-UPF1 from okadaic acid-treated human embryonic
kidney 293T (HEK293T) cells. Seven lines of evidence
justify using p-UPF1(S1116), which to our knowledge is
the only available antibody capable of efficiently immu-
noprecipitating p-UPF1 [see the Supplemental Material
for details], as a highly specific assay for p-UPF1 function
during NMD [Supplemental Fig. S1A–G]. Under the
conditions used, okadaic acid generated an approximately
fivefold accumulation of cellular p-UPF1 [Supplemental
Fig. S1F].

We immunoprecipitated cell lysates using anti-p-UPF1
(S1116) or, as a control, rabbit IgG [rgG] [Fig. 1A] and
subsequently generated RNA fragments that were pri-
marily <100 nt using limited RNase I digestion. As a
second control [Ctl], cell lysates were generated without
immunoprecipitation or RNase I treatment. Immunopre-
cipitated complexes were eluted from antibody-bound
beads using denaturing buffer. Eluted RNA fragments
were subjected to denaturing polyacrylamide gel electro-
phoresis, and those of ~25–40 nt were purified for cDNA
library construction [Fig. 1].

We obtained 1.6 million to 9.7 million single-end reads
from each of the six cDNA libraries; i.e., biological
replicates of three libraries. Of these, ~20%–25% of the
reads in Ctl and p-UPF1 samples could be mapped to a
unique genomic sequence, in contrast to only ~2% of the
reads in rgG samples, indicating low nonspecific
binding of RNAs to rgG. We plotted the ratio of RNA
fragment abundance [reads per kilobase per million
mapped reads [RPKM]] in p-UPF1 samples to those in
Ctl samples using what we call bona fide NMD targets.
These bona fide NMD targets were defined based on
a minimum of two criteria: up-regulation upon UPF1
down-regulation plus a longer half-life upon UPF1 down-
regulation [Supplemental Table S1]. Results demon-
strated that these NMD targets had significantly more
p-UPF1 binding compared with other transcripts [P = 9 ×
10−7, Kolmogorov–Smirnov test] [Fig. 1B]. In contrast,
the level of steady-state UPF1 binding to the same bona fide
NMD targets using data provided in Gregersen et al.
[2014] or Zünd et al. [2013] failed to show significantly
more UPF1 binding compared with other transcripts
[Supplemental Fig. S1H,J]. An enrichment of p-UPF1
binding similar to what we observed for these bona fide
NMD targets was obtained using putative NMD targets
defined by Tani et al. [2012] [group B], which were defined
based on an elongated half-life upon UPF1 down-regulation
[Fig. 1C], Mendell et al. [2004] or Yepiskoposyan et al. [2011],
which were defined based on an increase in abundance upon
UPF1 down-regulation [Fig. 1D,E], orYepiskoposyan et al.
[2011], which were defined based an increase in abundance
upon SMG6 or SMG7 down-regulation [Supplemental Fig.
S1J,K]. However, for unclear reasons, putative NMD targets
defined by Viegas et al. [2007] or Wittmann et al. [2006]
based on, respectively, UPF1 or UPF2 down-regulation were
not enriched in p-UPF1 binding [Supplemental Fig. S1L,M].

Following our global analysis of p-UPF1 binding to the
HEK293T cell transcriptome using RIP-seq, we examined
the distribution of p-UPF1 on selected bona fide NMD
targets. After subtracting background p-UPF1 binding,
data revealed that p-UPF1 is enriched on those NMD
target 3’ UTRs examined [Fig. 1F–K; data not shown for
other NMD targets]. This pattern of p-UPF1 binding
requires mRNA translation, without which only insig-
nificant levels of p-UPF1 binding are observed [see below].

Cell cycle-regulated histone mRNAs and Staufen-me-
diated mRNA decay [SMD] targets might also be expected
to be enriched in the p-UPF1 RIP-seq [Maquat and
Schoeneng and Maquat 2012]. While histone cluster 1
H1e [HIST1H1E] and the JUN proto-oncogene SMD target
are enriched, HIST1H1C, HIST2H2BE, and HIST1H1D
mRNAs as well as SMD targets ADP ribosylation factor 1
and ring finger protein168 mRNAs are only slightly
enriched, and other cell cycle-regulated histone mRNAs
and SMD targets are not enriched. Possibly, detectable
enrichment of histone mRNAs requires cell synchroni-
zation and accumulation at the end of S phase, when
histone mRNAs are degraded, and detectable enrichment
of SMD targets requires a cell type that supports SMD
more efficiently than do HEK293T cells [Ricci et al. 2014;
our unpublished data].

NMD targets are enriched in p-UPF1, SMG5,
and SMG7 but not UPF2, UPF3X, SMG1, SMG6,
eRF1, or eRF3

Our finding that p-UPF1 is enriched on NMD targets led
us to ask whether other mRNA proteins are likewise
enriched. Before assaying other proteins, we initially
tested the prediction, deriving from our RIP-seq data
[Fig. 1], that p-UPF1 would be enriched on the 3’ UTRs
of β-globin [Gl] Ter [i.e., PTC-containing] mRNA and
glutathione peroxidase 1 (GPx1) Ter mRNA compared
with their corresponding PTC-free counterparts. We pre-
pared lysates of HEK293T cells transiently expressing
either GI Norm mRNA + GPx1 Ter mRNA or GI Ter
mRNA + GPx1 Norm mRNA [Fig. 2A], each together
with mRNA that encodes the major urinary protein
[MUP] and controls for variations in cell transfection
efficiencies and RNA recovery [Kurosaki and Maquat
2013]. A fraction of each cell lysate was immunoprecip-
itated using anti-UPF1, which reacts with all of cellular
UPF1 regardless of its phosphorylation status (Supplemental Fig. S1F,G), or anti-p-UPF1(S1116).

Western blotting revealed that for each sample, anti-UPF1 immunoprecipitated comparable amounts of UPF1 (Fig. 2B), and anti-p-UPF1(S1116) immunoprecipitated comparable amounts of p-UPF1(S1116) (Fig. 2C). RT–PCR demonstrated that the levels of Gl and GPx1 Ter mRNAs were, respectively, ~40% and ~30% of the corresponding PTC-free mRNAs prior to immunoprecipitation (Fig. 2B, C). RT–qPCR data, see Fig. 2E, F). An approximately fivefold to sixfold increase in UPF1 binding to PTC-containing compared with PTC-free mRNA was also found after anti-HA immunoprecipitation of lysates of cells coexpressing HA-tagged MS2 coat protein and either Gl Norm mRNA or Gl Ter mRNA, each harboring six copies of the MS2 coat protein-binding site in its 3′ UTR (Supplemental Fig. S2A,B). Note that here we are comparing UPF1 binding to a PTC-containing transcript with UPF1 binding to its PTC-free counterpart, and the first transcript differs from the latter by having a longer 3′ UTR and a 3′ UTR EJC that cumulatively enhance UPF1 binding (Kurosaki and Maquat 2013). This comparison is distinct from the comparison of cellular mRNAs that are and are not NMD targets—a comparison that failed to show total cell UPF1 enrichment on NMD targets (Zünd et al. 2013; Gregersen et al. 2014) partly because the
Figure 2. p-UPF1, SMG5, and SMG7 preferentially bind to PTC-containing mRNAs compared with their PTC-free counterparts. (A) Diagrams of spliced Gl and GPx1 PTC-free [Norm] and Gl and GPx1 PTC-containing [39 Ter and 46 Ter, respectively] mRNAs. Boxes represent coding regions, vertical lines within boxes show spliced junctions, and horizontal lines denote UTRs. (B) HEK293T cells (8 \times 10^7 per 150-mm dish) were transiently transfected with 2 \mu g of phCMV-MUP and either 4 \mu g of pmCMV-Gl Norm + 4 \mu g of pmCMV-GPx1 Ter or 4 \mu g of pmCMV-Gl Ter + 4 \mu g of pmCMV-GPx1 Norm. Immunoprecipitations of lysates were performed using anti-UPF1 [\alpha-UPF1]. (Top) Western blotting (WB) before (-) or after immunoprecipitation (IP) using anti-UPF1 or, as a control for nonspecific immunoprecipitation, normal rabbit serum (NRS), where lanes below the wedge analyze serial threefold dilutions of lysate. (Bottom) RT–PCR, where the level of Gl mRNA or GPx1 mRNA before and after immunoprecipitation was normalized to the level of MUP mRNA, the normalized level after immunoprecipitation was calculated as a ratio of the normalized level before immunoprecipitation, and the ratio for Gl Norm mRNA or GPx1 Norm mRNA is defined as 100%. Lanes below the wedge analyze serial twofold dilutions of lysate RNA. (C) As in B, only immunoprecipitation was performed using anti-p-UPF1[S1116] or, as a control, rlgG. (D) As in B, only immunoprecipitation was performed using anti-SMG5. (E) As in B, except immunoprecipitation was performed using anti-SMG7. (F) RT-qPCR of Gl mRNA from samples analyzed in B–E and Supplemental Figure S2. (G) As in F for GPx1 mRNA. All quantitations derive from three to four independently performed experiments and represent the mean plus standard deviations.
NMD-inducing features of the bulk of cellular NMD targets are often diverse and undefined.

Anti-p-UPF1[\text{S1116}] immunoprecipitations demonstrated that Gl and GPx1 Ter mRNAs coimmunoprecipitated approximately fourfold to fivelofold more efficiently with p-UPF1 than did their PTC-free counterparts. Based on the finding that Gl and GPx1 Ter mRNAs coimmunoprecipitated with SMG5 and SMG7 approximately twofold to threefold more efficiently than their PTC-free counterparts [Fig. 2D-G], at least some of this p-UPF1 appears to be bound by SMG5 and SMG7, each of which preferentially associates with cellular p-UPF1 [Ohnishi et al. 2003; Okada-Katsuhata et al. 2012; Jonas et al. 2013; Loh et al. 2013]. We conclude that, under the conditions analyzed, steady-state UPF1 and p-UPF1 preferentially bind PTC-containing NMD targets relative to their PTC-free counterparts to the same order of magnitude.

Like SMG5 and SMG7, UPF2, UPF3X, SMG1, eRF1, and eRF3 form complexes with UPF1 at various stages of the NMD pathway [Serin et al. 2001; Kadlec et al. 2004, 2006; Clerici et al. 2009; Kashima et al. 2006]. However, unlike SMG5 and SMG7, each protein was not enriched on PTC-containing relative to PTC-free mRNAs (the slight 1.3–1.4-fold increase observed with UPF3X was not pursued) [Fig. 2F,G; Supplemental Fig. S2C-H]. In control experiments, CBP20 was also not enriched on PTC-containing mRNAs [Fig. 2F,G; Supplemental Fig. S2F], as expected. As additional controls, p-UPF1, SMG5, and SMG7, unlike UPF2, UPF3X, SMG1, eRF1, PP2Ac, or CBP20, were enriched on endogenous mRNA for growth arrest and DNA damage-inducible 45A [GADD45A], which is an NMD target [Mendell et al. 2004; Wittmann et al. 2006; Viegas et al. 2007; Tani et al. 2012], compared with endogenous β-actin mRNA, which is not an NMD target [Supplemental Fig. S2I]. Under the conditions used, neither PTC-containing nor PTC-free mRNAs detectably coimmunoprecipitated with SMG6 (see the Discussion; data not shown), which also forms a complex with UPF1 [Chiu et al. 2003; Eberle et al. 2009; Franks et al. 2010].

**SMG1 is not required for preferential UPF1 binding to PTC-containing vs. PTC-free mRNAs**

Since anti-UPF1 measures both hypophosphorylated UPF1 and p-UPF1, our finding that UPF1 binding and p-UPF1 binding are enhanced to the same extent on PTC-containing mRNAs does not tell us whether enhanced UPF1 binding occurs before or after SMG1-mediated UPF1 phosphorylation. Furthermore, the degrees to which other mRNP constituents influence UPF1 or p-UPF1 binding have never been characterized. We therefore down-regulated to <15% of their normal levels individual mRNA-associated proteins that are known to form a complex with UPF1 either before or after UPF1 phosphorylation [Fig. 3A,B]. We then used RT-qPCR to quantitate the levels of Gl and GPx1 Norm or Ter mRNAs before and after immunoprecipitation using anti-UPF1 or, as a control for nonspecific immunoprecipitation, normal rabbit serum [NRS] [Fig. 3C,D], where NMD efficiencies were much higher than in Fig. 2, making the level of UPF1-binding to PTC-containing vs. PTC-free mRNA higher than in Fig. 2).

Analyses of samples after immunoprecipitation revealed that the coimmunoprecipitation [co-IP] of Gl and GPx1 Ter mRNAs with UPF1 was reduced approximately twofold by UPF2 siRNA, approximately threefold to fourfold by UPF3X siRNA, approximately fivefold by eRF4A3 siRNA, or approximately threefold by eRF3 siRNA when compared with their co-IP in the presence of Ctl siRNA [Fig. 3C,D]. These findings are consistent with our previous results demonstrating that UPF1 binding to an NMD target 3’ UTR is augmented by the interaction between UPF1 and eRF3 at a PTC and between UPF1 and a 3’ UTR EJC [Kurosaki and Maquat 2013]. Our finding that SMG1 siRNA failed to influence UPF1 binding to either NMD target [Fig. 3C,D] may indicate that cellular UPF1 phosphorylation is not required for enhanced UPF1 binding to PTC-containing versus PTC-free mRNAs [see below] and suggests that hypophosphorylated UPF1 initially binds to NMD target 3’ UTRs. In contrast, the co-IP of Gl and GPx1 Ter mRNAs with UPF1 was reduced approximately twofold to threefold by SMG5 siRNA. Considering that SMG5 is enriched on NMD targets [Fig. 2D,F,G], SMG5 may physically stabilize UPF1 binding to NMD targets—in particular the p-UPF1 fraction of cellular UPF1 [see below]. In support of these data and the conclusions that we draw from them, similar but less dramatic results were obtained when the co-IP of GADD45A mRNA with UPF1 was quantitated [relative to co-IP of β-actin mRNA with UPF1] in the presence of siRNA to each of the six mRNA-associated proteins [Supplemental Fig. S3A].

**SMG5 and SMG7 augment UPF1 and p-UPF1 binding to NMD targets**

To further investigate the finding that SMG5 siRNA reduces UPF1 binding to Gl and GPx1 Ter mRNAs, we undertook similar assays of UPF1 binding in the presence of SMG6 siRNA, SMG7 siRNA, or PP2Ac siRNA, the latter of which down-regulates the catalytic subunit of PP2A [Fig. 3E,F]. Results revealed that SMG7 but neither SMG6 nor PP2Ac significantly contributes to the preferential binding of UPF1 to PTC-containing mRNAs [Fig. 3G,H] or the GADD45A cellular NMD target [Supplemental Fig. S3B]. Since SMG5 and SMG7 form a stable heterodimer that is critical for NMD [Ohnishi et al. 2003; Okada-Katsuhata et al. 2012; Jonas et al. 2013; Loh et al. 2013] and disrupting the SMG5–SMG7 heterodimer greatly reduces SMG5 binding to UPF1 [Loh et al. 2013], our finding that either SMG5 siRNA or SMG7 siRNA reduces UPF1 binding to an NMD target suggests that it is the heterodimer that associates with and enhances the binding of UPF1 to PTC-containing mRNAs.

Given that SMG5 and SMG7 interact with p-UPF1, we next asked whether SMG5 and/or SMG7 enhance(s) p-UPF1 binding to NMD targets. Using lysates of cells in which SMG5, SMG6, or SMG7 was down-regulated to <10% of normal [Fig. 3I], after anti-p-UPF1[\text{S1116}]
immunoprecipitation (Fig. 3J), SMG5 and SMG7, unlike SMG6, were found to contribute to p-UPF1 binding to Gl and GPx1 Ter mRNAs (Fig. 3K,L). We propose from these results and similar results obtained in assays of cellular GADD45A compared with β-actin mRNA (Supplemental Fig. S3C) that the SMG5–SMG7 heterodimer stabilizes p-UPF1 binding to an NMD target.

**Figure 3.** SMG5 and SMG7 augment p-UPF1 binding to NMD targets. (A) Western blotting prior to |−| immunoprecipitation (IP) of lysates of HEK293T cells (5 × 10⁶ per well of six-well plate) transiently transfected with 60 pmol of the specified or Ctrl siRNA per well and, 1 d later, 0.4 μg of pmCMV Gl Norm or Ter, 0.4 μg of pmCMV-GPx1 Norm or Ter, and 0.2 μg of phCMV-MUP. (B) As in A only after immunoprecipitation using anti-UPF1 (α-UPF1) or, as an immunoprecipitation control, NRS. (C) RT-qPCR of Gl mRNA as in Figure 2F. The ratio for Gl Norm mRNA in Ctrl siRNA down-regulation is defined as 100%. (D) RT-qPCR of GPx1 mRNA as in C. (E) As in A using the specified siRNAs. (F) As in B using the specified siRNAs. (G) As in C. (H) As in D. (I) As in A using the specified siRNAs. (J) As in B using the specified immunoprecipitations and siRNAs. (K) Gl mRNA was quantitated as in C but normalized to the level of immunoprecipitated p-UPF1 as exemplified in J. (L) As in K but for GPx1 mRNA. Quantitations derive from three independently performed experiments and represent the mean plus standard deviations.

immunoprecipitation [Fig. 3J], SMG5 and SMG7, unlike SMG6, were found to contribute to p-UPF1 binding to Gl and GPx1 Ter mRNAs [Fig. 3K,L]. We propose from these results and similar results obtained in assays of cellular GADD45A compared with β-actin mRNA [Supplemental Fig. S3C] that the SMG5–SMG7 heterodimer stabilizes p-UPF1 binding to an NMD target.

**DNA oligo-directed RNase H cleavage demonstrates that p-UPF1 binds NMD target 3′ UTRs**

UPF1 binds along the length of the 3′ UTRs of translationally active NMD targets [Hurt et al. 2013; Kurosaki and Maquat 2013; Zünd et al. 2013; Gregersen et al. 2014]. Thus, it seemed reasonable that the p-UPF1 that we
observed bound to Gl and GPx1 Ter mRNAs (Fig. 2C, F, G) would likewise reflect a translation-dependent 3' UTR association. To test this hypothesis and examine p-UPF1 distribution along the 3' UTR, we used HeLa cells that stably express IRE-Gl Ter mRNA (Fig. 4A), which harbors in its 5’ UTR the iron-responsive element [IRE] from ferritin heavy chain mRNA [Thermann et al. 1998; Sato and Maquat 2009, Kurosaki and Maquat 2013]. Cells were cultured for 1 d in medium containing the iron chelator deferoxamine mesylate [Df], which represses IRE-Gl.
mRNA translation, and were subsequently washed and cultured for 8 h in Df or hemin, the latter of which promotes IRE-Gl mRNA translation [Kuroasaki and Maquat 2013]. Lysates of cells cultured in the presence of hemin were subsequently immunoprecipitated using anti-UPF1 (or, as a control, NRS) or anti-p-UPF1(S1116) (or, as a control, rlgG). After extensive washing, UPF1-bound RNAs were incubated with RNase H in the absence or presence of one of three antisense DNA oligonucleotides (Fig. 4A) while associated with antibody–protein A-agarose beads. By so doing, cleavage products [CPs] that do not associate with UPF1 can be distinguished from those that do. However, this experimental approach does not quantify the amount of bound UPF1. IRE-Gl Ter mRNA 5′-CPs consist of 185, 320, or 434 nt and contain, respectively, 0, 114, or 228 nt of 3′ UTR (where 3′ UTR nucleotide 1 is defined as the nucleotide immediately downstream from Ter) (Fig. 4A). The corresponding 3′-CPs consist of 439, 303, or 190 nt, all of which derive from the 3′ UTR (except for the Oligo 1 3′-CP, which contains 5 nt of coding region plus Ter) (Fig. 4A).

In control experiments performed before immunoprecipitation, Df (but not hemin) increased the level of IRE-Gl Ter mRNA prior to RNase H-mediated cleavage [i.e., inhibited NMD] (Fig. 4B) and also blocked the production of CPs that do not associate with UPF1 can be distinguished from those that do. This is consistent with UPF1 removal from the 5′ end of decay intermediates that is primarily hypophosphorylated (Chiu et al. 2002; Kashima et al. 2006; Isken et al. 2008). Their relative phosphorylation status was found to be MYC-dependent Fig. S3), consistent with UPF1 phosphorylation occurring after UPF1 binding to an NMD target, and (3) effects on mRNP composition. Lysates were generated from HEK293T cells transiently expressing MYC alone; MYC-UPF1(WT), which is primarily hypophosphorylated [Chiu et al. 2003; Ohnishi et al. 2003; Isken et al. 2008; Okada-Katsuhata et al. 2012], or one of five MYC-UPF1 variants [Fig. 5A] that have been shown to inhibit NMD (Sun et al. 2003; Ohnishi et al. 2003; Isken et al. 2008; Okada-Katsuhata et al. 2012); or one of five MYC-UPF1 variants and analyzed their (1) phosphorylation status and helicase activities

Neither SMG1 siRNA nor PP2A siRNA detectably affect the differential binding of UPF1 to NMD targets compared with their PTC-free counterparts (Fig. 3; Supplemental Fig. S3), consistent with UPF1 phosphorylation occurring after UPF1 binding to an NMD target. To further examine how and when UPF1 phosphorylation occurs during NMD, we constructed a series of UPF1 variants and analyzed their [1] phosphorylation status using anti-p[S/T]Q, [2] relative binding to PTC-containing versus PTC-free mRNAs, and [3] effects on mRNP composition. Lysates were generated from HEK293T cells transiently expressing MYC alone; MYC-UPF1(WT), which is primarily hypophosphorylated [Chiu et al. 2003; Ohnishi et al. 2003; Isken et al. 2008; Okada-Katsuhata et al. 2012], or one of five MYC-UPF1 variants [Fig. 5A] that have been shown to inhibit NMD (Sun et al. 1998; Kashima et al. 2006; Isken et al. 2008). Their relative phosphorylation status was found to be MYC-
Figure 5. UPF1 recognition of NMD targets requires ATPase and helicase activities. (A) Diagrams of UPF1 variants denoting the CH region and the region containing the four serine–glutamine (SQ) SMG1 phosphorylation sites. Horizontal arrows denote amino acid changes at positions specified by downward-facing arrowheads. (B) HEK293T cells \(8 \times 10^7\) per 150-mm dish were transiently transfected with 0.6 \(\mu\)g of pmCMV-MYC-UPF1(WT), 2 \(\mu\)g of MYC-UPF1(C126S), 1 \(\mu\)g of MYC-UPF1(4SA), 0.5 \(\mu\)g of MYC-UPF1(dNT), 4 \(\mu\)g of MYC-UPF1(G495R/G497E), 0.8 \(\mu\)g of MYC-UPF1(R843C), or, as a negative control, 0.5 \(\mu\)g of pmCMV-MYC. Cell lysates were treated with (+) or without (−) RNase A and subsequently immunoprecipitated using anti-MYC (α-MYC). Western blotting before (−) or after (+) immunoprecipitation. For samples after immunoprecipitation, fivefold less immunoprecipitate was loaded in the analysis of MYC-UPF1 compared with the analysis of other proteins. (C) HEK293T cells were transiently transfected as described in B except that transfections included 0.5 \(\mu\)g of phCMV-MUP and 1 \(\mu\)g each of pmCMV-Gl and pmCMV-GPx1, either Norm or Ter. Cell lysates were immunoprecipitated using anti-MYC and analyzed by Western blotting. (D) RT-qPCR of full-length Gl or GPx1 mRNA from samples in C. The level of Gl or GPx1 mRNA before immunoprecipitation was normalized to the level of MUP mRNA, and, given the comparable immunoprecipitation efficiencies, the normalized level of Gl Norm or GPx1 Norm mRNA after immunoprecipitation that coimmunoprecipitated with MYC-UPF1(WT) is defined as 1. Diagrams are of Gl mRNA or GPx1 mRNA as in Figure 2A, and the arrows specify PCR primer positions. Quantitations derive from two to three independently performed experiments and represent the mean plus standard deviations.
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UPF1[G495R/G497E] >> MYC-UPF1[ΔNT] > MYC-UPF1

[WT] > MYC-UPF1[C126S] = MYC-UPF1[4SA] = MYC-

UPF1[R843C] [Fig. 5A,B, lanes 2–13; Supplemental Fig. S4A].

EJC-interacting UPF2 coimmunoprecipitated in a largely

RNase A-insensitive fashion with MYC-UPF1[WT] and all

variants except for MYC-UPF1[C126S] [Fig. 5B, lanes 2–13],

which harbors a point mutation in the cytochrome–
histidine-rich (CH) domain that disrupts UPF2 binding

[Chamieh et al. 2008; Chakrabarti et al. 2011; Shigeoka

et al. 2000; Chamieh et al. 2008; Chakrabarti et al. 2011; Bhattacharya

et al. 1998; Bhattacharya et al. 2000; Cheng et al. 2007; Isken et al. 2008] and its binding to mRNA was not

significantly enhanced by the presence of a PTC in either

Gl or GPx1 mRNA [Fig. 5C,D], we suggest that binding is

largely nonspecific. In support of this idea, the abnor-

mally elevated amounts of SMG5, SMG6, and SMG7 that

coimmunoprecipitated with MYC-UPF1[G495R/G497E] were

incapable of degrading mRNA. Assays of Escherichia

coli-produced human UPF1 [Supplemental Fig. S4D] re-

vealed that the G495R/G497E mutations inhibit UPF1

ATPase and helicase activities [Supplemental Fig. S4E,F].

FRET analyses showed that UPF1 is efficiently released

from RNA in the presence of ATP compared with ADP or

the nonhydrolyzable ATP analog AMP/PNP or ADP-

Be₃ [Supplemental Fig. S4G–I]. This was determined using human UPF1[295–914] that was site-specifically

modified by Sortase labeling [Popp et al. 2007] so that its

C terminus harbors a Cy5 dye [Supplemental Fig. S4G,H] and

generates a FRET signal when the Cy5 dye is in close

proximity to a donor Cy3-labeled 50-nt RNA [Supplemen-
	tal Fig. S4I]. Hence, ATP hydrolysis, rather than simply

ATP binding, leads to dissociation of UPF1 from RNA, in

keeping with the hypothesis that UPF1 is a helicase that

translocates along RNA in a 5′-to-3′ direction [Bhattacharya

e t al. 2000; Chamieh et al. 2008; Chakrabarti et al. 2011; Shigeoka et al. 2012; Park et al. 2013]. We suggest that the large decrease in FRET observed in the presence of ATP is
dueto movement of UPF1 away from the donor fluorophore
attached to RNA and/or dissociation of UPF1 from the 3′
end of donor-labeled RNA [see Supplemental Fig. S4] for
stop–flow kinetic FRET analyses.

As expected from the failure of mRNP constituents to
detectably coimmunoprecipitate with hypophosphorylated

MYC-UPF1[R843C] [Fig. 5B, lanes 12,13], Gl and GPx1

Norm and Ter mRNAs also failed to detectably coimmu-
noprecipitate [Fig. 5C,D]. Thus, the observed interaction

between MYC-UPF1[R843C] and EJC constituents likely
occurs while not bound to RNA. The R843C mutation

inhibits UPF1 helicase but not ATPase activity [Supple-

mental Fig. S4E,F]. Just as MYC-UPF1[G495R/G497E] is

hypophosphorylated because it fails to dissociate from
RNA, MYC-UPF1[R843C] is hypophosphorylated because it
fails to detectably bind RNA. Thus, the R843C mutation

apparently uncouples ATPase activity from RNA binding.

We conclude that, under the conditions used, UPF1

binds both PTC-containing and PTC-free mRNAs indis-
iminately in the absence of ATP and requires the ability
to cleave ATP to recognize an NMD target. The enhanced

binding of UPF1 to PTC-containing relative to PTC-free

mRNAs requires that UPF1 have an intact CH domain,

which allows interaction with EJCs, and also possess

helicase activity, which requires ATP hydrolysis [Weng

et al. 1998; Bhattacharya et al. 2000; Cheng et al. 2007; Chamieh et al. 2008; Chakrabarti et al. 2011; Shigeoka

et al. 2012; Park et al. 2013].
**PP2Ac depletion up-regulates the level of p-UPF1 without inhibiting NMD**

Current models indicate that PP2A is responsible for dephosphorylating p-UPF1 during NMD despite the absence of data showing that PP2A directly contributes to mRNA-degradative activities and/or restores UPF1 to its largely hypophosphorylated steady state (Anders et al. 2003; Chiu et al. 2003; Ohnishi et al. 2003; Kashima et al. 2006). To assay for PP2A function in NMD, three individual siRNAs targeting PP2Ac were separately used to down-regulate the level of PP2Ac to <10%–25% of normal without affecting the level of cellular UPF1 but increasing the level of cellular p-UPF1 approximately twofold to fourfold (Fig. 6A). Remarkably, no PP2Ac siRNA inhibited the NMD of Gl or GPx1 Ter mRNA (Fig. 6B,C) or endogenous NMD targets GADD45A or GADD45B mRNA (Fig. 6D). In striking contrast, down-regulating SMG1 to ~10% of normal, which decreased the level of cellular p-UPF1 ~2.5-fold, efficiently inhibited the NMD of the two reporter and the two endogenous NMD targets [Fig. 6B–D]. Furthermore, immunoprecipitations using anti-PP2Ac revealed that PP2Ac is not enriched on Gl or GPx1 Ter mRNA [Fig. 6E] or GADD45A mRNA [Supplemental Fig. S2I]. While it is possible that, despite siRNA-mediated down-regulation, residual levels of PP2Ac were sufficient to fully support NMD and/or that another phosphatase functionally replaced PP2Ac, PP2Ac may only indirectly function in NMD via its role in p-UPF1 recycling to a hypophosphorylated status. At least under the experimental conditions tested, the level of hypophosphorylated UPF1 did not limit UPF1 phosphorylation and thus NMD.

**Discussion**

Here, we establish using transcriptome-wide deep sequencing of cellular RNAs that p-UPF1 provides the first reliable marker of cellular NMD targets (Fig. 1; Supplemental Fig.
A complete inventory of the cellular mRNAs targeted by NMD has been lacking for a number of reasons: UPF1 binding does not provide a marker of cellular NMD targets (Zünd et al. 2013; Gregersen et al. 2014), direct NMD targets form a collection with myriad incompletely defined cis-acting NMD triggers (uORFs, 3’ UTR EJCs, 3’ UTR length, etc.), and experimentally down-regulating NMD factors by nature identifies both direct and indirect NMD targets. Thus, our finding that p-UPF1 serves as the discriminating mark of direct cellular NMD targets is essential toward elucidating how NMD regulates a multitude of cellular processes. Our results indicate that SMG5 and SMG7 binding should also identify direct NMD targets (Fig. 2; Supplemental Fig. S2I). Our finding a lack of UPF2, UPF3X, SMG1, eRF1, and eRF3 enrichment on NMD targets (Fig. 2; Supplemental Fig. S2) suggests, but certainly does not prove, that (1) SURF forms only transiently on NMD targets, (2) the peripheral EJC constituents UPF2 and UPF3X spend comparable time on NMD targets and their PTC-free counterparts, and (3) SMG1-mediated UPF1 phosphorylation occurs through transient interactions of SMG1 with NMD target-bound UPF1. Notably, either p-UPF1 or UPF1 can be used as an NMD target marker when analyses are confined to 3’ UTRs provided that (1) a PTC-containing mRNA is compared with its PTC-free counterpart, or (2) an mRNA that terminates translation normally and has a long 3’ UTR that triggers NMD is compared with the same mRNA with a short 3’ UTR that fails to trigger NMD (Figs. 2, 4, 5; Hogg and Goff 2010; Hurt et al. 2013; Kurosaki and Maquat 2013; Zünd et al. 2013; Gregersen et al. 2014).

We also examine the role of ATP hydrolysis on UPF1 binding to RNA. While it was known that UPF1 switches between ATP- and ADP-bound states, how the cycle of ATP hydrolysis is involved in NMD remained unresolved. Early biochemical data suggested that ATP binding significantly decreases the affinity of UPF1 to RNA (Weng et al. 1998; Bhattacharya et al. 2000; Cheng et al. 2007; Chamieh et al. 2008; Chakrabarti et al. 2011). Here, we demonstrate using ensemble FRET and immunoprecipitations of UPF1 variants from cellular lysates that UPF1 dissociation from RNA requires the binding and hydrolysis of ATP (Fig. 5; Supplemental Fig. S4G–J). When expressed in HEK293T cells, neither MYC-UPF1[R843C], which manifests ATPase but not helicase activity, nor MYC-UPF1[G495R–G497E], which manifests neither ATPase nor helicase activity, can discriminate between mRNAs that are and are not NMD targets (Fig. 5). Since MYC-UPF1[G495R/G497E] cannot dissociate from non-specific interactions with RNA, it coimmunoprecipitates with three to four orders of magnitude more RNA than does MYC-UPF1[R843C], which fails to detectably bind RNA (Fig. 5). Furthermore, since MYC-UPF1[G495R/G497E] is hyperphosphorylated, whereas MYC-UPF1[R843C] is hypophosphorylated (Fig. 5), and since UPF1 phosphorylation occurs on RNA (Fig. 5), we conclude that prolonged UPF1 binding to RNA results in UPF1 hyperphosphorylation.

Taken together, our findings unveil the following model in which a number of important new links have been added (Fig. 7A). Steady-state hypophosphorylated UPF1 associates and dissociates from mRNAs that are and are not NMD targets nonspecifically in a mechanism that depends on ATP binding and hydrolysis (Fig. 5; Supplemental Fig. S4G–J). Translating ribosomes also regulate the distribution of UPF1 along mRNAs to remove nonspecifically bound UPF1 from the 5’ UTRs and coding regions and specifically recruit UPF1 to the 3’ UTRs of NMD targets (Figs. 1–4; Hurt et al. 2013; Kurosaki and Maquat 2013; Zünd et al. 2013; Gregersen et al. 2014). Data indicate that UPF1 moves along the mRNA 3’ UTR in a 5’-to-3’ direction (Bhattacharya et al. 2000; Chamieh et al. 2008; Chakrabarti et al. 2011; Shigeoka et al. 2012; Park et al. 2013), facilitating mRNA scanning (Shigeoka et al. 2012).

In later steps (Fig. 7B), NMD targets are marked as such by regulated SMG1-mediated UPF1 phosphorylation (Fig. 6A; Supplemental Fig. S1G), which is enhanced by cap-bound CBP80–CBP20 and UPF factors bound to an EJC that is situated downstream from the PTC (Figs. 3, 5; Kashima et al. 2006; Hwang et al. 2010; Franks et al. 2010). Notably, UPF1 phosphorylation occurs while UPF1 is bound to the 3’ UTR via a transient association with SMG1 (Figs. 2, 5, 6; Supplemental Fig. S1G). How many UPF1 molecules load onto a 3’ UTR and which are converted to p-UPF1 molecules remain to be fully understood. p-UPF1 recruits the endonuclease SMG6 and also SMG5–SMG7, the latter of which stabilizes p-UPF1 binding to an NMD target 3’ UTR (Figs. 2, 3, 5) and recruits mRNA degradative activities (Lejeune et al. 2003; Omishi et al. 2003; Fukuhara et al. 2005; Eberle et al. 2009; Okada-Katsuhata et al. 2012; Jonas et al. 2013; Loh et al. 2013). Although NMD targets cannot be detected in immunoprecipitations of SMG6 (data not shown), they are cleaved by SMG6 (Fig. 4J,K). Thus, it appears that SMG6 spends relatively little time on an NMD target prior to its decay, although other explanations are possible. Provided that experimental results using PP2Ac siRNAs are not confounded by levels of residual PP2Ac that are sufficient for NMD or the activity of a phosphatase that functionally substitutes of PP2Ac, our data indicate that p-UPF1 dephosphorylation occurs after the initiation of mRNA decay (Fig. 6). The ratio of UPF1 to p-UPF1 that moves along the mRNA 3’ UTR to facilitate mRNP remodeling and decay (Franks et al. 2010) remains to be determined.

Taken together, our data elucidate how UPF1 ATPase and helicase activities contribute to the identification of NMD targets, which are marked by regulated UPF1 phosphorylation. Accordingly, UPF1 phosphorylation is tightly controlled by the remaining NMD factors.

Materials and methods

Cell transfections and lysis

HEK293T cells or HeLa cells stably expressing IRE-Gl Ter mRNA (Kurosaki and Maquat 2013) were propagated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum. DMEM for HeLa cell growth additionally
 contained 100 μM hemin (Sigma) or Df (Sigma) as described before (Kurosaki and Maquat 2013). When specified, cells were treated with 100 nM wortmannin (Caymen Chemicals) or 200 nM okadaic acid (Sigma) or transiently transfected with 20–30 nM control siRNA (Ambion) or an experimental siRNA (Dharmacon) (Supplemental Table S3) using Lipofectamine RNAiMAX (Life Technologies) and/or plasmid DNAs using Lipofectamine 2000 (Life Technologies). Cell lysates were prepared using hypotonic gentle lysis buffer (10 mM Tris at pH 7.4, 10 mM NaCl, 10 mM EDTA, 0.5% [w/w] Triton X-100) (Kurosaki and Maquat 2013) with protease inhibitor cocktail (Roche). Protein was analyzed after the addition of NaCl to 150 mM, and RNA was extracted and purified using TRIzol reagent (Life Technologies).

Figure 7. Model for the dynamics of UPF1 binding to cellular mRNAs. (A) Steady-state UPF1 binds and hydrolyzes ATP as a means to dissociate from nonproductive mRNA binding, e.g., binding that does not lead to UPF1 phosphorylation and the subsequent p-UPF1-mediated recruitment of degradative activities and mRNA decay. UPF1 binding to translationally active mRNAs is largely restricted to 3' UTRs at least in part because translating ribosomes remove UPF1 from 5' UTRs and coding regions. (B) Regulated UPF1 phosphorylation by transiently associating SMG1 requires UPF1 recognition of a termination codon as one that triggers NMD, e.g., as one situated sufficiently upstream of a 3' UTR EJC (not shown). p-UPF1 recruits SMG6 and/or SMG5–SMG7, which directly [solid arrow] or indirectly [dotted arrows] trigger(s) mRNA decay, respectively. While the binding of SMG6 is sufficiently transient to be undetectable, SMG5–SMG7 stabilize p-UPF1 binding to NMD target 3' UTRs. It may be that PP2A returns p-UPF1 to a dephosphorylated state after mRNA decay is initiated.

p-UPF1-bound RIP and fragmentation for cDNA library construction

HEK293T cells (24 × 10^7 in three 150-mm dishes) were incubated in 200 nM okadaic acid for 3 h. Cellular RNAs bound by p-UPF1 were immunoprecipitated using anti-p-UPF1[S1116] (Millipore, anti-phospho-Upf1[Ser1127]) and Dynabeads protein A magnetic beads (Life Technologies). The RNA in bead-bound RNA–p-UPF1[S1116] complexes was digested to primarily <100 nt by incubation for 30 min at 4°C with 1 U/μL RNase I (Life Technologies); for RNA size estimations after RNase I digestion, 200 ng of RNA was radiolabeled using g^{32}PATP (Perkin Elmer) and T4 polynucleotide kinase (New England Biolabs) and subsequently visualized using a Typhoon 9410 variable mode imager (GE Healthcare) after electrophoresis in 6M urea–15% polyacrylamide. To prepare RNA for cDNA library construction, after extensive washing, bound complexes were eluted using immunoprecipitation elution buffer (Ishigaki et al. 2001), and RNA fragments were separated in 6M urea–15% polyacrylamide in parallel with a DynaMarker prestain marker for small RNA (BioDynamics Laboratory). Small-range RNAs (~25–40 nt) were excised from 6 M urea–15% polyacrylamide and agitated overnight at 25°C in RNA extraction buffer (20 mM Tris, 300 mM sodium acetate, 2 mM EDTA, 0.2% [v/v] SDS). RNAs were eluted from 6 M urea–15% polyacrylamide gel using a Coaster Spin-X column (Corning) and further purified using TRIzol reagent followed by ethanol precipitation. In parallel, control immunoprecipitations using rIgG were performed. Additional control samples were prepared without immunoprecipitation.

cDNA library construction for RIP-seq

Purified 25- to 40-nt RNA fragments were treated with recombinant shrimp alkaline phosphatase (New England Biolabs) to remove 3' phosphates and were subsequently phosphorylated at 5'-hydroxyl groups using T4 polynucleotide kinase (New England Biolabs). Phosphorylated RNA fragments were purified with RNeasy mini columns (Qiagen). A 3' adenylated adapter was ligated to the phosphorylated RNA fragments using truncated T4 RNA ligase (New England Biolabs). An RT primer was annealed to the adapted RNAs to prevent adapter self-ligation, followed by 5' RNA adapter ligation using T4 RNA ligase (New England Biolabs). After RT of adapter-ligated RNAs, cDNAs were amplified using 15 PCR cycles. Amplified cDNAs were purified in 8% polyacrylamide, and the quality and quantity of cDNAs were assessed using an Agilent Bioanalyzer and qPCR. cDNAs were then sequenced using the Illumina HiSeq 2500 platform.
Computational analysis of RIP-seq data

The 3’ adapter sequence was first removed, and reads with a length of <15 nt were discarded. Reads were mapped to the human genome (hg19) using Bowtie2 [local mode]. Reads with a mapping quality score [MAPQ] of ≥10 were selected for further analysis. mRNA abundance was measured using RPKM based on exonic regions of RefSeq sequences. The 3’ ends of genes in HEK293 cells were defined using the 3’ READS method [Hoque et al. 2013].

For plasmid constructions, immunoprecipitations, Western blotting, RT–PCR, RT-qPCR, 5’-RACE, mass spectrometry, in vitro UPF1 helicase and ATPase activity assays, protein production, solid-phase peptide synthesis, and GGGC(Cy5) Sortase labeling of hUPF1(295–914)-Srt, or FRET measurements and data analyses, see the compatible probe construction, Sortase labeling of hUPF1(295–914)-Srt, or FRET measurements and data analyses, see the Supplemental Material.

Accession number

The sequencing data have been deposited in the Gene Expression Omnibus (GEO) database under accession number GSE60045.

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References


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A post-translational regulatory switch on UPF1 controls targeted mRNA degradation

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