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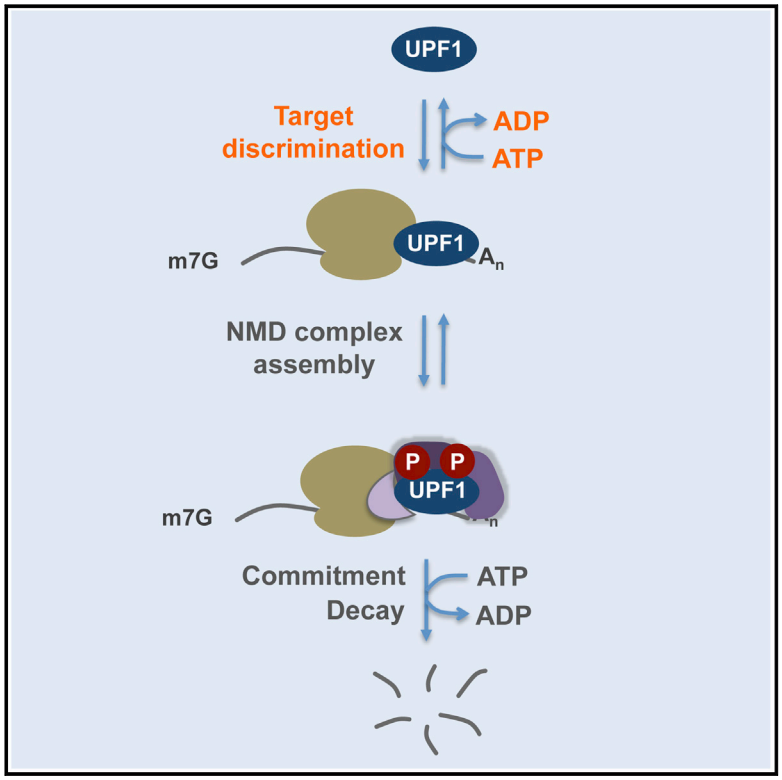
Target Discrimination in Nonsense-Mediated mRNA Decay Requires Upf1 ATPase Activity

Graphical Abstract



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In Brief

RNA quality-control pathways serve to rid the cell of aberrant transcripts, yet the mechanisms for target specificity have yet to be fully elucidated. Lee et al. show that in the nonsense-mediated decay pathway, target specificity is achieved by ATP hydrolysis-dependent release of the central regulator Upf1 from non-target mRNAs.

Highlights

- Upf1 association with mRNA inversely correlates with Upf1 ATPase activity
- Upf1 mutants deficient in ATP binding and hydrolysis lose NMD target specificity
- Translation and PABP require Upf1 ATPase to modulate Upf1-mRNA association
- Upf1 ATPase activity is required for its preferential release from non-target mRNA

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Target Discrimination in Nonsense-Mediated mRNA Decay Requires Upf1 ATPase Activity

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SUMMARY

RNA quality-control pathways get rid of faulty RNAs and therefore must be able to discriminate these RNAs from those that are normal. Here we present evidence that the adenosine triphosphatase (ATPase) cycle of the SF1 helicase Upf1 is required for mRNA discrimination during nonsense-mediated decay (NMD). Mutations affecting the Upf1 ATPase cycle disrupt the mRNA selectivity of Upf1, leading to indiscriminate accumulation of NMD complexes on both NMD target and non-target mRNAs. In addition, two modulators of NMD—translation and termination codon-proximal poly(A) binding protein—depend on the ATPase activity of Upf1 to limit Upf1-non-target association. Preferential ATPase-dependent dissociation of Upf1 from non-target mRNAs in vitro suggests that selective release of Upf1 contributes to the ATPase dependence of Upf1 target discrimination. Given the prevalence of helicases in RNA regulation, ATP hydrolysis may be a widely used activity in target RNA discrimination.

INTRODUCTION

The dynamic interaction of RNA binding proteins (RBPs) with RNA is critical to every aspect of RNA metabolism (Moore, 2005). Yet how do RNA regulators faithfully distinguish their target RNAs from non-targets in the cell? Most models for RBP target specificity invoke RBP affinity for target-specific RNA sequences, structures, or bound proteins (Ankö and Neugebauer, 2012; Glisovic et al., 2008). However, for RNA quality-control pathways, which detect and destroy faulty or non-functional RNAs, target-specific mechanisms for RBP recruitment are harder to envision, as aberrant RNAs have the potential to differ widely in sequence and associated proteins (van Hoof and Wagner, 2011; Porrua and Libri, 2013).

The first mRNA quality-control pathway discovered was nonsense-mediated decay (NMD) (Leeds et al., 1991; Losson and Lacroute, 1979; Maquat et al., 1981; Pulak and Anderson, 1993). Conserved in eukaryotes, this translation-dependent pathway degrades transcripts whose termination codons are recognized as premature. In this way, NMD prevents the accumulation of truncated polypeptides arising from aberrant mRNAs bearing premature termination codons (PTCs). NMD also affects the accumulation of select naturally occurring mRNAs, affecting up to 10% of protein-coding genes in diverse eukaryotes (Schweingruber et al., 2013).

The key RNA binding regulator in NMD is the Superfamily 1 (SF1) RNA helicase Upf1. Target degradation involves assembly of Upf1 with other NMD factors, such as Upf2, Upf3, and, in most eukaryotes studied to date, Smg1 kinase and one or more of Smg5-7 (Kervestin and Jacobson, 2012; Schweingruber et al., 2013). In humans, Smg1 phosphorylates Upf1 in a manner stimulated by Upf2, Upf3, and the exon junction complex (EJC) (Kashima et al., 2006). This promotes association of phospho-binding proteins Smg5 and Smg7, as well as general mRNA decay factors (Chakrabarti et al., 2014; Cho et al., 2013; Loh et al., 2013; Okada-Katsuhata et al., 2012). Smg6 is itself an endonuclease, which has both phospho-dependent and phospho-independent interactions with Upf1 (Chakrabarti et al., 2014; Eberle et al., 2009; Nicholson et al., 2014; Okada-Katsuhata et al., 2012). In addition, the adenosine triphosphatase (ATPase) activity of Upf1 has been implicated in a late step of target mRNA degradation: remodeling the messenger ribonucleoprotein (mRNP) to enhance nuclease access (Franks et al., 2010).

Despite the wealth of information regarding NMD target degradation, a fundamental, yet poorly understood, aspect of NMD is what enables Upf1 to distinguish targets from non-targets in the first place. Well-studied NMD targets share in common the fact that translation termination occurs at an unusual position in the mRNA (Schweingruber et al., 2013). One model for NMD target recognition is that stalled or aberrant termination complexes recruit Upf1 (Kervestin and Jacobson, 2012; Schweingruber et al., 2013). Indeed, ribosome toe-printing in *S. cerevisiae* extracts and rabbit reticulocyte lysates revealed that ribosome dissociation at an NMD-inducing PTC is stalled aberrantly (Amrani et al., 2004; Peixeiro et al., 2012). Although it remains to be fully elucidated how termination at a PTC becomes aberrant, the absence of proximal mRNP factors that promote normal termination, such

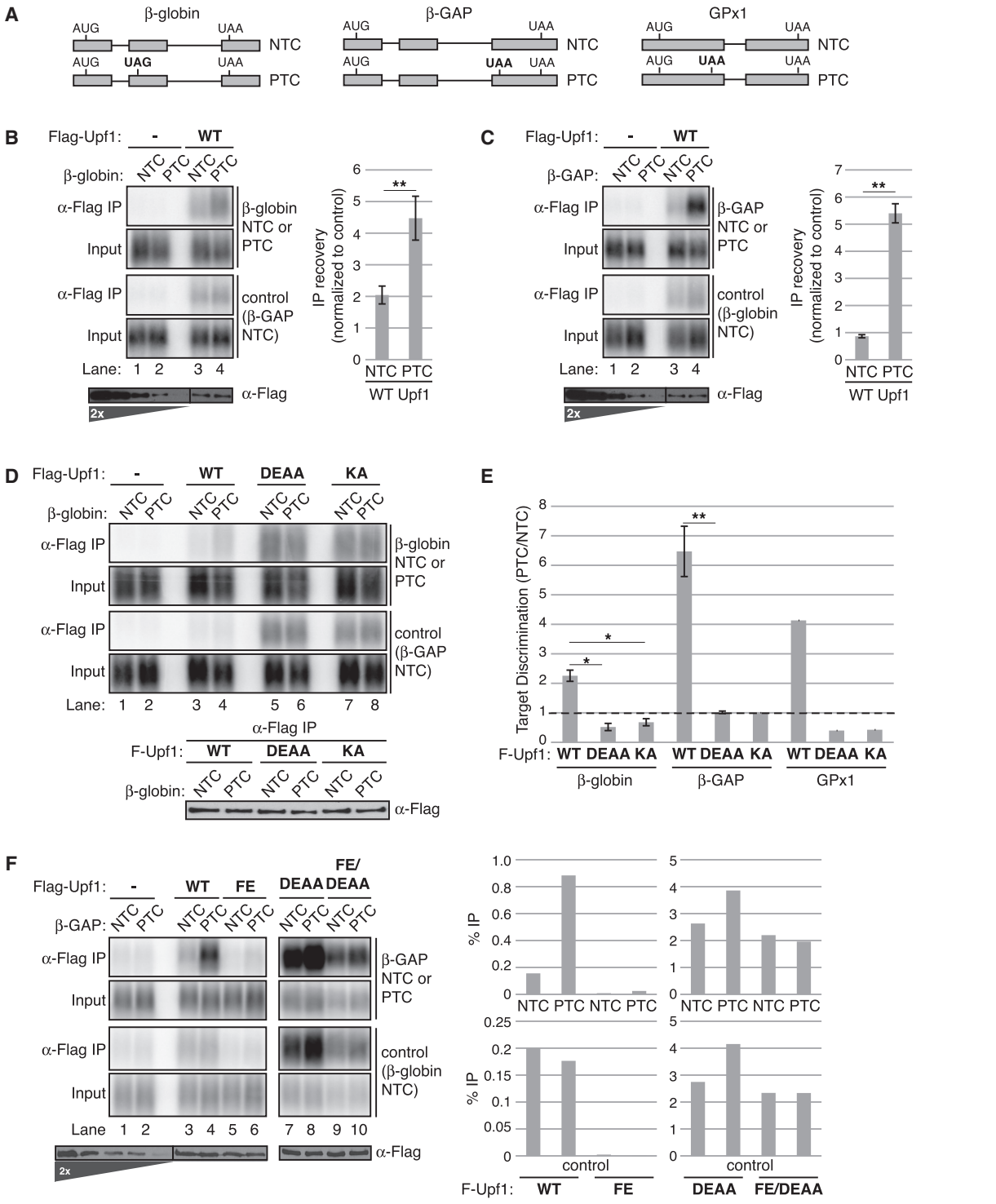

Figure 1. Upf1 ATPase Cycle Mutants Are Defective in Selective NMD Target Association

(A) NMD target (PTC) and non-target (NTC) mRNA reporter pairs used in RIP assays based on human β-globin, β-globin with an insertion from GAPDH (β-GAP), or GPx1. The NMD-inducing termination codon is denoted in bold.

(B) Northern blots of β-globin and control mRNAs in input (0.5%) samples or coprecipitated with Flag-Upf1 using anti-Flag antibody (α-Flag IP). Flag-Upf1 recovered in IPs is shown alongside a 2-fold titration of input on anti-Flag western blots below northern blots. The graph on the right represents recovery of β-globin mRNAs (NTC or PTC) with Flag-Upf1 over input normalized to recovery of the internal control (lanes 3 and 4), after subtraction of background in negative control IPs (lanes 1 and 2). Data are represented as mean ± SEM for five biological replicates.

(legend continued on next page)

as poly(A) binding protein, has been suggested to distinguish NMD targets from non-targets (Cosson et al., 2002b; Ivanov et al., 2008; Kervestin and Jacobson, 2012; Uchida et al., 2002). Notably, Upf1 and the ribosome release factors, eRF1 and eRF3, have been found to interact in yeast and human cells (Czaplinski et al., 1998; Ivanov et al., 2008; Kashima et al., 2006; Singh et al., 2008).

However, recent reports cloud the simple view that Upf1 recruitment to aberrantly stalled termination complexes accounts fully for NMD target specificity. For example, Upf1 associates with both target and non-target mRNAs even in the absence of translation, and the manner and degree to which translation seems to affect Upf1-mRNA accumulation vary among different mRNAs (Hogg and Goff, 2010; Kurosaki and Maquat, 2013; Kurosaki et al., 2014; Zünd et al., 2013). Additionally, genome-wide crosslinking-immunoprecipitation (CLIP) studies showed that translation inhibitors cause Upf1 binding sites along mRNAs to expand from a bias for 3′ UTRs to be more broadly distributed (Gregersen et al., 2014; Hurt et al., 2013; Zünd et al., 2013). This suggests that translation influences where Upf1 associates along an mRNA's length, in addition to how strongly it associates with NMD target and non-target mRNAs overall.

Thus, the mechanisms involved in how Upf1 distinguishes differences in target and non-target mRNPs have remained unclear. Here we present evidence for a critical role for Upf1 ATPase activity in NMD target discrimination, with preferential ATPase-dependent release of Upf1 from non-target mRNAs as part of the underlying mechanism.

RESULTS

Upf1 Mutants Unable to Bind or Hydrolyze ATP Are Defective in NMD Target Discrimination

Previous studies have demonstrated that Upf1 target discrimination is reflected in Upf1-RNA immunoprecipitation (RIP) assays, which yield greater Upf1 copurification of NMD target than non-target mRNAs at steady state (Hwang et al., 2010; Johansson et al., 2007; Johns et al., 2007; Silva et al., 2008). Thus, to examine determinants of human Upf1 target specificity, we established stable cell lines in which endogenous Upf1 could be depleted by RNAi and complemented with small interfering RNA (siRNA)-resistant Flag-Upf1 induced to near endogenous levels (Figure S1A) for use in RIP assays. We examined the binding specificity of Flag-Upf1 using gene reporters previously shown to be NMD sensitive, because of a PTC, or NMD insensitive, because of a normal termination codon (NTC) (Singh et al., 2008) (Figure 1A). Non-target mRNAs differing in size were used as internal controls.

Consistent with expectation, RIP assays performed with Flag-tagged wild-type (WT) Upf1 exhibited ~2.3- to 6.5-fold greater recovery of PTC mRNAs over their NTC counterparts, as seen in Figures 1B, 1C, and S1B (compare lane 4 with lane 3 in the top panels). This mirrors the range in half-life differences for these PTC-NTC pairs (Singh et al., 2008). Although Upf1 immunoprecipitations (IPs) enriched PTC-containing mRNA, both PTC and NTC mRNAs copurified with Upf1 above background (compare lanes 3 and 4 with lanes 1 and 2), consistent with previous observations that Upf1 can associate with both target and non-target mRNAs (Hogg and Goff, 2010; Hwang et al., 2010; Johns et al., 2007; Silva et al., 2008; Zünd et al., 2013). Importantly, lysate mixing controls (Mili and Steitz, 2004) confirmed that the observed Upf1-mRNA associations reflect interactions occurring in intact cells, rather than after cell lysis (Figure S1C).

Because Upf1 mutations that block the Upf1 ATPase cycle render Upf1 inactive for NMD (Bhattacharya et al., 2000; Cheng et al., 2007; Franks et al., 2010; Kashima et al., 2006), we wanted to examine the impact of these mutations on mRNA association and target discrimination. We therefore repeated our RIP assays with mutant Upf1 defective in two steps of the ATPase cycle, Upf1 K498A (Upf1 KA; deficient in ATP binding) and Upf1 D637A/E638A (Upf1 DEAA; deficient in ATP hydrolysis) (Figures S1A and S1D). In striking contrast to WT Upf1, both mutants exhibited a complete loss in target discrimination and, in fact, recovered increased levels of both target and non-target mRNAs compared with WT Upf1 (Figure 1D; quantified in Figure 1E). Interestingly, two of the three target-non-target pairs assayed exhibited slightly greater association with NTC mRNA over PTC mRNA with Upf1 DEAA and Upf1 KA (target discrimination < 1 in Figure 1E). This apparent reverse discrimination was eliminated upon depletion of Smg6 (Figures S1D and S1E), likely reflecting a role for Smg6-mediated cleavage in reducing target mRNA levels in IPs. We conclude that Upf1 mutants defective in ATP binding or ATP hydrolysis exhibit increased association with both target and non-target mRNAs and lack the ability to discriminate between them.

A Hyperactive Upf1 ATPase Mutant Is Defective in Binding to Both Target and Non-target mRNAs

To examine how mRNA association might be affected in a third Upf1 mutant with a perturbed Upf1 ATPase cycle, we turned to the hyperactive mutant F192E (Upf1 FE). Structural and biochemical studies have shown that the cysteine/histidine-rich domain partially inhibits Upf1 ATPase, and an F192E mutation within this domain renders bacterially expressed Upf1 hyperactive in its ATPase activity and less stably bound to RNA in vitro (Chakrabarti et al., 2011; Chamieh et al., 2008). Our RIP assays of Upf1 FE with the β-GAP PTC-NTC pair revealed indiscriminate

(C) Similar to (B) for RIPs of β-GAP mRNAs.

(D) Similar to (B), comparing IPs with Flag-Upf1 WT, D637A/E638A (DEAA), or K498A (KA). Western blot of recovered Flag-Upf1 variants is shown below northern blots.

(E) Graph representing the ratio of normalized IP recovery for NMD target (PTC) to non-target (NTC) mRNAs with the indicated Upf1 variants. A value of 1, denoted by the dotted line, reflects an absence of discrimination between target and non-target. Data are represented as mean ± SEM for three to four biological repeats.

(F) Similar to (C) but without normalization, comparing percentage IP recovery of β-GAP mRNAs and the internal control by Flag-Upf1 WT, F192E (FE), DEAA, or DEAA/FE mutants.

*$p \leq 0.05$, **$p \leq 0.001$ (paired Student's t test, two-tailed). See also Figure S1.

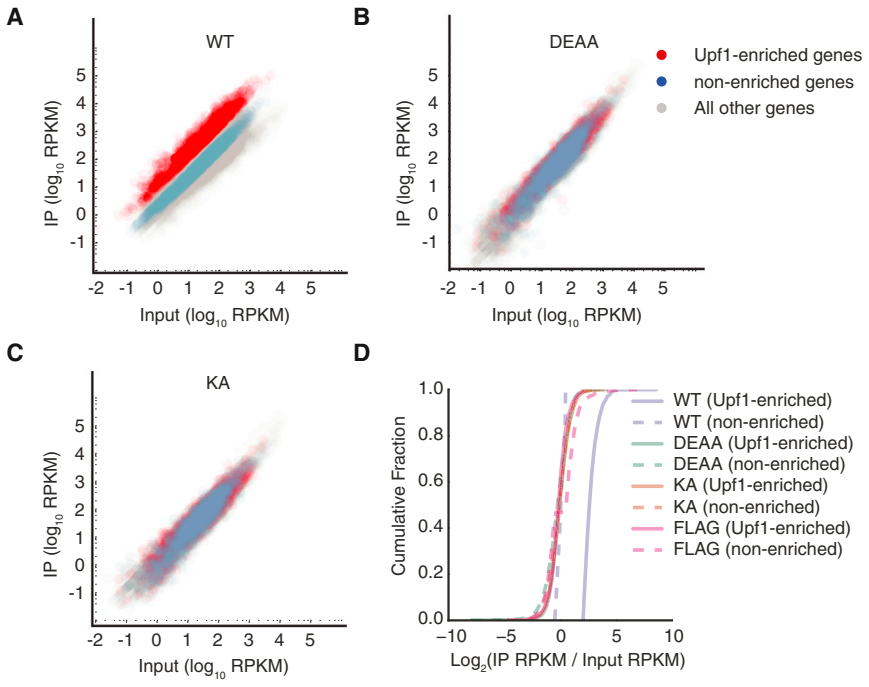

Figure 2. Transcriptome-wide Loss in mRNA Selectivity for Upf1 ATPase Cycle Mutants

(A–C) Scatterplots of RPKM from RNA-seq of input samples versus IPs for Flag-Upf1 WT (A), DEAA (B), and KA (C). Genes with IP/input ratios for WT Upf1 of greater than 2.05 (cutoff based on 5% FDR established using cells expressing Flag epitope only; Figure S2) are shown in red (Upf1-enriched), while genes with $\log_2$ (IP/input) between −0.5 and +0.5 are shown in blue (non-enriched). All remaining genes are shown in gray.

(D) Cumulative fraction of Upf1-enriched and non-enriched genes with IP enrichment represented as $\log_2$ (IP RPKM/input lysate RPKM) for Flag-Upf1 WT, KA, and DEAA, along with Flag only. Difference between WT Upf1 (Upf1-enriched) curve compared with all other curves was statistically significant ($p < 0.05$ for all comparisons, KS test). See also Figure S2 and Table S1.

loss in association with both target and non-target mRNA compared with WT Upf1 (Figures 1F and S1G, compare lanes 5 and 6 with lanes 3 and 4; quantified on the right). Importantly, this loss was predominantly dependent on Upf1 ATPase activity because mRNA association was largely recovered when the FE mutant was rendered deficient in ATP hydrolysis (Upf1 FE/DEAA, Figures 1F and S1G, lanes 7–10; quantified on the right). ATPase assays performed in vitro with Upf1 from our cell lines confirmed Upf1 FE hyperactivity and Upf1 FE/DEAA ATPase deficiency (Figure S1F). Together, these findings for ATPase-hyperactive and ATPase-deficient Upf1 indicate that the steady-state mRNA association of Upf1 inversely correlates with Upf1 ATPase activity and that Upf1 target discrimination is critically dependent on a normal Upf1 ATPase cycle.

Upf1-mRNA Selectivity Is Lost on a Transcriptome-wide Level in Upf1 ATP-Binding and ATP-Hydrolysis Mutants

To examine the contribution of Upf1 ATPase activity to mRNA selectivity among endogenous mRNAs, we applied RIP sequencing (RIP-seq) (RIP followed by strand-specific high-throughput sequencing) to Flag-Upf1 WT, DEAA, and KA expressed at endogenous levels (Figures 2 and S2A). A parental cell line expressing no exogenous Upf1 was used as a negative control. RIP-seq libraries were sequenced to a mean depth of 23 million reads, and approximately 20,000 genes had >0.1 reads per kilobase transcript per million mapped reads (RPKM) per library. Importantly, Upf1 WT and mutant RIPs were all highly enriched for transcripts annotated as protein coding, with a smaller fraction derived from pseudogenes (Table S1), indicating that the ATPase mutations do not disrupt Upf1 preference for mRNAs over non-coding transcripts.

Using the background of negative control IPs to establish a 5% false-discovery rate (FDR) (Figure S2B), a distinct population of 2,040 Upf1-associated RNAs was found to be enriched by at least 2-fold over input levels (Figure 2A, Upf1-enriched genes indicated in red; solid purple line denotes WT in Figure 2D). On the basis of observations by others (Hogg and Goff, 2010; Kurosaki et al., 2014; Zünd et al., 2013), these RNAs likely include a mixture of NMD-sensitive mRNAs and mRNAs that are less sensitive to NMD but limited in downstream steps of the NMD pathway. In striking contrast to WT Upf1, RIPs for Upf1 DEAA and KA did not show enrichment for any RNAs when subjected to the same FDR cutoff (Figure S2B). Accordingly, the population of WT Upf1-enriched RNAs was not enriched in DEAA and KA RIPs over a WT Upf1-non-enriched RNA population, defined by 0.97- to 1.03-fold enrichment in WT RIPs over inputs (Figures 2A–2C, compare red and blue; Figure 2D). These global findings generalize our observations for individual mRNA reporters to the human transcriptome, supporting the conclusion that mRNA selectivity is lost in Upf1 mutants deficient in ATP binding or hydrolysis.

ATP Hydrolysis-Deficient Upf1 Is Phosphorylated and Assembles Smg5-7 Proteins on Both Target and Non-target mRNAs

Our finding that Upf1 mutants blocked in ATP binding and ATPase activity exhibit elevated and indiscriminate mRNA association (Figures 1 and 2) raised the possibility that the Upf1 ATPase cycle plays a critical role in preventing NMD complex assembly on non-target mRNAs. To investigate this, we used RIP assays to compare the phosphorylation levels of WT Upf1 and Upf1 DEAA and their ability to support assembly of Smg5-7 on target and non-target mRNAs.

To examine Upf1 phosphorylation, we used an antibody specific for Upf1 phosphorylated on serine 1116, a Smg1-phosphorylation site shown to be important for efficient NMD (Kurosaki et al., 2014; Yamashita et al., 2001). As seen in Figure 3A and quantified in Figure 3B, α-phospho-Upf1 RIPs from cells treated with okadaic acid prior to harvest to promote recovery

of phosphorylated Upf1 (Kurosaki et al., 2014; Yamashita et al., 2001) exhibited a pattern similar to RIPs with a pan-Upf1 antibody: greater recovery of PTC mRNA over NTC mRNA from WT Upf1-expressing cells and equal recovery of both types of mRNA from Upf1 DEAA-expressing cells. RIPs with resin only and λ-protein phosphatase pre-treated lysates served as negative controls for α-Upf1 and α-phospho-Upf1, respectively (Figure 3A, right, and Figure S3). We note that target recovery preference in α-Upf1 RIPs of WT Upf1 exceeds that observed in α-phospho-Upf1 RIPs (Figure 3B, compare top left and bottom left graphs), perhaps reflecting greater phospho-epitope masking by phospho-Upf1 binding proteins on targets than on non-targets or a population of target-bound Upf1 that has not undergone phosphorylation. Regardless, these observations suggest that indeed, mRNA-associated Upf1 DEAA is phosphorylated and to an extent indistinguishable between target and non-target mRNAs.

We had previously observed that Upf1 DEAA supports assembly of Smg5-7 proteins on NMD target mRNA (Franks et al., 2010). Examining whether this occurs also on non-target mRNAs, we observed that all three Smg5-7 proteins coprecipitated PTC mRNA to a greater extent than NTC mRNA in the presence of Upf1 WT (Figure 3C, lanes 1–8), exhibiting a degree of target discrimination equal to or slightly less than that exhibited by Upf1 WT alone (Figure 3D; quantified in Figure 3E, left). In contrast, target discrimination was completely lost for Smg5-7 in the presence of Upf1 DEAA, with equal recovery of PTC and NTC mRNA (Figure 3C, lanes 9–16; quantified in Figure 3E, right). Taken together, these observations suggest that Upf1 ATPase activity serves a critical role in limiting the assembly of NMD complexes containing phosphorylated Upf1 on non-target mRNAs.

Translation Prevents Upf1 Accumulation on Non-target mRNAs in a Upf1 ATPase-Dependent Manner

Active translation is critical for NMD, and recent studies have suggested a role for translation in reducing the overall level of Upf1 association with non-target mRNAs (Zünd et al., 2013), while promoting or leaving unchanged the level of Upf1 association with target mRNAs (Hogg and Goff, 2010; Kurosaki et al., 2014). Consistent with this, we observed increased Upf1 recovery of NTC mRNAs and decreased recovery of a PTC mRNA upon global repression of translation elongation with cycloheximide or puromycin (Figure S4). To test whether these effects of translation are dependent on Upf1 ATPase activity, we introduced a stable hairpin (HP) into the 5′ UTRs of our PTC-NTC reporter pairs that inhibits translation (Kozak, 1989), efficiently inhibiting polysome formation (Franks and Lykke-Andersen, 2007) and preventing NMD (Belgrader et al., 1993; G. Singh and J.L-A., unpublished data). RIP assays revealed that HP-induced translational repression reduced PTC mRNA association with both WT Upf1 and Upf1 DEAA, with the magnitude of reduction slightly smaller for DEAA than for WT (Figures 4A and 4B; quantified on the right). This suggests that for target mRNAs, translation promotes Upf1 accumulation in a manner largely independent of Upf1 ATPase activity, consistent with models in which ribosomes contribute to Upf1 recruitment (Czaplinski et al., 1998; Ivanov et al., 2008; Kashima et al., 2006; Min et al., 2013). In contrast, on NTC mRNAs, HP-induced translational repression increased mRNA association with WT Upf1, while reducing Upf1 DEAA association to a similar extent as on PTC mRNAs (Figures 4A and 4B; compare quantifications for NTC mRNAs with or without HP on the right). Therefore, translation limits Upf1 accumulation on non-target mRNAs by a mechanism that is dependent on Upf1 ATPase activity.

PTC-Proximal Poly(A) Binding Protein Limits Upf1-mRNA Accumulation in a Manner Dependent on Upf1 ATPase Activity

mRNA reporters subject to NMD have been found to partially evade NMD when modified to bring PABP proximal to the PTC (Amrani et al., 2004; Behm-Ansmant et al., 2007; Eberle et al., 2008; Fatscher et al., 2014; Ivanov et al., 2008; Joncourt et al., 2014; Silva et al., 2008; Singh et al., 2008). To test if PTC-proximal PABP affects Upf1-mRNA association, and, if so, requires Upf1 ATPase activity to do so, we repeated our Upf1 RIP assays with two reporters previously shown to evade NMD because of PTC-proximal PABP (Singh et al., 2008) (Figures 5A and 5B).

As seen in Figure 5A (left), tethering MS2-PABPC1 to a β-GAP PTC variant containing six MS2 binding HPs (PTC-6xMS2) downstream of the PTC reduced mRNA reporter association with WT Upf1 relative to tethering MS2 alone or when the reporter was coexpressed with untethered PABPC1 (quantified on the right), despite similar levels of IP recovery of tethered and untethered PABP (Figure S5). Significantly, this reduction is dependent on Upf1 ATPase activity, as it was not observed in Upf1 DEAA RIPs (Figure 5A, lanes 4–6; quantified on the right). Similar observations were seen for an mRNA bearing a PTC-proximal tract of 30 adenosines predicted to recruit PABPC1 (Figure 5B). These observations suggest that termination-proximal PABPC1 reduces Upf1 accumulation on mRNA in a Upf1 ATPase-dependent manner.

ATP Binding and ATPase-Deficient Upf1 Accumulate on mRNA 3′ UTRs and Are Enriched near Termination Codons and 3′ Ends

Our observations suggest that Upf1 ATPase activity is required for preventing Upf1 from accumulating and promoting NMD complex formation on translated non-target mRNAs. Recent studies using UV crosslinking and Upf1 IP followed by high-throughput sequencing (CLIP-seq) have reported that although Upf1 binding sites can be found all along the length of mRNAs, the overall distribution has a distinct 3′ UTR bias (Gregersen et al., 2014; Hurt et al., 2013; Zünd et al., 2013). To gain insight into how a disrupted Upf1 ATPase cycle might affect Upf1 binding site distribution, we performed CLIP-seq on Flag-Upf1 WT, DEAA, and KA expressed at near endogenous levels (Figures S6A–S6C).

Notably, the distributions of Upf1 DEAA and KA binding were nearly identical to each other and similar to WT Upf1 in 3′ UTR bias (Figures 6A, S6D, and S6E) and preferential binding to mRNAs over non-coding RNAs (Table S2). However, Upf1 mutants exhibited an even greater average accumulation in 3′ UTRs than WT Upf1 (Figure 6A; compare read densities in 3′ UTRs for DEAA, KA with WT) across all genes, as seen by

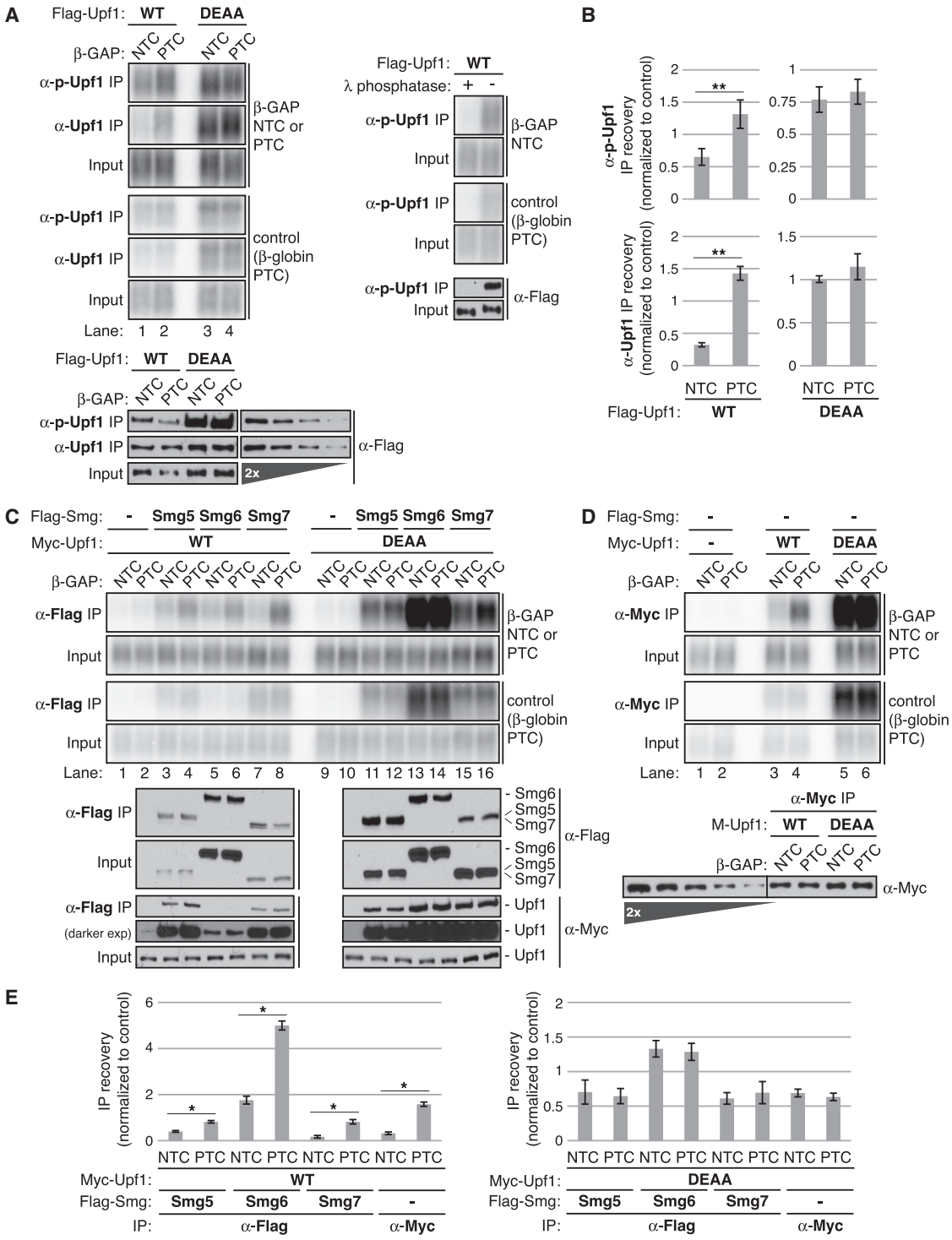

Figure 3. ATP Hydrolysis-Deficient Upf1 Accumulates as a Phosphoprotein in Complexes with Smg Proteins on Both Target and Non-target mRNA

(A) Northern blots of β-GAP and control reporter mRNAs in inputs (0.3%) or RIPs with α-phospho-S1116 Upf1 (α-p-Upf1) or α-Upf1 antibodies from okadaic acid-treated cells expressing Flag-Upf1 WT or DEAA. Control RIPs performed with α-phospho-Upf1 from lysates treated with or without λ protein phosphatase are shown on the right. Western blots of Flag-Upf1 recovered in IPs alongside a 2-fold titration of Flag-Upf1 WT input are shown below northern blots.

(B) Graphs representing mean IP recovery of β-GAP mRNAs normalized by recovery of the internal control ± SEM for triplicate biological repeats of α-phospho-Upf1 RNA-IPs and duplicates of α-Upf1 RNA-IPs.

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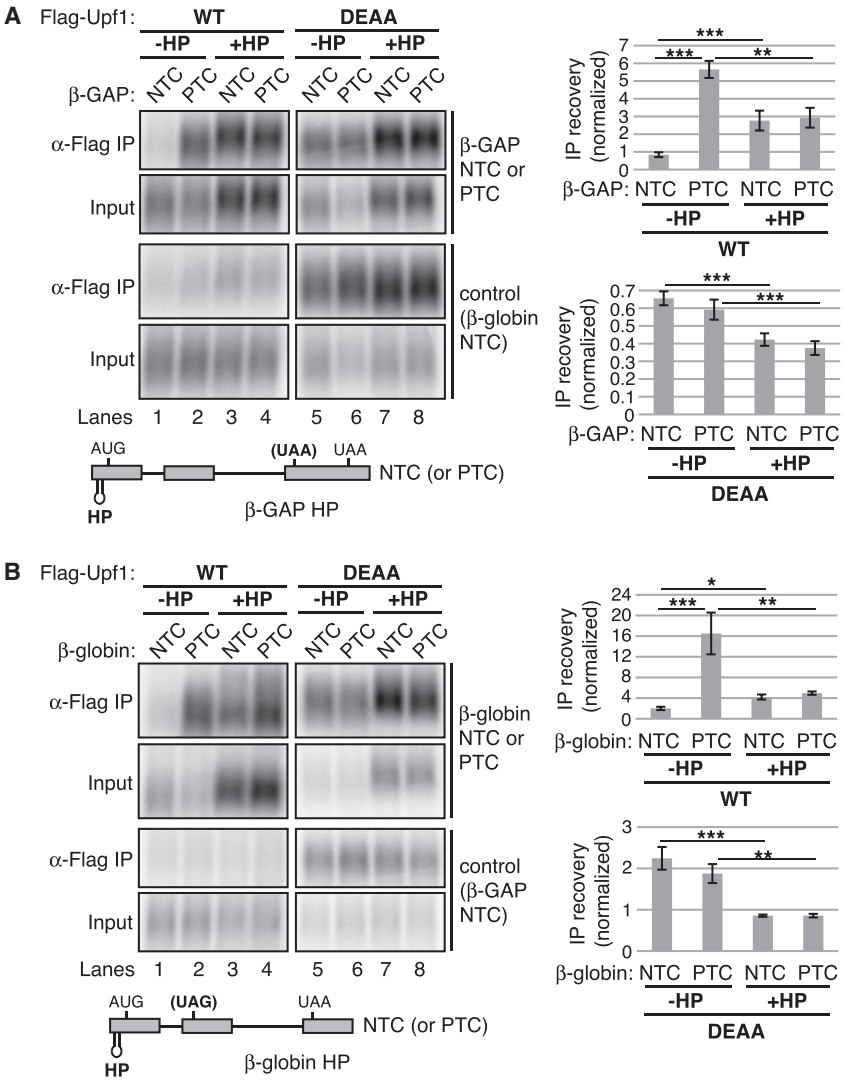

Figure 4. Translation Prevents Upf1 Accumulation on Non-target mRNA in a Upf1 ATPase-Dependent Manner

(A) Northern blots for β-GAP and control reporter mRNAs in inputs (0.5%) or coprecipitated with Flag-Upf1. Schematic of the β-GAP mRNAs used in RIPs is shown below northern blots. HP denotes a stable RNA HP in the 5′ UTR that blocks translation. Graphs on the right represent mean IP recovery over input of β-GAP mRNA normalized by recovery of the internal control after subtraction of background from negative control IPs, ± SEM for triplicate biological repeats.

(B) Similar to (A) for β-globin RIPs, except performed in the presence of Smg6 depletion to prevent Smg6-mediated cleavage of the β-globin PTC mRNA (see Figure S1).

*p ≤ 0.1, **p ≤ 0.05, ***p ≤ 0.01 (paired Student's t test, two-tailed). See also Figure S4.

the increased fraction of 3′ UTR-derived reads per mRNA normalized to length in DEAA and KA CLIPs compared with WT (Figure 6B, left graph, see right-shifted curves for DEAA, KA compared with WT; $p < 0.05$, Kolmogorov-Smirnov [KS] test). Comparison of read distributions for WT Upf1-enriched and non-enriched RNA subpopulations identified by RIP-seq (Figure 2) revealed that for WT Upf1, a greater fraction of enriched mRNAs had a stronger 3′ UTR distribution bias than non-enriched mRNAs, while for Upf1 mutants, both sets of mRNAs were indistinguishable in their enhanced 3′ UTR bias (Figure 6B, right graph, compare dashed and solid lines). These findings suggest that Upf1 ATPase activity limits Upf1 accumulation in 3′ UTRs to an extent that is greater for non-target than target mRNAs.

Examination of CLIP-seq read density at nucleotide resolution around translation termination codons and at mRNA 3′ ends revealed two peaks that are stronger for Upf1 DEAA and KA than WT Upf1. The first centers around 45 nucleotides downstream of the termination codon (Figure 6C, left), while the second peak occurs upstream of transcript 3′ ends (Figure 6C, right). This difference between mutant and WT Upf1 in binding site distribution proximal to the termination codon and the poly(A) tail was seen for both Upf1-enriched and non-enriched mRNAs (Figure S6F). These findings suggest that Upf1 ATPase activity is needed to limit Upf1 accumulation at these specific 3′ UTR sites.

Upf1 Dissociates Faster from Non-target mRNA than Target mRNA in an ATPase- and Translation-Dependent Manner in Vitro

The findings described above are consistent with a mechanism by which Upf1 target binding specificity depends on Upf1 ATPase activity to limit accumulation of Upf1 and assembly of NMD complexes on non-target mRNAs, particularly in 3′ UTRs. One possible mechanism accounting for this Upf1 ATPase dependence is that Upf1 ATP hydrolysis is needed

(C) Northern blots of β-GAP and control mRNAs in inputs (0.6%) or coprecipitated with Flag-Smg5, Flag-Smg6, or Flag-Smg7 in cells coexpressing Myc-Upf1 WT or DEAA. Westerns of Flag-Smg proteins recovered in IPs, along with copurifying Myc-Upf1 are shown below northern blots.

(D) Similar to (C) for RIPs with Myc-Upf1 WT or DEAA, with 2% inputs loaded. Western blot of Myc-Upf1 recovered in IPs alongside a 2-fold titration of Myc-Upf1 WT input is shown below northern blots.

(E) Quantifications, similar to those in (B), for RIPs shown in (C) and (D) with SEM for triplicate (Smg5, Smg7, Upf1) or duplicate (Smg6) biological repeats.

*p ≤ 0.05, **p ≤ 0.01 (paired Student's t test, two-tailed). See also Figure S3.

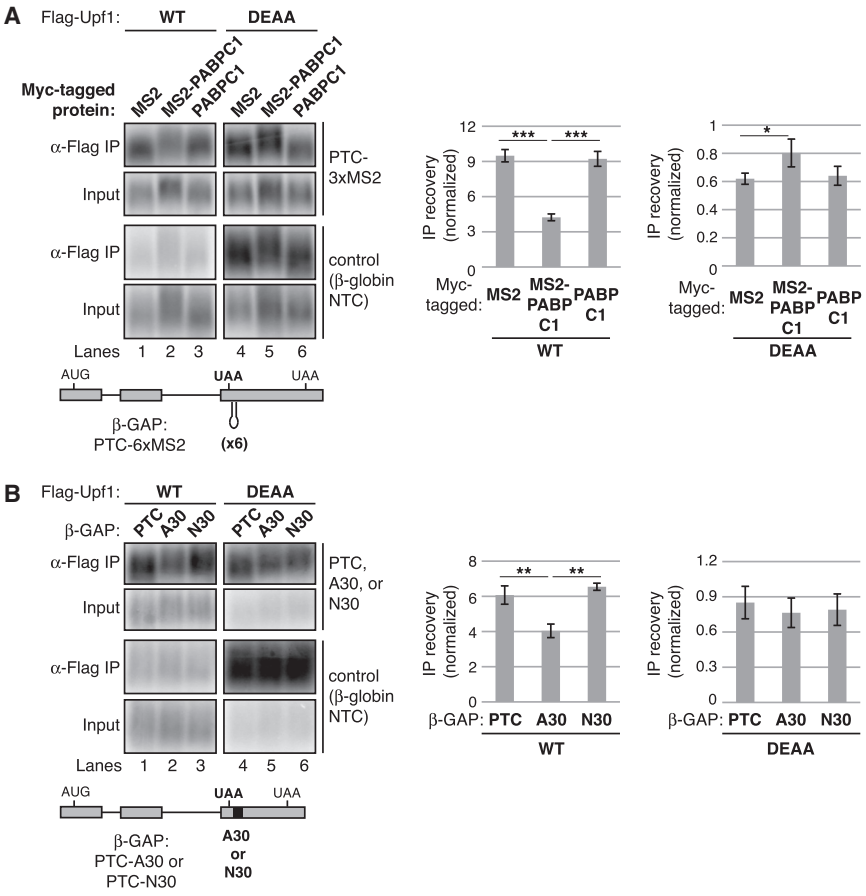

Figure 5. PTC-Proximal Poly(A) Binding Protein Prevents Upf1 Accumulation on mRNA in a Upf1 ATPase-Dependent Manner

(A) Northern blots for β-GAP and control reporter mRNAs in inputs (0.3%) or coprecipitated with Flag-Upf1 WT or DEAA from cells coexpressing Myc-tagged proteins as indicated. MS2 denotes fusion with MS2 coat protein. Schematic of the β-GAP PTC-6xMS2 mRNA used is shown below northern blots. Graphs on the right represent mean IP recovery of reporter mRNA normalized by recovery of the internal control after subtraction of background from negative control IPs, ± SEM for triplicate biological repeats.

(B) Similar to (A) for β-GAP PTC reporter mRNAs containing 30 adenosines (A30) or a 30-nucleotide degenerate sequence (N30) instead of MS2 coat protein binding sites (schematic shown below northern blots), with 0.5% inputs loaded on northern blots and IPs performed in the absence of MS2 fusion proteins.

*p ≤ 0.1, **p ≤ 0.05, ***p ≤ 0.01 (paired Student's t test, two-tailed). See also Figure S5.

for preferential dissociation of Upf1 from non-targets. Indeed, Upf1 binding to synthetic RNAs is disrupted upon incubation with ATP but not ADP or non-hydrolyzable ATP analogs (Kurosaki et al., 2014; Weng et al., 1998). To test if Upf1 release from non-target mRNAs differs from target mRNAs, we developed in vitro RNA release assays that measure the relative release kinetics of Upf1 from reporter mRNAs. RIP for Flag-Upf1 was performed from cell extracts with a low level of EDTA to repress the Upf1 ATPase and subsequently supplemented at regular intervals during IPs with Mg^{2+} and ATP to stimulate Upf1 ATPase activity.

As seen in Figure 7A, Upf1 dissociates from both target and non-target mRNAs over time in the presence of Mg-ATP, despite even IP recovery of Upf1 (Figure S7A) and unchanged overall mRNA levels (Figure S7B). Intriguingly, quantification of the Upf1-mRNA dissociation rate after normalization to the PTC-containing internal control revealed that release of NTC mRNA was faster than its PTC counterpart (Figure 7A, graph below). To avoid significant RNA degradation (S.R.L., unpublished data), the release assays were performed at a low temperature (4°C). Although we note that the rate of release in these assays is slow, ATP hydrolysis by Upf1 under the same conditions occurred at a comparably slow rate (Figure S7C).

The preferential Mg-ATP-dependent release of NTC-mRNA was abolished for ATPase-deficient Upf1 DEAA (Figure S7D) and upon replacement of ATP with a non-hydrolysable ATP analog, AMP-PNP (Figure 7B). In fact, addition of AMP-PNP yielded a small but significant increase in NTC mRNA retention by WT Upf1 relative to the PTC internal control, perhaps because of AMP-PNP competition with cellular ATP in the extract. These findings suggest that Upf1 ATP hydrolysis supports faster Upf1 release from non-target mRNAs than from targets.

Because translation limits Upf1 accumulation on non-target mRNA (Figure 4), we next tested the possibility that preferential release of non-target mRNAs by Upf1 is dependent on translation. As shown in Figure 7C, mRNAs translationally repressed by an HP structure were blocked in ATP hydrolysis-dependent release, in contrast to their unrepressed counterparts. It is important to note that this result cannot distinguish whether mRNA release from Upf1 is affected by translation occurring in the extract or instead by translation-dependent mRNP remodeling in cells. Regardless, these results suggest that Upf1 release of non-target mRNAs is faster than targets and requires Upf1 ATP hydrolysis and translation.

DISCUSSION

The Upf1 ATPase Cycle Is Required for NMD Target Discrimination

In this study, we present multiple lines of evidence that target mRNA specificity in the NMD pathway is critically dependent on the ATPase cycle of the central NMD factor Upf1 (Figure 7D). First, Upf1 mutations that interfere with distinct aspects of the Upf1 ATPase cycle render Upf1 defective in its preferential association with NMD target mRNAs. This is evidenced by the loss of mRNA specificity for mutant Upf1 blocked in ATP binding or ATP

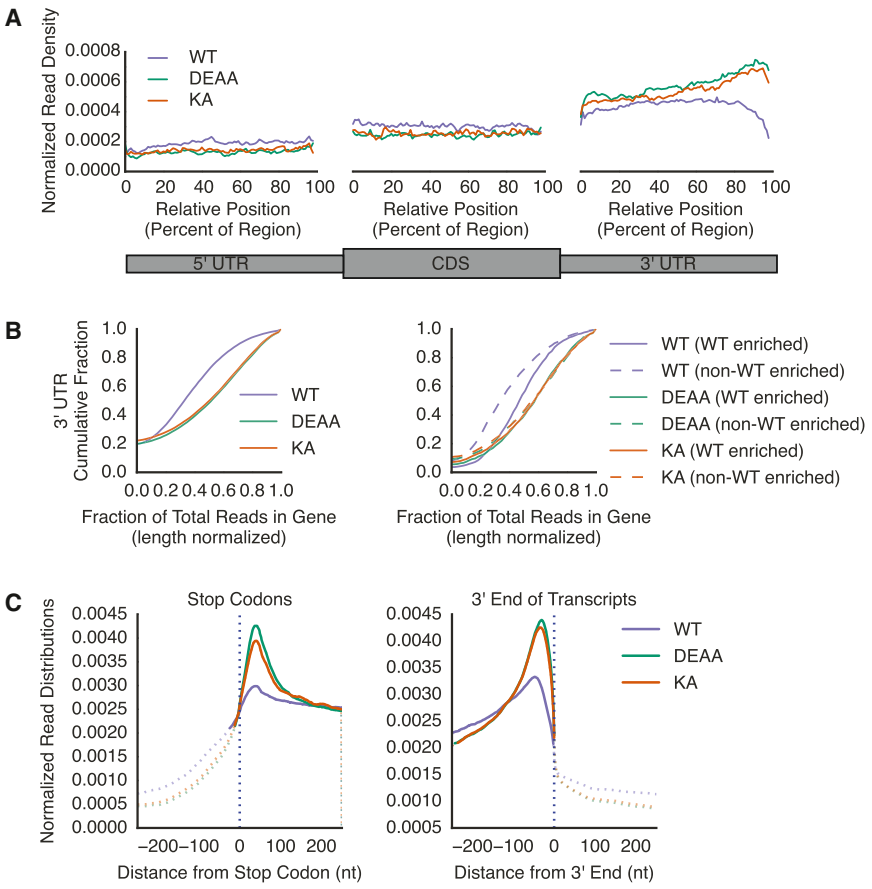

Figure 6. Upf1 ATPase Mutants Accumulate in 3′ UTRs with Enhanced Binding Downstream of Termination Codons and Near 3′ Ends

(A) Mean read density for Upf1 WT and mutant CLIP assays across the metagene, normalized to the total number of reads per gene.

(B) Cumulative fraction of genes with 3′ UTR read abundance represented as a fraction of total reads in the gene, normalized to nucleotide length, shown for all mRNAs on the left, and for WT-enriched and non-WT-enriched mRNAs, as defined in Figure 2, on the right. Differences between WT and mutant curves were statistically significant ($p < 0.001$; KS statistic 0.30 for both DEAA and KA compared with WT for non-WT-enriched and 0.20 and 0.19 for DEAA and KA, respectively, compared with WT for WT enriched).

(C) Mean read densities, shown as percentages of total reads mapped in the region depicted per mRNA, around the first nucleotide of the stop codon, shown on the left, and the 3′ end of annotated transcripts, shown on the right. Solid lines represent regions where differences were found to be significant with a p value < 0.05 (Bonferroni corrected).

See also Figure S6 and Table S2.

hydrolysis steps of the ATPase cycle (Figures 1 and 2) and the failure of ATPase-hyperactive Upf1 to accumulate on mRNA (Figure 1). Consistent with our findings, a recent study also reported loss of selective target mRNA association for Upf1 G495A/G497E, which also lacks ATPase activity (Kurosaki et al., 2014). Second, processes central to target discrimination by the NMD pathway affect Upf1 specificity in an ATPase-dependent manner, as both translation and termination-proximal PABPC1 require Upf1 ATPase activity to prevent Upf1 accumulation on non-target mRNAs (Figures 4 and 5). Third, WT Upf1 is preferentially retained on target over non-target mRNA in vitro in a manner dependent on ATP hydrolysis (Figures 7A–7C). Thus, our findings from mutational interference with three distinct aspects of the Upf1 ATPase cycle as well as the effect of a non-hydrolyzable ATP analog on WT Upf1 support the conclusion that the Upf1 ATPase cycle is critical for Upf1 target discrimination.

Upf1 ATPase Activity Prevents Accumulation of NMD Complexes on Non-target mRNAs

How does Upf1 ATPase activity contribute to Upf1 target specificity? Surprisingly, the principal contribution appears to be in preventing accumulation of Upf1 and NMD complexes on non-target mRNAs. This is evidenced by the observation that Upf1 mutants blocked in the ATPase cycle accumulate in a phosphorylated form along with downstream Smg5-7 NMD factors on non-target mRNAs (Figures 1 and 3). Moreover, Upf1 ATPase activity was required for translation- and PABPC1-dependent inhibition of Upf1 accumulation on non-target mRNAs (Figures 4 and 5). Notably, WT Upf1 also associates with non-target mRNAs, but to a lesser degree than Upf1 mutants blocked in the ATPase cycle (Figure 1), suggesting that these Upf1 mutants are stalled at a normal step in the mRNA binding dynamics of Upf1. Despite the increased accumulation of NMD complexes on non-target mRNAs in the presence of Upf1 ATP binding or ATP hydrolysis mutants, we did not observe evidence for endonucleolytic cleavage or accelerated decay of these mRNAs (S.R.L. and J.L.-A., unpublished data), consistent with previous observations for ATPase-deficient yeast Upf1 (Sheth and Parker, 2006). Thus, either the large number of mRNPs with which these mutant Upf1 proteins associate renders the availability of Upf1 or Upf1-interacting factors limiting or Upf1 ATP hydrolysis may have additional roles in downstream steps of the NMD pathway after NMD complex formation, consistent with our previous observations for target mRNAs (Franks et al., 2010) (Figure 7D). Alternatively, as proposed previously by others (Hogg and Goff, 2010; Sheth and Parker, 2006), a second discrimination step based on NMD target features may occur after NMD complex assembly and be required for degradation (commitment step in Figure 7D). In metazoans, this could include Smg6-mediated endonucleolytic cleavage stimulated by 3′ UTR-associated EJCs (Boehm et al., 2014), which are absent from the non-target mRNAs tested in this study. Regardless, our observations suggest that the Upf1 ATPase cycle plays a key role in ensuring NMD complex assembly specifically on NMD substrates, thereby preventing NMD

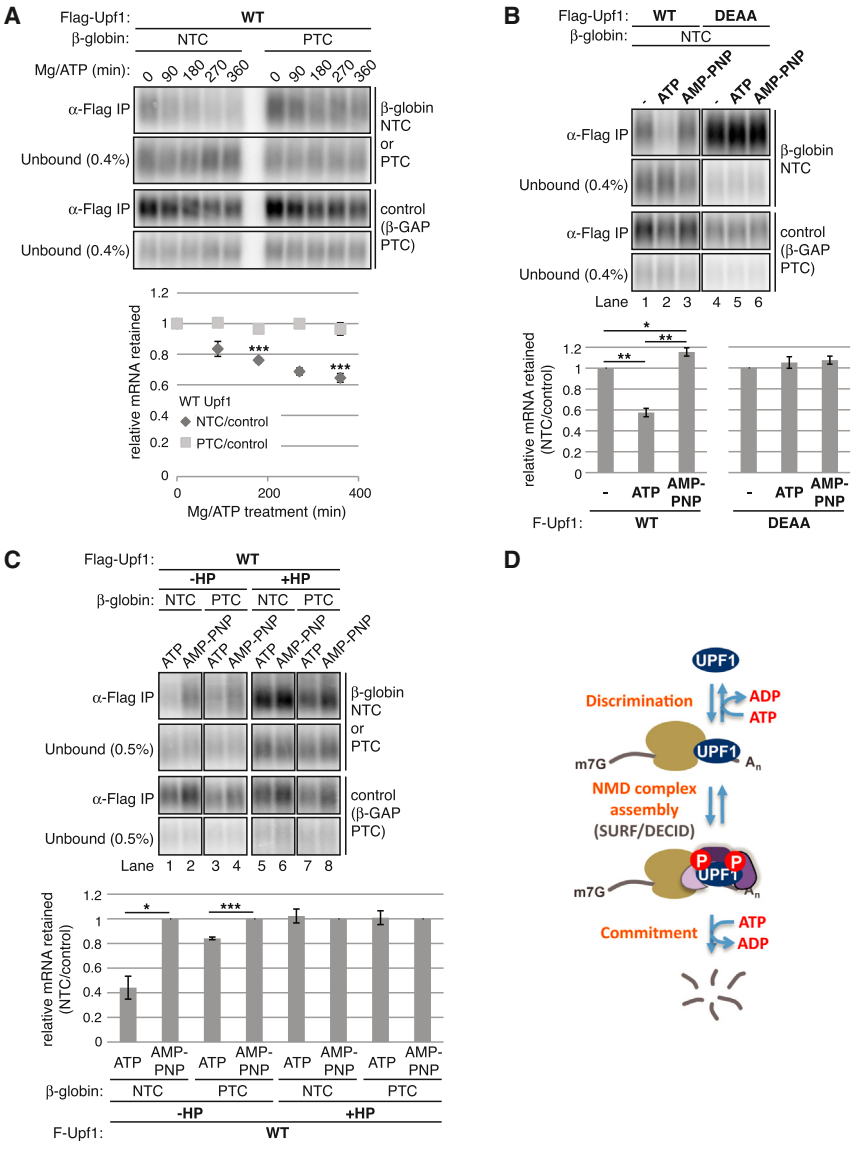

Figure 7. Upf1 ATP Hydrolysis Is Required for Faster Release of Upf1 from Non-target over Target mRNA

(A) Northern blots for β-globin and control reporter mRNAs in unbound fractions or coprecipitated with Flag-Upf1 WT from cell lysates treated with $MgCl_2$/ATP for the number of minutes indicated above lanes. Graph under northern blots shows the ratio of β-globin mRNA recovery in IPs to the internal control β-GAP PTC mRNA after subtraction of background from negative control Flag IPs as a function of $MgCl_2$/ATP treatment duration, normalized to values at time 0. Data are represented as the normalized mean ratio ± SEM for two to three biological replicates. P value calculations were restricted to time points with triplicate measurements.

(B) Northern blots of β-globin and control mRNAs in unbound fractions or coprecipitated with Flag-Upf1 from untreated lysates (–) or lysates treated with $MgCl_2$/ATP or $MgCl_2$/AMP-PNP for 360 min. Graphs under northern blots represent mean ratios ± SEM for triplicate biological repeats calculated as in (A), normalized to values for untreated lysate samples.

(C) Similar to (B) comparing release of β-globin ± HP mRNAs used in Figure 4B. Graphs under northern blots are from triplicate biological repeats normalized to values for AMP-PNP-treated samples.

(D) Model depicting the ATPase-dependent mRNA discrimination step by Upf1 preceding NMD complex assembly. A second mRNA-selective commitment step might occur prior to mRNA degradation. See text for details.

In (A)–(C), *p ≤ 0.1, **p ≤ 0.05, ***p ≤ 0.01 (paired Student's t test, two-tailed). See also Figure S7.

complexes from accumulating on non-target mRNAs and titrating out cellular NMD machinery.

Translation and PABP as Modulators of Upf1 ATPase-Dependent mRNA Specificity

Manipulating translation revealed that translation promotes Upf1 accumulation on target mRNAs, but inhibits accumulation on non-targets (Figure 4). The latter observation is consistent with previous observations for another non-target mRNA reporter (Zünd et al., 2013); our findings indicate that this effect is dependent on Upf1 ATPase activity (Figure 4). How translation affects Upf1 accumulation on mRNAs may reflect both direct interactions between Upf1 and translational machinery and translation-dependent impacts on mRNP composition. For example, direct contacts of Upf1 with the 40S ribosomal subunit (Min et al., 2013) and/or ribosome release factors (Czaplinski et al., 1998; Ivanov et al., 2008; Kashima et al., 2006; Singh et al., 2008) may contribute to the accumulation of Upf1 on target mRNAs. Conversely, the process of translational elongation may, as previously proposed (Gregersen et al., 2014; Hurt et al., 2013; Zünd et al., 2013), interfere with accumulation of Upf1 on translated regions of mRNAs. Additionally, our observation that termination-proximal PABP inhibits Upf1-mRNA accumulation in an ATPase-dependent manner (Figure 5) raises the possibility that coupling between translation termination and PABP, or PABP-associated factors, prevents Upf1-mRNA association in a manner dependent on the Upf1 ATPase. Indeed, interactions between PABP and eRF3 are well established (Cosson et al., 2002a; Hoshino et al., 1999; Kozlov and Gehring, 2010) and NMD targets are thought to undergo aberrant termination at least in part because they lack termination-proximal PABP and/or PABP-associated factors (Amrani et al., 2004; Fatscher et al., 2014; Joncourt et al., 2014; Kervestin and Jacobson, 2012; Peixeiro et al., 2012). The relative contributions of translation elongation and normal termination in limiting Upf1 accumulation on non-target mRNAs in an ATPase-dependent manner, and the mechanism by which PABP acts, remain important issues for future study.

How Does Upf1 ATPase Activity Affect Upf1-mRNA Selectivity?

Although it is possible that Upf1 ATPase activity affects its recruitment to NMD targets, our observations point instead to a target discrimination mechanism by which Upf1 ATPase activity promotes Upf1 release preferentially from non-target mRNAs (Figure 7D). This is evidenced by the dramatic increase in non-target mRNA accumulation for Upf1 mutants blocked in the ATPase cycle (Figure 1) and the ATPase-dependent release of WT Upf1 that occurs preferentially from non-target over target mRNA in vitro (Figure 7). At this point, it remains to be resolved whether the faster dissociation of Upf1 from non-target mRNA reflects direct release or involves translocation along the mRNA. A striking observation from our CLIP assays is that Upf1 mutants blocked in the ATPase cycle exhibit greater binding in mRNA 3′ UTRs compared with WT Upf1, particularly in regions just downstream of translation termination sites and at mRNA 3′ ends (Figure 6). This suggests that Upf1 ATPase activity is particularly important for release from these sites. Taken together, our observations suggest a mechanism by which Upf1 is initially recruited to both target and non-target mRNAs but is preferentially released from non-targets by a mechanism that requires activation of the Upf1 ATPase and is dependent on translation. Differential release of an RNA binding regulator for target discrimination may be particularly advantageous in RNA quality-control pathways, as it could permit rapid inspection of an entire RNA population under surveillance.

Deeper insight into the Upf1 ATPase dependence of Upf1 target specificity is likely to come through future study of the specific mechanisms by which target and non-target mRNP components differentially affect Upf1-mRNA accumulation and the Upf1 ATPase. Interestingly, *S. cerevisiae* translation release factors eRF1 and eRF3 have been found to inhibit the Upf1 ATPase in vitro (Czaplinski et al., 1998), an observation we were able to reproduce with human eRF1 (Figures S7E and S7F). This raises the possibility that Upf1 ATPase repression by release factors could cause retention of Upf1 on mRNA by a mechanism influenced by the efficiency of translation termination, which is thought to differ on NMD target and non-target mRNAs, as discussed above. Another factor reported to influence Upf1 ATPase activity in vitro is Upf2, which stimulates Upf1 ATPase activity by a mechanism inhibited by the Upf1 C terminus (Chakrabarti et al., 2011; Chamieh et al., 2008; Fiorini et al., 2013), a finding we confirmed with full-length human Upf2 (Figure S7G). Future studies should reveal whether Upf2 contributes to the Upf1 ATPase-dependent step required for target discrimination described in this study or if Upf2 promotes a step in the NMD pathway downstream of Upf1-target mRNA binding, as has been previously implicated in yeast NMD (Sheth and Parker, 2006). Collectively, our findings suggest that a key determinant for NMD is a delay in the activation of Upf1 ATPase specifically on target mRNAs, which allows NMD complexes to form and initiate degradation prior to Upf1 ATPase-mediated complex disassembly and complete mRNA degradation (Figure 7D).

EXPERIMENTAL PROCEDURES

For RIP and release assays, human Flp-In 293-T-Rex cells (with or without stably expressed Upf1 variants as indicated in figures) were transfected with CMV promoter-driven reporter mRNA constructs, as well as protein expression constructs and, if used, siRNAs for protein depletion, 48–72 hr before cell harvest. At 20–24 and 3–3.5 hr before harvest, Flag-Upf1 expression in stable cell lines was induced at close to endogenous levels with tetracycline, and cells used in Figure 3 were treated with okadaic acid, respectively. Cell extracts prepared in isotonic lysis buffer were subjected to IP with indicated antibodies (see Supplemental Experimental Procedures for details). Phosphatase Arrest I (G Biosciences) was included in all extracts prepared for Figure 3 except those pre-treated with λ protein phosphatase. Protein and Trizol (Invitrogen)-extracted RNA samples prepared from input and IP samples were subjected to western and northern or RNA-seq analysis, respectively. RIP-seq libraries were prepared using an Illumina TruSeq Stranded Total RNA HT Sample Prep Kit with Ribo-Zero Gold for rRNA depletion. CLIP-seq libraries were prepared as described previously (Yeo et al., 2009), with slight modifications (see Figure S6A and Supplemental Experimental Procedures for details).

ACCESSION NUMBERS

The expression number for the sequencing data reported in this paper is GEO: GSE69586.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2015.06.036.

AUTHOR CONTRIBUTIONS

S.R.L. and J.L.-A. conceived of the study and wrote the manuscript, with input from G.W.Y., G.A.P., and F.J.M. G.W.Y., G.A.P., and F.J.M. carried out RIP-seq and CLIP-seq. J.L.-A. performed in vitro ATPase assays. G.W.Y. and G.A.P. conducted the bioinformatics analyses. S.R.L. executed all other experiments.

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