

A conserved role for cytoplasmic poly(A)-binding protein 1 (PABPC1) in nonsense-mediated mRNA decay

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The nonsense-mediated mRNA decay (NMD) pathway degrades mRNAs with premature translation termination codons (PTCs). The mechanisms by which PTCs and natural stop codons are discriminated remain unclear. We show that the position of stops relative to the poly(A) tail (and thus of PABPC1) is a critical determinant for PTC definition in *Drosophila melanogaster*. Indeed, tethering of PABPC1 downstream of a PTC abolishes NMD. Conversely, natural stops trigger NMD when the length of the 3' UTR is increased. However, many endogenous transcripts with exceptionally long 3' UTRs escape NMD, suggesting that the increase in 3' UTR length has co-evolved with the acquisition of features that suppress NMD. We provide evidence for the existence of 3' UTRs conferring immunity to NMD. We also show that PABPC1 binding is sufficient for PTC recognition, regardless of cleavage or polyadenylation. The role of PABPC1 in NMD must go beyond that of providing positional information for PTC definition, because its depletion suppresses NMD under conditions in which translation efficiency is not affected. These findings reveal a conserved role for PABPC1 in mRNA surveillance.

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Introduction

Nonsense-mediated mRNA decay (NMD) is a conserved mRNA quality control mechanism (surveillance) that ensures the fidelity of gene expression by detecting and degrading mRNAs containing premature translation termination codons (PTCs, nonsense codons). In this way, NMD safeguards cells from accumulating potentially deleterious truncated proteins (reviewed by Conti and Izaurralde 2005; Lejeune and Maquat, 2005; Amrani *et al*, 2006).

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The NMD pathway serves not only to degrade mRNAs containing PTCs (as a consequence of mutations or errors in transcription or mRNA processing), but also regulates the expression of about 3–10% of the transcriptome in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and human cells. These natural NMD targets play a role in biological processes as diverse as transcription, cell proliferation, the cell cycle, telomere maintenance, cellular transport and organization, and metabolism (reviewed by Rehwinkel *et al*, 2006).

NMD is triggered by premature translation termination, which leads to the assembly on the mRNA of a so-called surveillance complex. The surveillance complex comprises the conserved NMD effectors UPF1, UPF2 and UPF3, and couples the premature translation termination event to mRNA decay by interacting with both eukaryotic translation termination factors (i.e. eRF1 and eRF3) and with mRNA degradation enzymes (Conti and Izaurralde 2005; Lejeune and Maquat, 2005; Amrani *et al*, 2006).

Stop codons are recognized as premature depending on their location relative to downstream sequence elements (DSEs) and associated proteins (Conti and Izaurralde 2005; Lejeune and Maquat, 2005; Amrani *et al*, 2006). In mammals, these downstream sequences are represented by exon–exon boundaries. Indeed, stop codons located at least 50–55 nt upstream of an exon–exon boundary are generally defined as premature, whereas most PTCs downstream of this point do not elicit decay (Nagy and Maquat, 1998).

Exon–exon boundaries are marked by the exon junction complex (EJC), which is deposited during splicing 20–24 nt upstream of a splice junction (Le Hir *et al*, 2000). Current models for mammalian NMD postulate that UPF3 associates with the EJC within the nucleus and recruits UPF2 following the export of the mRNA to the cytoplasm. When translating ribosomes encounter a stop codon upstream of an EJC, the recruitment of UPF1 by translation release factors leads to an interaction with the UPF2 and UPF3 proteins bound to the downstream EJC, and thus to the assembly of the surveillance complex and to mRNA degradation (reviewed by Lejeune and Maquat, 2005).

In *S. cerevisiae* and *D. melanogaster*, PTC recognition occurs independently of splicing, and different models have been proposed to explain what distinguishes premature from natural stops in these organisms. One model proposes that mRNAs harbor loosely defined DSEs with a function analogous to that of mammalian exon junctions (reviewed by Amrani *et al*, 2006). Alternative models suggest that a generic feature of the mRNA, such as the poly(A) tail, or a mark deposited during the cleavage and polyadenylation reaction could provide positional information to discriminate premature from natural stop codons (Hilleren and Parker, 1999; Muhrad and Parker, 1999; Palaniswamy *et al*, 2006).

Yet another model, the ‘faux 3' UTR model’, proposes that premature translation termination is intrinsically aberrant

because the stop codon is not in the appropriate context (Amrani *et al*, 2004, 2006). According to this model, natural 3' UTRs are marked by a specific set of proteins that influence translation termination. Termination is efficient at natural stops because terminating ribosomes are able to interact with these 3' UTR-bound proteins. In contrast, translation termination would be impaired or too slow at premature stops, because of the inability of the terminating ribosome to establish these interactions. In this case, the surveillance complex is assembled, leading to the rapid degradation of the mRNA (Amrani *et al*, 2004, 2006).

In support of the 'faux 3' UTR model', experiments in *S. cerevisiae* have shown that translation termination is aberrant at premature stop codons, and prematurely terminating ribosomes are not released efficiently (Amrani *et al*, 2004, 2006). This effect is abolished by flanking the nonsense codon with a normal 3' UTR. Moreover, tethering the poly(A)-binding protein (Pab1p) downstream of a PTC, which is likely to mimic a normal 3' UTR, leads to efficient translation termination and abolishes NMD (Amrani *et al*, 2004, 2006). This suggests that proximal Pab1p binding defines natural stops in *S. cerevisiae*. Consistently, most *S. cerevisiae* 3' UTRs are homogeneous in length (ca. 100 nt; Graber *et al*, 1999), and aberrant transcripts with exceptionally long 3' UTRs (due to errors in 3'-end processing) are regulated by NMD (Muhlrad and Parker, 1999).

In multicellular organisms, 3' UTR length distribution ranges from a few to several thousand nucleotides, raising the question of whether the faux 3' UTR model could account for PTC recognition. We have investigated the mechanism of PTC recognition in *D. melanogaster*. We show that the cytoplasmic poly(A)-binding protein (PABPC1) provides positional information discriminating premature from natural stops in this organism. Consistently, natural stops can be made to trigger NMD by increasing the 3' UTR length. However, a large proportion of naturally occurring transcripts with exceptionally long 3' UTRs are not NMD substrates, suggesting that some of these 3' UTRs may have evolved features to avoid NMD. Supporting this possibility, we show that 3' UTRs specifying rapid decay also confer immunity to NMD. Finally, we demonstrate that depletion of PABPC1 inhibits NMD, revealing a new role for this protein in mRNA surveillance.

Results

Position effects of nonsense codons in *D. melanogaster*

To gain further insights into the mechanism of PTC recognition in *D. melanogaster*, we introduced stop codons over the whole length of the *D. melanogaster* alcohol dehydrogenase (*adh*) open reading frame (ORF) C terminally fused to a V5 epitope. The *adh*-V5 ORF was cloned downstream of the actin 5C promoter and upstream of a polyadenylation site derived from SV40 (Figure 1A). The natural stop in this construct is located at codon 289. *D. melanogaster* Schneider cells (S2 cells) were transiently transfected with the reporters and a plasmid encoding a truncated version of *adh* (*adhΔ*), which served as a transfection control. In the experiments described below, the steady-state levels of the reporter mRNA were analyzed by Northern blot and normalized to those of the *adhΔ* mRNA.

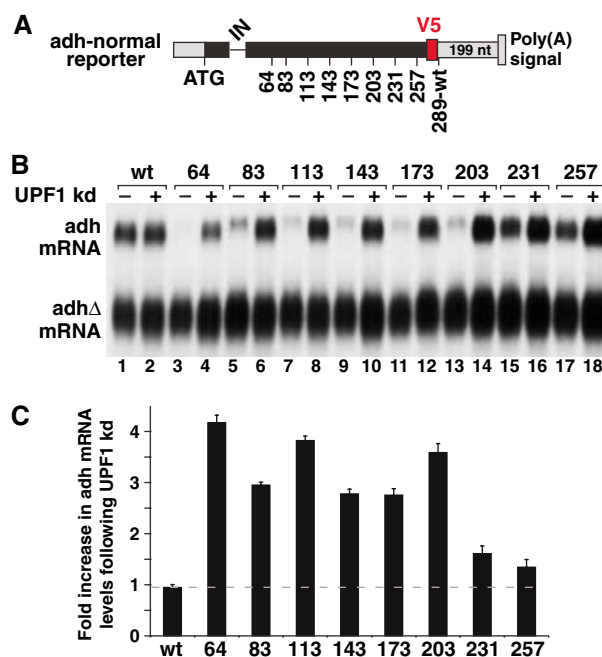


Figure 1 Boundary-dependent NMD in *D. melanogaster*. (A) Schematic representation of the *adh* reporters. Black boxes: exons; gray boxes: sequences derived from vector pAc5.1B; red box: V5 epitope; IN: intron. (B, C) S2 cells were transfected with vectors expressing *adh*-wt or the PTC reporters indicated above the lanes. A truncated version of *adh* (*adhΔ*) served as a transfection control. Transfected cells were then divided into two pools and treated with either GFP dsRNA (-) or UPF1 dsRNA (+). Total RNA samples were analyzed by Northern blot (B). The levels of the *adh* reporters were normalized to the levels of *adhΔ* mRNA. For each reporter, the normalized values obtained in UPF1-depleted cells were divided by those obtained in control cells (C). Mean values \pm s.d. of three independent experiments are shown. Kd: knockdown.

Wild-type *adh* mRNA (*adh*-wt) was expressed at higher levels than *adh* mRNAs carrying PTCs at codon 64 or 83 (*adh*-64 or *adh*-83; Figure 1B, lane 1 versus lanes 3 and 5), as reported before (Gatfield *et al*, 2003). Similarly, the presence of PTCs at codons 113, 143, 173 and 203 led to a significant reduction of mRNA reporter levels, indicating that PTCs at codon 203 and upstream of it promote mRNA decay (Figure 1B, lanes 7, 9, 11 and 13). In contrast, mRNAs containing PTCs at codon 231 or 257 were expressed at levels comparable to those of the wild-type mRNA (Figure 1B, lanes 15 and 17), indicating that these PTCs are impaired in promoting mRNA degradation.

To confirm that differences in reporter expression levels reflect genuine NMD, we analyzed whether these levels could be restored by depletion of the essential NMD effector UPF1. For the reporters carrying PTCs at codon 203 and upstream, depletion of UPF1 resulted in a three- to four-fold increase of mRNA levels (Figure 1B and C). The expression levels of *adh*-231 and *adh*-257 increased ca. 1.5-fold, whereas those of the wild-type mRNA did not change significantly (Figure 1B and C). These results indicate that the PTC-containing reporters are downregulated by NMD. They also show that there is a clear boundary effect rather than a polar effect of PTCs in this reporter, such that PTCs at codon 203 or upstream are efficient NMD triggers and those downstream cause modest NMD. In support of this, mRNAs with PTCs at codon 64 or 203 have identical half-lives (see Figure 2F). Thus, the extent

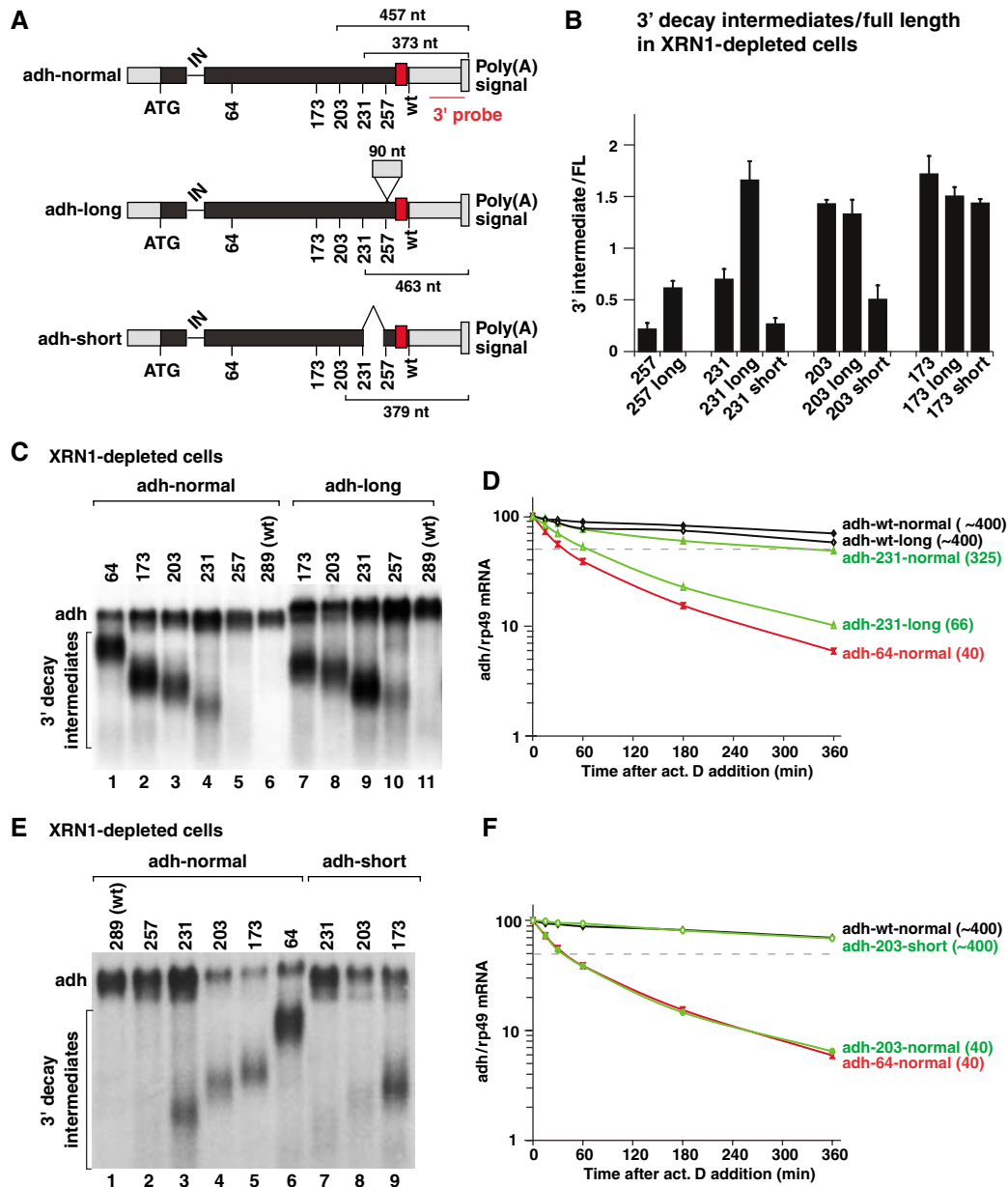


Figure 2 The ability of a PTC to trigger NMD depends on its distance from the 3' UTR. (A) Schematic representation of the adh reporters. Symbols are as in Figure 1A. The distance of some PTCs to the polyadenylation site, as well as the positions of the insertion and deletion in adh-long and adh-short, respectively, are indicated. (B, C, E) S2 cells depleted of XRN1 were transfected with the reporters indicated above the lanes. RNA samples were analyzed by Northern blot (C, E) using a probe hybridizing to the 3' UTR of the reporters (as shown in panel A). The levels of the 3' decay intermediates were normalized to the levels of the full-length transcript (B). Mean values \pm s.d. of three independent experiments are shown. (D, F) S2 cells expressing the indicated adh reporters were treated with actinomycin D. Total RNA samples were collected at the indicated time points and analyzed by Northern blot. The levels of the adh reporters normalized to the levels of rp49 mRNA are plotted as a function of time. mRNA half-lives are indicated in brackets.

of mRNA degradation depends on the position of the stop codon relative to downstream sequences.

The ability of a nonsense codon to trigger mRNA degradation is modulated by changing its distance to the 3' UTR

The positional effect of PTCs suggests that definition of a stop codon as premature depends on its position relative to either the 3' UTR or to a DSE, which in our reporter should be between codons 203 and 231. In previous studies, we have shown that specific DSEs are unlikely to occur in *D. melanogaster*

as PTC-containing mRNAs from bacterial origin are subject to NMD (Gatfield *et al*, 2003). If the distance to the 3' UTR is the critical determinant for NMD, increasing the distance of PTC 231 or 257 to the 3' UTR should increase the efficiency of these PTCs in eliciting mRNA decay. Conversely, decreasing the distance of PTCs 203 or 231 to the 3' UTR should stabilize the transcript. To test this hypothesis, we inserted a 90 nt DNA fragment derived from the 3' UTR of SV40 immediately downstream of PTC 257 and upstream of the natural stop. This DNA fragment has no stop codons, and in the wild-type context extends the adh ORF by

30 aa (Figure 2A, adh-long). In other words, relative to the poly(A) tail this insertion brings PTC 231 to the position occupied by PTC 203 in the normal context (Figure 2A).

To measure NMD efficiency, we took advantage of the observation that NMD in *D. melanogaster* is initiated by endonucleolytic cleavage in the vicinity of the PTC. The resulting 3' decay intermediate is digested exonucleolytically by the 5' to 3' exonuclease XRN1 (Gatfield and Izaurralde, 2004). Consequently, in cells depleted of XRN1, this fragment is stabilized and the extent of its accumulation relative to the full-length transcript is proportional to NMD efficiency. Using the relative accumulation of the 3' decay intermediates in XRN1-depleted cells, we observed that increasing the distance of PTCs 231 or 257 to the 3' UTR (adh-long reporter) triggered NMD more efficiently than in the normal context (Figure 2B and C, lanes 9 and 10 versus 4 and 5).

These results were confirmed by measuring the half-lives of a transcript carrying a PTC at codon 231 in the normal or longer reporter (adh-231-normal or adh-231-long). The half-life of adh-231-normal mRNA was ca. 325 min (Figure 2D). When the distance of this PTC to the 3' UTR was increased, its half-life was reduced to 66 min, comparable to that of a normal transcript with a PTC at codon 64 (Figure 2D). The insertion did not affect significantly the half-life of the adh wild-type reporter (Figure 2D).

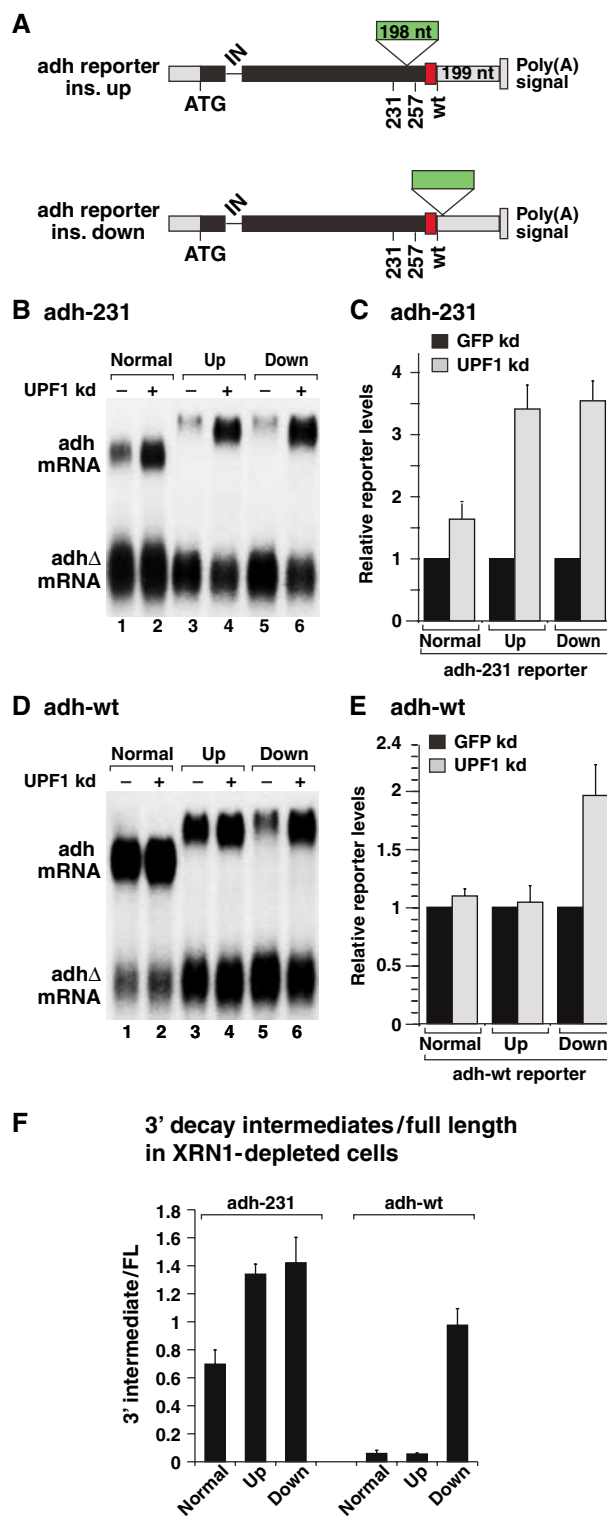
We next tested whether reducing the distance to the 3' UTR could convert an efficient PTC into an inefficient trigger of NMD. To this end, we deleted a 78 nt DNA fragment between codons 231 and 257 (Figure 2A, adh-short). This deletion shifts PTC-203 relative to the 3' end to the equivalent position of PTC-231 in the normal reporter (Figure 2A). Using the accumulation of the 3' decay intermediates in XRN1-depleted cells, we observed that decreasing the distance of PTC 203 or PTC 231 to the 3' UTR inhibited NMD (Figure 2B and E, lanes 7 and 8 versus 3 and 4, respectively), whereas the potential to elicit decay of the PTC 173 further upstream was unaltered (Figure 2B and E, lanes 5 and 9). These results were also confirmed by measuring mRNA half-lives. The half-life of adh-203 mRNA in the natural context was ca. 40 min. When the distance to the 3' UTR was decreased, the half-life increased to more than 400 min (Figure 2F). The half-life of adh wild-type mRNA was not affected significantly by the deletion (data not shown).

These results show that there is no DSE between codons 203 and 231, and clearly establish that it is the distance of the stop codon to the poly(A) tail (or other features of the 3' UTR) that plays a critical role in NMD.

Figure 3 Redefinition of a natural stop as premature by increasing the length of the 3' UTR. (A) Schematic representation of the adh reporters having an insertion upstream (ins. up) or downstream (ins. down) of the natural stop (green boxes). Symbols are as in Figure 1A. (B–E) S2 cells were transfected with vectors expressing the indicated reporters. Plasmid adhΔ served as a transfection control. Transfected cells were treated with GFP (–) or UPF1 (+) dsRNAs. Panels B and D show representative Northern blots. In panels C and E, the levels of the adh mRNA reporters were normalized to the levels of adhΔ mRNA in three independent experiments. For each reporter, these ratios were set to 1 in control cells treated with GFP dsRNA (black bars). Mean values ± s.d. are shown. (F) The levels of the 3' decay intermediates accumulating in XRN1-depleted cells were normalized to the levels of the full-length transcript. Mean values ± s.d. of three independent experiments are shown.

Natural stops are redefined as premature when the length of the 3' UTR is increased

If the distance of the PTC to the poly(A) tail of the transcript plays a critical role in PTC recognition, one could also expect to convert a natural stop into a premature stop by increasing the length of the 3' UTR. To test this possibility, we inserted a 198 nt cDNA fragment derived from the bacterial β-lactamase gene, upstream or downstream of the natural stop, thus



doubling the length of the 3' UTR (Figure 3A, ins. up or ins. down). Insertion of this fragment either upstream or downstream of the natural stop increased the efficiency of PTC-231 in triggering NMD (Figure 3B, C and F). This lends further support to the conclusion that specific DSEs within the ORF are unlikely to provide positional information for PTC-definition in *D. melanogaster*, as shown in Figure 2.

Remarkably, out-of-frame insertion of the β -lactamase cDNA fragment downstream of the natural stop decreased the expression levels of the wild-type reporter in a UPF1-dependent manner (Figure 3D and E). That this reporter is indeed subject to NMD was confirmed by analyzing the accumulation of the corresponding 3' decay intermediate in cells depleted of XRN1 (Figure 3F). Altogether, these results indicate that extending the 3' UTR length does indeed result in the redefinition of the natural stop as premature.

Binding of PABPC1 provides positional information for PTC-definition regardless of cleavage or polyadenylation

The experiments described above provide strong evidence for a role of 3' UTRs in PTC recognition: either the cleavage and polyadenylation reaction deposits a mark on the mRNA to provide positional information (see Palaniswamy *et al*, 2006) or PABPC1 itself plays a role in this process. To discriminate between these possibilities, we designed adh reporters in which the cleavage and polyadenylation signal is replaced either by the *D. melanogaster* histone H4 3' stem-loop structure (Adamson and Price, 2003) or by a self-cleaving hammerhead ribozyme element (Dower *et al*, 2004; Figure 4A; adh-SL and adh-HhR reporters).

When the 3' end of the transcript was generated by the histone 3'-end-processing machinery, a reporter bearing a PTC at codon 64 was no longer regulated by NMD, as judged by the observation that the steady-state levels of the reporter did not change upon UPF1 depletion (64-SL reporter, Figure 4B, lanes 7 and 8; Figure 4C). Similar results were obtained for the reporter containing the hammerhead ribozyme (adh-HhR reporter). Indeed, the expression levels of adh-64-HhR mRNA (carrying a PTC at codon 64) were unaffected by UPF1 depletion (Figure 4D and E, lanes 3 and 4).

Importantly, wild-type mRNAs whose 3' ends are generated by ribozyme cleavage or by the histone 3'-end-processing machinery are exported to the cytoplasm and translated, as judged by the detection of the ADH-V5 fusion protein by Western blot (Figure 4F and G; Duvel *et al*, 2002; Dower *et al*,

2004). Furthermore, when we compared the expression levels of the ADH-V5 protein with the corresponding mRNA levels, we saw that the translation efficiency of these non-polyadenylated mRNAs was comparable to that of the mRNAs having a poly(A) tail (Figure 4G). This indicates that the absence of NMD for PTC-containing, unadenylated mRNAs cannot be attributed to reduced translation efficiency.

To determine whether susceptibility to NMD could be restored by binding of PABPC1, we followed two approaches.

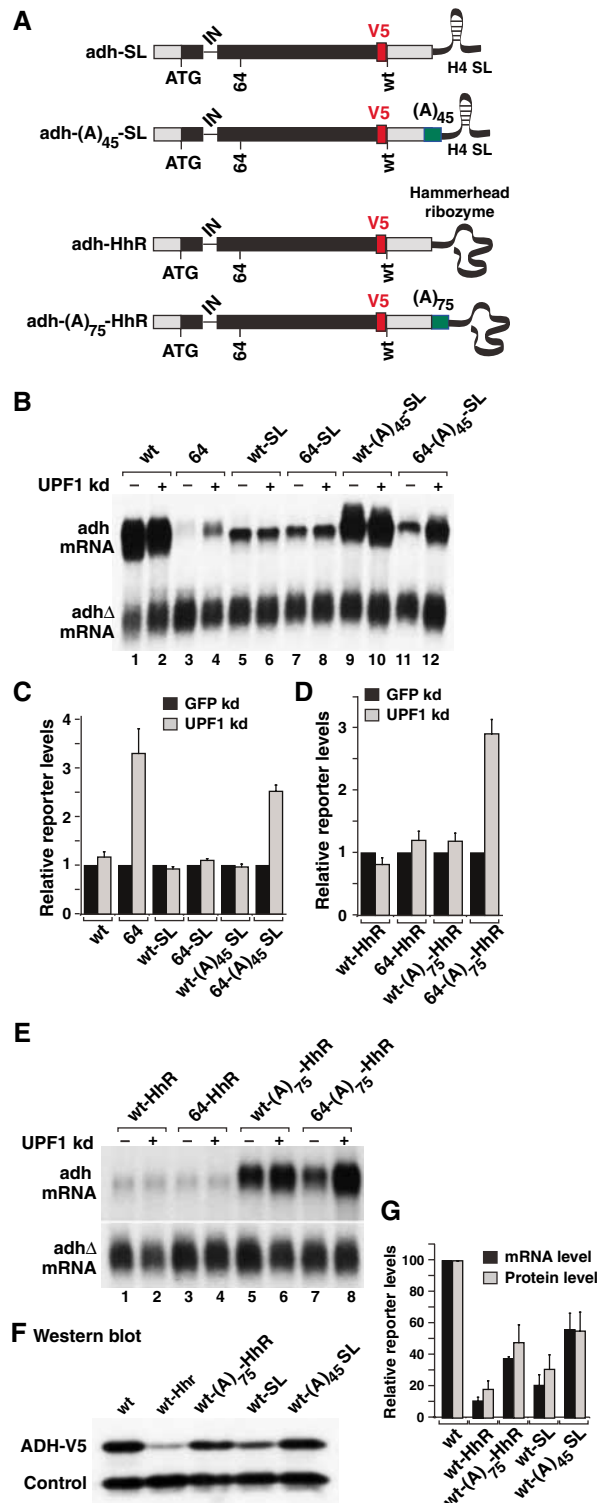


Figure 4 Binding of PABPC1, regardless of cleavage and polyadenylation, is sufficient for PTC definition. (A) Schematic representation of the adh reporters in which the polyadenylation signal of SV40 is replaced by the histone H4 3' stem-loop (SL) or a self-cleavable hammerhead ribozyme (HhR). Symbols are as in Figure 1A. The insertion of a poly(A) stretch is indicated by a green box. (B-E) S2 cells transfected with the reporters indicated above the panels were treated with GFP (-) or UPF1 (+) dsRNAs. Panels B and E show representative Northern blots. In panels C and D, the levels of the adh reporters were normalized to the levels of adh Δ mRNA in three independent experiments and analyzed as described in Figures 3C and E. (F, G) S2 cells were transfected with the indicated adh reporters. Total protein and RNA samples were analyzed by Western and Northern blots, respectively, and quantitated in three independent experiments.

First, we inserted a stretch of DNA-encoded adenosine residues immediately upstream of the histone stem-loop structure or of the ribozyme element (Figure 4A; adh-(A)₄₅-SL and adh-(A)₇₅-Hhr). It has previously been shown that a stretch of at least 45 adenosines is sufficient for PABPC1 binding *in vivo* (Dower *et al*, 2004). The artificial poly(A) tails rescued the ability of PTC-64 to promote NMD in both cases (Figures 4B–E). The insertion of the poly(A) stretch increased steady-state mRNA levels (Figure 4B and E), but had no effect on the translation efficiency of the corresponding wild-type mRNAs (Figure 4F and G). This observation supports the conclusion that the failure of PTC-64 to trigger mRNA decay in the reporters lacking a stretch of adenosine residues is not caused by inefficient translation.

In the second approach, we used a tethering assay based on the high-affinity interaction between the RNA-binding peptide derived from the bacteriophage-λN protein (λN-peptide) and an RNA hairpin known as BoxB element (Gehring *et al*, 2003). A chimeric protein consisting of the λN-peptide fused to *D. melanogaster* PABPC1 was coexpressed with adh reporter mRNAs in which five BoxB elements were inserted immediately upstream of the hammerhead ribozyme. Tethering of λN-PABPC1 increased mRNA levels and partially rescued the ability of PTC 64 to promote NMD (Supplementary Figure 1) as with the insertion of an artificial poly(A) tail. In summary, these results indicate that PABPC1 is sufficient to provide positional information for PTC recognition, regardless of 3'-end cleavage and polyadenylation.

PABPC1 tethered downstream of a PTC abolishes NMD

We next tested whether tethering PABPC1 downstream of a PTC could convert it into an NMD-insensitive stop. To this end, we coexpressed λN-PABPC1 with adh reporter mRNAs in which four BoxB elements were inserted at codon 117 (Figure 5A, 4BoxB). To control for nonspecific effects that may arise by overexpressing λN-PABPC1, we coexpressed untaged PABPC1 with the reporters.

Tethering of PABPC1 downstream of PTCs 64, 83 and 113 suppressed NMD in a polar manner: the closer the PTC to the tethering site, the better the suppression by PABPC1 (Figure 5B and C). Consistently, the half-life of adh-113 mRNA increased when PABPC1 was tethered immediately downstream of this PTC (Figure 5D). In contrast, PABPC1 did not suppress NMD for PTCs located downstream of the tethering site (PTCs 143 and 173; Figure 5B and C).

To examine whether the effects of PABPC1 are specific, we also tested the effect of tethering the nuclear poly(A)-binding protein 1 (PABPN1) downstream of adh-113. In contrast to PABPC1, which suppresses NMD, PABPN1 has no effect, despite both proteins being expressed at comparable levels (Figure 5E–G).

The results obtained for the adh reporter were validated with a second reporter based on the bacterial chloramphenicol acetyl transferase (CAT) gene (Figure 6). Indeed, tethering of PABPC1 downstream of codon 129 suppresses NMD triggered by PTCs upstream, but not downstream, of the tethering site (Figure 6A–C). In agreement with these observations, the half-life of CAT-126 increased when PABPC1 was tethered immediately downstream (Figure 6D and E). We conclude that, as in *S. cerevisiae* (Amrani *et al*, 2004, 2006), tethering of PABPC1 downstream of a PTC in *D. melanogaster* abolishes NMD in a polar manner.

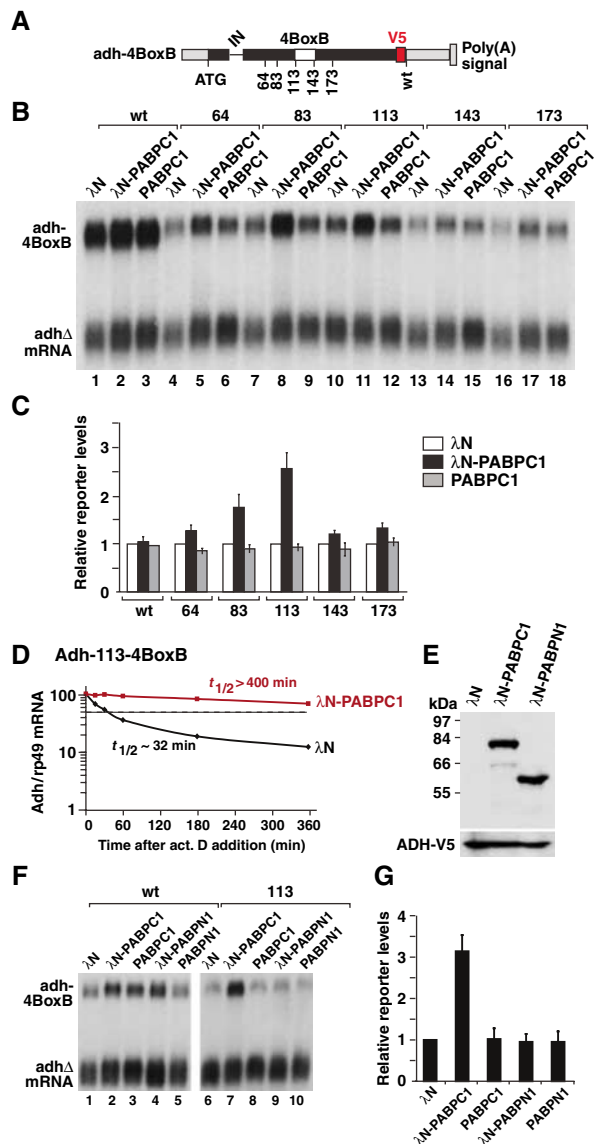


Figure 5 Tethering of PABPC1 suppresses NMD in a polar manner. (A) Schematic representation of the adh-4BoxB reporters. Symbols are as in Figure 1A. White box: 4BoxB tethering sites. (B, C) S2 cells were transfected with the adh-4BoxB reporters, adhΔ and plasmids encoding the λN-peptide, λN-PABPC1 or untaged PABPC1 as indicated. Total RNA samples were analyzed by Northern blot (B). The levels of the adh reporters were normalized to the levels of adhΔ mRNA. For each reporter, these levels were set to 1 in cells expressing the λN-peptide alone (C, white bars). Mean values ± s.d. of three independent experiments are shown. (D) S2 cells expressing Adh-113-4BoxB in the presence of the λN-peptide or λN-PABPC1 were treated with actinomycin D. Samples were collected at the indicated time points and analyzed by Northern blot. The levels of the adh mRNA normalized to the levels of rp49 mRNA are plotted as a function of time. The half-lives of the adh mRNA are indicated. (E–G) S2 cells were transfected with adh-wt-4BoxB or adh-113-4BoxB and plasmids encoding the proteins indicated above the lanes. Samples were analyzed as described in panel C. In panel E, the expression levels of PABPC1 and PABPN1 were analyzed by Western blot. ADH-V5 served as a transfection control.

The polar effect of tethered PABPC1 is in contrast with the boundary effect observed when the PTC is moved in the context of a natural poly(A) tail (Figure 1). This suggests that tethering of PABPC1 may not fully reproduce its normal function when it binds directly to poly(A) tail of the mRNA.

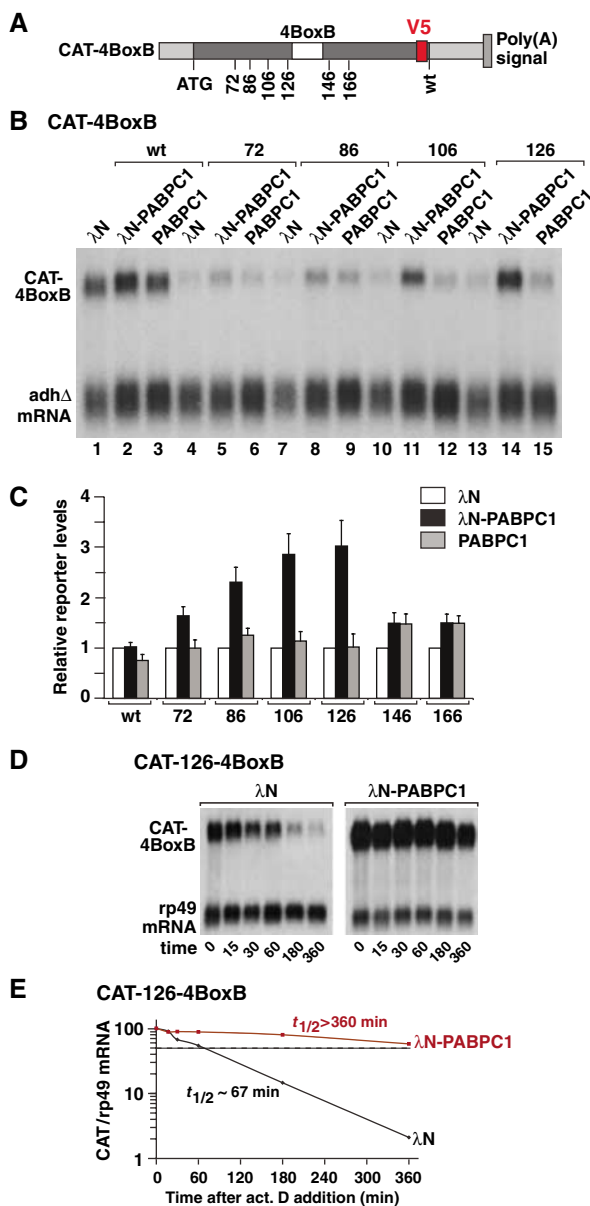


Figure 6 Tethered PABPC1 suppresses NMD. (A) Schematic representation of the CAT-4BoxB reporters. Symbols are as in Figure 5A. (B, C) S2 cells were transfected with vectors expressing CAT-4BoxB reporters and plasmids encoding the proteins indicated above the lanes. Total RNA samples were analyzed by Northern blot (B). In panel C, the levels of the CAT reporters were analyzed as described in Figure 5C. (D, E) S2 cells expressing CAT-126-4BoxB in the presence of the λN-peptide or λN-PABPC1 were treated with actinomycin D. Samples were analyzed as described in Figure 5D.

Indeed, only four PABPC1 molecules can be tethered to the reporter; they do not contact the RNA directly, and may not interact with each other as they normally would (Bernstein and Ross, 1989).

Most endogenous transcripts with long 3' UTRs are not regulated by NMD

The results described above indicate that PABPC1 plays a critical role in NMD but do not determine completely whether it is the distance to PABPC1 alone that distinguishes a premature from a normal stop or whether sequence context

also plays a role. If the distance to the PABPC1-binding site were alone sufficient to discriminate normal versus premature stops, transcripts with 3' UTRs longer than average would be expected to be regulated by NMD. In a previous study in which we identified endogenous NMD targets by expression profiling of cells depleted of NMD factors, we reported that NMD targets are not enriched significantly with transcripts having long 3' UTRs (Rehwinkel *et al*, 2005). Indeed, detectable transcripts in S2 cells have an average 3' UTR length of 450 nt, whereas the average 3' UTR length of NMD targets is 520 nt. When we analyzed the length distribution of 3' UTRs in windows of 500 nt, we saw that NMD targets are slightly enriched (ca. two-fold) in transcripts having 3' UTRs longer than 1500 nt, relative to all detectable transcripts (data not shown). Nevertheless, many transcripts with long 3' UTRs are not substrates for NMD, indicating that length is not the only determinant.

The 3' UTR of hsp70 mRNA confers immunity to NMD

One possible explanation for the observation that not all transcripts with long 3' UTRs are regulated by NMD is that some of these 3' UTRs may have evolved sequences that confer immunity to NMD. In human cells, PTCs in histone or heat-shock mRNAs do not elicit NMD, because these transcripts derive from intronless genes and are devoid of EJC (Maquat and Li, 2001). Figure 4 shows that PTCs in histone transcripts are unlikely to elicit NMD in *D. melanogaster*. We therefore speculate that the heat-shock mRNAs may also escape NMD in *D. melanogaster* and that their 3' UTRs are good candidates for having features that antagonize NMD.

To test this possibility, we replaced the 3' UTR of the adh and CAT reporters described above by the 3' UTR of hsp70, which is comparable in length (268 nt). We observed that PTCs that elicited NMD efficiently in the normal context (e.g. CAT-72 or adh-64) no longer decrease the expression levels of the reporter carrying the 3' UTR of hsp70 (Figure 7A–D). Consistently, the steady-state levels of PTC-containing transcripts having the hsp70 3' UTR were unaffected by UPF1 depletion (Figure 7A–D). These results provide strong evidence for the existence of 3' UTRs immune to NMD.

3' UTRs specifying rapid decay confer immunity to NMD

The results described so far show that at least three different classes of nonsense mRNAs escape NMD: (1) ribozyme-terminated mRNAs; (2) unadenylated mRNAs whose 3' ends are generated by the histone 3'-end-processing machinery; and (3) mRNAs with a 3' UTR derived from hsp70. In *S. cerevisiae*, ribozyme-terminated mRNAs are rapidly degraded by the cytoplasmic exosome and/or XRN1 (Dower *et al*, 2004; Meaux and van Hoof, 2006). Moreover, the hsp70 3' UTR contain several AU-rich elements that promote rapid decay. To determine whether the immunity of these transcripts to NMD is due to their intrinsic instability, we measured the half-lives of the reporters. We observed that in all three cases, the half-lives of wild-type and the corresponding nonsense transcripts were nearly identical (Supplementary Figure 2), providing further evidence that these transcripts are not NMD substrates. A surprising result is that these half-lives (ca. 20 min) are similar to the half-lives of PTC-containing reporters with a normal 3' UTR (e.g. adh-64). Thus, immunity to NMD is not due to the presence of stabilizing sequence elements that antagonize degradation.

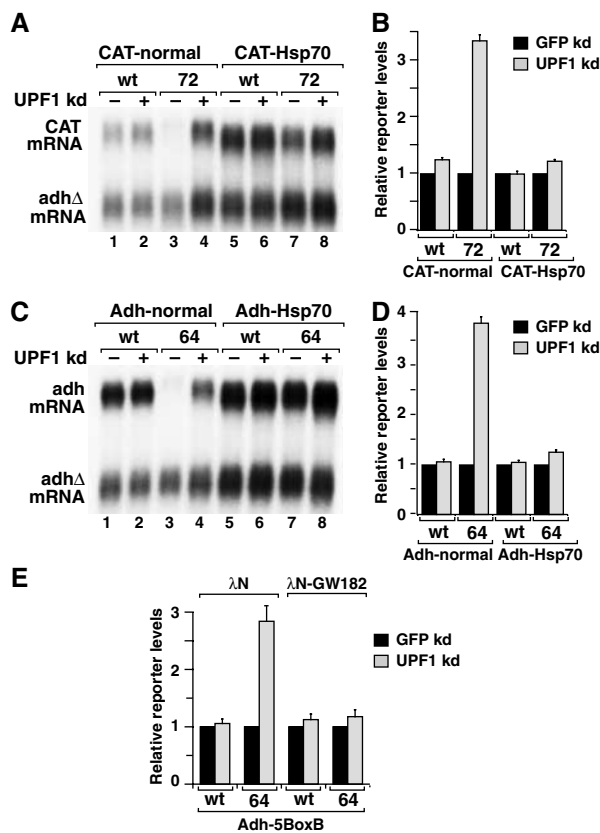


Figure 7 Hsp70 3' UTR counteracts NMD. (A–D) S2 cells transfected with vectors expressing the reporters indicated above the panels were treated with GFP (–) or UPF1 (+) dsRNAs. Panels A and C show representative Northern blots. The levels of the adh or CAT reporters were normalized to the levels of adhΔ mRNA in three independent experiments (B, D). For each reporter, these normalized values were set to 1 in cells treated with GFP dsRNA (black bars). Mean values ± s.d. are shown. (E) Cells were transfected with vectors expressing adh or adh-64 reporters carrying 5BoxB elements inserted in the 3' UTR, adhΔ and plasmids encoding λN or λN-GW182. Transfected cells were treated with GFP or UPF1 dsRNAs as indicated. Total RNA samples were analyzed by Northern blot (not shown). The levels of the adh reporters were normalized to the levels of the adhΔ transfection control. For each reporter, these levels were set to 1 in control cells treated with GFP dsRNA (black bars). Mean values ± s.d. of three independent experiments are shown.

Insertion of a poly(A) stretch upstream of the ribozyme element significantly increases the half-life of the adh-Hhr reporter (from 12 to 85 min; Supplementary Figure 2A and B), in agreement with the increased steady-state levels (Figure 4E), and render the reporter susceptible to NMD (Figure 4E and Supplementary Figure 2B). Thus, it is possible that ribozyme-terminated mRNAs as well as mRNAs with the hsp70 3' UTR are not subject to NMD because these transcripts are degraded very rapidly, and are therefore immune to any further increase of their decay rates by PTCs.

To validate this hypothesis, we made use of the adh wild-type and adh-64 reporters having five BoxB elements inserted upstream of the SV40 polyadenylation site. These reporters were expressed with the λN peptide or with a λN fusion of the protein GW182, which is a P-body component and strongly reduces half-lives of bound mRNAs by promoting deadenylation and decapping (Behm-Ansmant *et al*, 2006). We observed that adh-64-5BoxB was subjected to NMD in cells

expressing the λN-peptide, as its levels were upregulated in UPF1-depleted cells (Figure 7E). Coexpression of λN-GW182 strongly reduced the expression levels of both the wild-type and NMD reporters to similar extents (data not shown). Furthermore, the expression levels of adh-64-5BoxB were no longer sensitive to UPF1 depletion (Figure 7E), indicating that this reporter is not subject to NMD. This illustrates that binding of proteins that destabilize transcripts confers immunity to NMD.

Depletion of PABPC1 abolishes NMD

In the experiment shown in Figure 4, insertion of a poly(A) stretch upstream of the histone stem-loop structure in the adh-SL reporters confers sensitivity to NMD without increasing dramatically mRNA half-lives (from 20 to 30 min; Supplementary Figure 2C and D), suggesting that PABPC1 may have a more direct role in NMD, as opposed to simply stabilizing the transcripts. To investigate this, we measured NMD in cells depleted of PABPC1.

In cells depleted of PABPC1, the ratio of adh-64 to adh-wt increased 2.8-fold relative to the ratio observed in control cells (Figure 8A and B), suggesting that reducing the cellular concentration of PABPC1 interferes with NMD. Importantly, translation efficiency (measured by the ratio of ADH-V5 protein relative to that of adh-wt mRNA) was unchanged (Figure 8C and D).

The requirement of PABPC1 for NMD was confirmed for an endogenous NMD target, the ODA mRNA encoding ornithine decarboxylase (Gatfield and Izaurralde, 2004; Rehwinkel *et al*, 2005). Relative to the levels of endogenous rp49 mRNA, the steady-state levels of ODA mRNA increased 3.5- and 2.5-fold in cells depleted of UPF1 and PABPC1, respectively (Figure 8E and F), indicating that NMD is impaired in these cells. Together, these results show that PABPC1 is required for NMD.

Discussion

We show that PABPC1 plays a critical role in the mechanism by which premature stops are distinguished from natural stops. We also show that a synthetic poly(A) tail of 45 adenosine residues (or tethering of PABPC1) is sufficient to confer sensitivity to NMD on unadenylated RNAs, indicating that the role of PABPC1 in NMD is independent of 3'-end cleavage and polyadenylation. Finally, we show that NMD is impaired in cells depleted of PABPC1. Our findings, together with previous studies in *S. cerevisiae* (Amrani *et al*, 2004, 2006), reveal a conserved role for PABPC1 in NMD. More generally, the observation that natural stops are redefined as premature by increasing 3' UTR length suggests that NMD is likely to have presented a selective constraint on the evolution of eukaryotic 3' UTRs.

A conserved role for PABPC1 in PTC recognition

What could be the role of PABPC1 in PTC recognition? As proposed by the faux 3' UTR model, proximal PABPC1 binding is likely to increase the efficiency of translation termination and of ribosome release (Amrani *et al*, 2004). Indeed, PABPC1 interacts with eRF3, and tethering of eRF3 downstream of a PTC also abolishes NMD, albeit less effectively than does tethering PABPC1 (Amrani *et al*, 2004; data not shown). PABPC1 may also recruit other translational

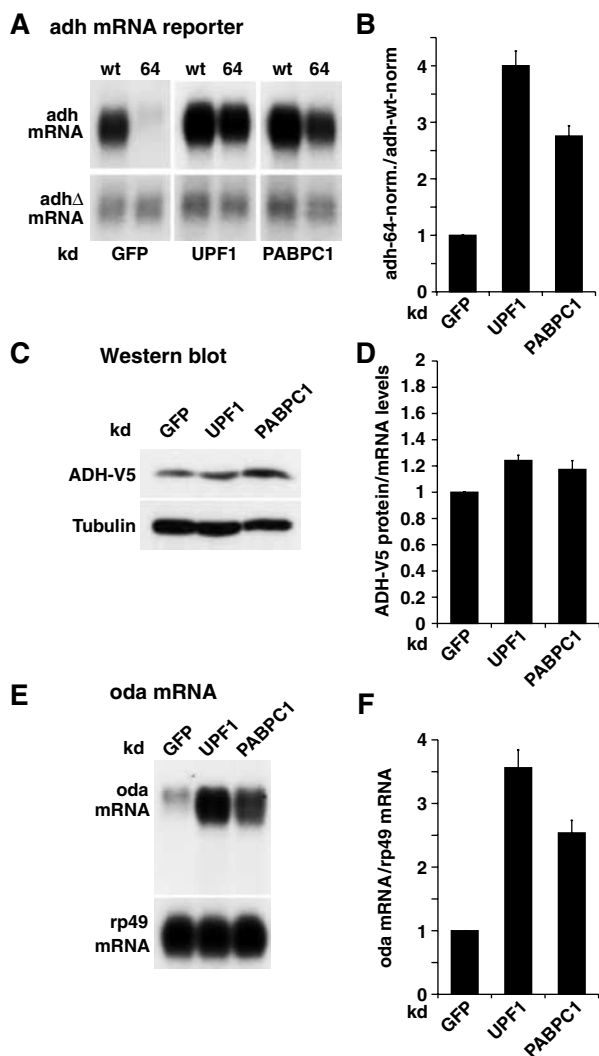


Figure 8 PABPC1 is required for NMD. (A–D) Cells transfected with the reporters indicated above the panels were treated with dsRNAs targeting GFP, UPF1 or PABPC1. Panel A shows a representative Northern blot. The levels of the adh mRNA reporters were normalized to the levels of adh Δ mRNA in three independent experiments. The normalized values obtained for adh-64 were divided by those obtained for adh-wt for each knockdown (to compensate for potential unspecific effects of the depletions), and set to 1 in cells treated with GFP dsRNA. Expression of ADH-V5 from the wild-type reporter was analyzed by Western blot (C). Tubulin served as a loading control. ADH-V5 protein levels were normalized to adh-wt mRNA levels in three independent experiments for each knockdown (D). (E, F) The levels of endogenous ODA mRNA in cells treated with GFP, UPF1 or PABPC1 dsRNAs were analyzed by Northern blot (E), and normalized to the levels of rp49 mRNA in three independent experiments. These ratios were set to 1 in cells treated with GFP dsRNA. Mean values \pm s.d. from three independent experiments are shown.

regulators that stimulate translation termination or proteins that prevent NMD when translation terminates at the physiological stop codon. Thus, increasing the distance of the stop codon to PABPC1 for a given mRNA would impair these interactions (as is the case for PTCs located towards the 5' end of the transcript) and translating ribosomes may not terminate translation efficiently, leading to the recruitment of the NMD machinery.

Similar analyses to those described here have been performed in human cells. In most cases, the positional depen-

dence of nonsense codons to trigger NMD exhibited a clear boundary effect, with PTCs upstream of the boundary eliciting mRNA degradation efficiently, whereas those close or downstream of the boundary being inefficient triggers of NMD. In contrast to the results in *D. melanogaster*, however, in mammalian cells the boundary corresponds to the 3'-most exon–exon junction (Nagy and Maquat, 1998). Indeed, for many reporters, nonsense codons should be located at least 50–55 nt upstream of the boundary to destabilize the transcript (an effect referred as to the '50 nt boundary rule'; Nagy and Maquat, 1998).

The 50 nt boundary rule is not absolute and in some mRNAs the spacing between the nonsense codon and the boundary can be smaller (Carter *et al*, 1996; Wang *et al*, 2002; Buhler *et al*, 2004, 2006). Moreover, there are an increasing number of examples of PTCs that elicit NMD despite the absence of a downstream intron, although steady-state mRNA levels are only slightly affected in this case (Chan *et al*, 1998; Zhang *et al*, 1998; Rajavel and Neufeld, 2001; Delpy *et al*, 2004; Buhler *et al*, 2006). These observations have been interpreted as an indication that in mammals, as in *S. cerevisiae*, PTC recognition depends on the distance to the poly(A) tail (Buhler *et al*, 2006). However, although PABPC1 binds to mRNAs subject to NMD (Hosoda *et al*, 2006), a role for the poly(A) tail, and hence for PABPC1, in PTC-definition in mammals has not been demonstrated directly. On the contrary, available evidence indicates that PABPC1 is dispensable for NMD in human cells, provided that the PTC is located upstream of an exon–exon boundary (Neu-Yilik *et al*, 2001). Indeed, Neu-Yilik *et al* (2001) reported that replacing a polyadenylation signal with a histone stem–loop structure does not affect NMD in human cells. The results of Neu-Yilik *et al* (2001) are in sharp contrast with our observations in *D. melanogaster* cells, where mRNAs terminated with a histone stem–loop structure are immune to NMD.

In addition to the differences mentioned above, several lines of evidence point to a significant divergence between the NMD pathway in mammals and invertebrates. For instance, as a consequence of the splicing-dependent mechanism for PTC definition, mammalian EJC components are required for NMD. These include the proteins Y14, MAGOH, eIF4AIII, Barentsz and RNPS1 (Conti and Izaurralde, 2005; Lejeune and Maquat, 2005). The *D. melanogaster* orthologs are not required for NMD (Gatfield *et al*, 2003). Moreover, there are no clear orthologs of these proteins in *S. cerevisiae*. It has been argued that EJC components may not play a direct role in PTC recognition in mammals, but may act as enhancers of NMD, PTC recognition being dependent on the distance to the poly(A) tail (Buhler *et al*, 2006). However, even the observation that EJC components and exon–exon boundaries enhance NMD triggered by PTCs located upstream in mammals, but not in *S. cerevisiae* or *D. melanogaster*, points to clear differences in the mechanisms of NMD between these organisms.

Although the NMD machinery is conserved, our results suggest that a change in the mechanism by which nonsense codons are defined occurred during evolution with a switch from PABPC1-dependent to a predominantly EJC-dependent mode. This may have created the opportunity for EJC components to functionally substitute for UPF2, as suggested by Gehring *et al* (2005). The release of functional constraints on NMD effectors may have in turn facilitated the acquisition of

additional functions. Indeed, mammalian UPF1, SMG1 and SMG5–7 have been implicated in cellular processes distinct from NMD (reviewed by Rehwinkel *et al*, 2006).

PABPC1 binding is sufficient for PTC recognition regardless of cleavage and polyadenylation

An important finding from our experiments is that replacing the cleavage and polyadenylation signal of nonsense mRNAs with a self-cleaving hammerhead ribozyme or a histone stem-loop structure suppresses NMD. These results are in agreement with those reported in *S. cerevisiae*, showing that nonsense ribozyme-terminated transcripts are not subject to NMD (Baker and Parker, 2006; Dower *et al*, 2004). However, in contrast to the results in *S. cerevisiae*, we show that immunity to NMD is not due to reduced translation efficiency, but may be at least in part due to the high decay rate of these mRNAs, which makes them resistant to any further acceleration of degradation. These short-lived, unadenylated mRNAs become susceptible to degradation by NMD when 45 or more adenosine residues are inserted upstream of the ribozyme cleavage site or the histone stem-loop structure, indicating that binding of PABPC1, independently of 3'-end processing, is sufficient to confer sensitivity to NMD.

PABPC1 is required for NMD

For ribozyme-terminated mRNAs, binding of PABPC1 stabilizes the transcript and this may in turn be sufficient to confer sensitivity to NMD. In contrast, the reporters terminated with a histone stem-loop structure become NMD-sensitive upon PABPC1 binding, although a small effect on mRNA half-lives is observed. This suggests that PABPC1 may have additional roles in NMD. In agreement with this, depletion of PABPC1 inhibits NMD, under conditions in which translation efficiency is not affected. A possible additional role for PABPC1 could be that of facilitating the recruitment of the NMD machinery. However, we could not detect a stable interaction between PABPC1 and NMD factors by co-immunoprecipitation assays (not shown), suggesting that if these interactions occur they may be indirect or transient. Alternatively, PABPC1 could act in translation termination and indirectly on NMD.

Modulation of NMD activity by specific RNA-binding proteins

The existence of a mechanism for PTC recognition based on the position of the stop relative to PABPC1 binding raises the question of how endogenous transcripts with exceptionally long 3' UTRs avoid NMD. There are several mechanisms by which mRNAs may escape NMD. We show that inherently unstable mRNAs are insensitive to NMD. Alternatively, long 3' UTRs may have sequence elements that are folded via RNA–RNA or RNA–protein interactions into structures that bring PABPC1 into close proximity with the natural stop. In this case, proximity to PABPC1 is not directly correlated to the length in nucleotides between the natural stop and the poly(A) tail.

An alternative mechanism to confer resistance to NMD may involve the presence of mRNA-stabilizing elements that prevent decay directly. Sequence elements that antagonize NMD have been reported in viral and *S. cerevisiae* transcripts. A sequence element that stabilizes unspliced Rous sarcoma virus RNAs also inhibits NMD when located downstream of a

PTC (Weil and Beemon, 2006). The *S. cerevisiae* *PGK1*, *GCN4* and *YAP1* mRNAs have sequence elements that prevent NMD in a heterologous context when positioned downstream of a PTC (Ruiz-Echevarria and Peltz, 2000). The effects of the *S. cerevisiae*-stabilizing elements are mediated by the RNA-binding protein PUB1 (Ruiz-Echevarria and Peltz, 2000). It is unclear whether PUB1 prevents decay or increases the efficiency of translation termination at nonsense codons located upstream of its binding site, but these two modes of action could be envisaged for different RNA-binding proteins. Immunity or susceptibility to NMD could be regulated if these proteins were to be expressed under specific physiological conditions or in a tissue-specific manner. In this way, NMD could contribute to the establishment of gene expression 'programs' in response to changes in physiological conditions or in specific tissues.

Materials and methods

NMD reporter constructs

The adh and CAT reporters have been described before (Gatfield *et al*, 2003; Gatfield and Izaurralde, 2004); additional PTCs were introduced in the wild-type reporters using an oligonucleotide-directed *in vitro* mutagenesis system (quick-change site-directed mutagenesis, Stratagene). The long-adh reporters were produced by in-frame insertion of the first 90 nt of the 3' UTR of the pAc5.1B vector downstream of codon 257. The short-adh reporters were obtained by deletion of the nucleotides between codons 231 and 257. The adh reporters with an insertion upstream or downstream of the natural stop codon were generated by inserting the first 198 nt of the coding sequence of the β -lactamase gene either downstream of codon 257 (restriction site *ApaI*) or downstream of the normal stop codon (restriction site *StuI*). The pAc5.1B-adh Δ was constructed by inserting the adh sequence (accession no. X78384; residues 2021–2119; 2185–2200) between the *EcoRI* and *XhoI* sites of vector pAc5.1B (Invitrogen).

H4 histone stem-loop was amplified by PCR using as template *D. melanogaster* histone H4 minigene (Adamson and Price, 2003), whereas the hammerhead ribozyme was amplified by PCR using plasmid TDH-GFP-RZ as template (Dower *et al*, 2004). These inserts were cloned in a unique *NotI* site introduced by mutagenesis in pAc5.1-adh-wt or pAc5.1-adh-64. This insertion deletes the SV40 polyadenylation signal present in the pAc5.1 vector. The poly(A) stretches were inserted upstream of the H4 histone stem-loop or of the hammerhead ribozyme. Reporters with the hsp70 3' UTR were generated by replacing the *XhoI*–*Sall* fragment corresponding to the 3' UTR of pAc5.1 vector by an *XhoI*–*Sall* cDNA fragment containing the hsp70 3' UTR. All constructs were sequenced.

Tethering assays

To generate the pAc5.1- λ N plasmid, a cDNA sequence encoding the λ N peptide was amplified by PCR using vector pCIneo- λ N (Gehring *et al*, 2003) as template and cloned between the *KpnI* and *EcoRI* restriction sites of pAc5.1B vector (Invitrogen). *D. melanogaster* cDNAs encoding PABPC1 (CG5119) and PABPN1 (CG2163) were amplified by PCR from an oligo(dT)-primed S2 cell cDNA library using primers containing appropriate restriction sites. Amplified cDNAs were then cloned into the vector pAc5.1- λ N or the vector pAc5.1A. All PCRs were performed with the Expand high-fidelity PCR system (Roche) and the constructs were sequenced.

To generate the adh-4BoxB and CAT-4BoxB plasmids, the pAc5.1-adh Δ I3 and pAc5.1-CAT vectors described above were mutated in two consecutive steps to introduce *NheI* and *SacII* sites between codons 119 and 125 and codons 132 and 138, respectively. These sites were used to insert 4BoxB sequences.

Cell transfections, RNA isolation and Northern blots

S2 cells were transfected with Effectene (Invitrogen). The pAc5.1-adh Δ plasmid served as a transfection control. Transfection mixtures contained 0.3 μ g of wild-type or NMD reporters and 0.7 μ g of pAc5.1-adh Δ plasmid. For the tethering assays, the transfection mixtures contained 0.15 μ g of pAc5.1 CAT-4BoxB or

adh-4BoxB, 0.2 µg of pAc5.1-λN and 0.65 µg of the pAc5.1 adhΔ plasmid. Total RNA was isolated using TRIzol Reagent (Life Technologies) and analyzed as described by Gatfield *et al* (2003). For determination of mRNA half-lives, cells were incubated with actinomycin D (5 µg/ml) for the times indicated in the figures.

RNA interference and Western blotting

RNA interference was performed essentially as described by Gatfield *et al* (2003). For Western blots, total cell extracts were prepared by addition of protein sample buffer to cells 24 h after transfection. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Western blotting was performed with a mouse monoclonal anti-V5 antibody (Invitrogen), using the CDP-Star chemiluminescent immunoblot system (PE Biosystems).

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Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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