

# Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways

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**Nonsense-mediated mRNA decay (NMD) represents a key mechanism to control the expression of wild-type and aberrant mRNAs. Phosphorylation of the protein UPF1 in the context of translation termination contributes to committing mRNAs to NMD. We report that translation termination is inhibited by UPF1 and stimulated by cytoplasmic poly(A)-binding protein (PABPC1). UPF1 binds to eRF1 and to the GTPase domain of eRF3 both in its GTP- and GDP-bound states. Importantly, mutation studies show that UPF1 can interact with the exon junction complex (EJC) alternatively through either UPF2 or UPF3b to become phosphorylated and to activate NMD. On this basis, we discuss an integrated model where UPF1 halts translation termination and is phosphorylated by SMG1 if the termination-promoting interaction of PABPC1 with eRF3 cannot readily occur. The EJC, with UPF2 or UPF3b as a cofactor, interferes with physiological termination through UPF1. This model integrates previously competing models of NMD and suggests a mechanistic basis for alternative NMD pathways.**

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## Introduction

Nonsense-mediated mRNA decay (NMD) represents one of the core mechanisms that help ensure fidelity of gene expression. NMD has been highly conserved during evolution, and identifies and limits the expression of transcripts with

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premature termination codons (Hentze and Kulozik, 1999; Maquat, 2004; Behm-Ansmant *et al.*, 2007b; Chang *et al.*, 2007; Isken and Maquat, 2007). Such transcripts encode C-terminally truncated proteins that may be non-functional or could exert an effect in a dominant-negative manner. The biological and medical significance of NMD is highlighted by an increasing number of hereditary and acquired genetic diseases that are modified by the activity of this mRNA surveillance pathway (Holbrook *et al.*, 2004; Neu-Yilik and Kulozik, 2004; Tarpey *et al.*, 2007). Furthermore, NMD also has an important function in the development of novel treatment strategies for diseases that are caused by premature termination codons (Durand *et al.*, 2007; Welch *et al.*, 2007; Linde *et al.*, 2007a, b).

Mechanistically, the mammalian NMD machinery distinguishes a normal stop codon from a premature termination codon in a splicing- and translation-dependent manner (Carter *et al.*, 1996; Thermann *et al.*, 1998; Le Hir *et al.*, 2000; Danckwardt *et al.*, 2002), although in a few cases NMD may also exert an effect independently from splicing (Buhler *et al.*, 2006). Splicing deposits a protein complex 20–24 nt upstream of the splice junction (Kataoka *et al.*, 2000; Le Hir *et al.*, 2000; Lykke-Andersen *et al.*, 2001), which is referred to as the exon junction complex (EJC). The core of the EJC consists of the proteins eIF4AIII, BTZ, Y14 and MAGOH, which is thought to recruit additional components, including UPF3b and UPF2 (Kataoka *et al.*, 2000; Le Hir *et al.*, 2000; Lykke-Andersen *et al.*, 2000, 2001; Gehring *et al.*, 2003, 2005; Chan *et al.*, 2004; Palacios *et al.*, 2004; Bono *et al.*, 2006). In case of premature translation termination, UPF2/UPF3b interact with UPF1 and the release factors eRF1 and eRF3 at the A-site of the terminating ribosome (Kashima *et al.*, 2006), promoting phosphorylation of UPF1 by the P13-kinase-like SMG1 kinase. This in turn recruits SMG5, SMG6 and SMG7, leading to degradation of the mRNA (Yamashita *et al.*, 2001; Ohnishi *et al.*, 2003; Grimson *et al.*, 2004; Unterholzner and Izaurralde, 2004; Glavan *et al.*, 2006).

This linear model of mammalian NMD is likely oversimplified because examples have been described where NMD can be UPF2 or UPF3b independent, or even occur independently of splicing (Gehring *et al.*, 2005; Buhler *et al.*, 2006; Chan *et al.*, 2007). Furthermore, inactivating UPF3b mutations in humans do not abolish NMD, suggesting the existence of alternative pathways (Tarpey *et al.*, 2007).

In yeast, physiological translation termination requires a permissive mRNP environment. A termination codon is recognized by the NMD machinery, if it is part of an inappropriate mRNP, that has been termed 'faux 3'-UTR'. Poly(A)-binding protein (Pab1p) appears to represent an important component of a termination-permissive 3' mRNP because the expression of NMD substrate mRNAs can be rescued by tethering Pab1p downstream of a premature stop codon (Amrani *et al.*, 2004). Tethered cytoplasmic poly(A)-binding

protein (PABPC1) has been shown to suppress NMD also in *Drosophila* cells (Behm-Ansmant *et al*, 2007a).

In this study, we analyse the interplay between the mammalian release factors eRF1 and eRF3, the NMD proteins UPF1, UPF2, UPF3b, the EJC and PABPC1. On the basis of our results and the published literature, we discuss an integrated model for mammalian NMD. As its novel features, it postulates that UPF1 inhibits the efficiency of translation termination, and that the functionally important phosphorylation of UPF1 is stimulated alternatively by its interaction with UPF2 or UPF3b. This model thus offers a biochemical basis for recently described alternative NMD pathways and for the redundancy of UPF3b in patients with inactivating mutations.

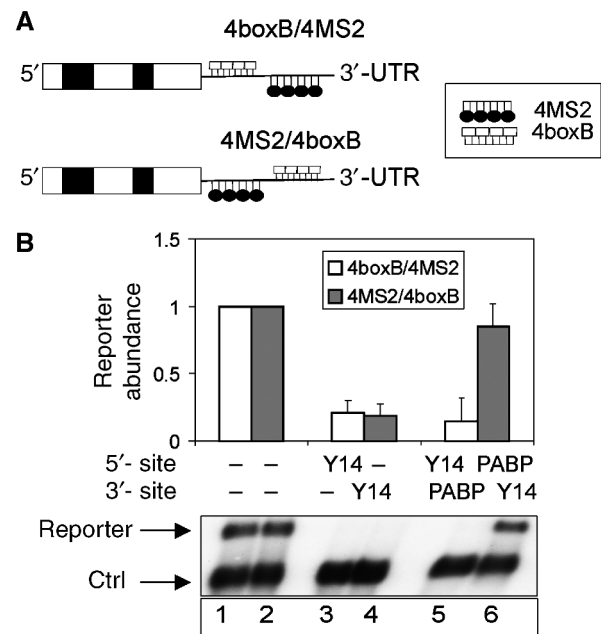
## Results

### **PABPC1 inhibits NMD in a position-dependent manner**

In yeast, efficient termination depends on the interaction between the terminating ribosome and a specific 3' mRNA including cytoplasmic Pab1p. Prematurely terminating ribosomes that encounter an improper, 'faux', 3' mRNA, are not released efficiently, and the mRNA is targeted to the NMD pathway. 'Faux' 3'-UTRs can be 'repaired' by tethering Pab1p sufficiently close to the translation termination codon (Amrani *et al*, 2004).

We first analysed the role of PABPC1 and its interplay with the EJC in human NMD. We designed a set of reporter constructs with 4MS2 and 4boxB-binding sites in their 3'-UTRs (Figure 1A), which allows to tether simultaneously two different,  $\lambda$ N- and MS2-tagged proteins to the mRNA in a position-defined manner. Importantly, tethering of the EJC proteins Y14, MAGOH and RNPS1 solidly downmodulates reporter mRNA expression in a predictable manner that is independent of the tag and the tethering site within the 3'-UTR (Figure 1B and Supplementary Figure 1). Furthermore, positioning of the multimerizing MS2 coat protein between the termination codon and a tagged EJC protein does not suppress NMD, indicating that the ability of a tethered EJC protein to trigger NMD is not suppressed by mere steric hindrance from a protein tethered between it and the stop codon (Figure 1B, lane 4). We next tested if positioning of PABPC1 between the tethered EJC protein and the termination codon would antagonize NMD. Simultaneous transfection of MS2-PABPC1 and  $\lambda$ NV5-Y14 together with the 4MS2/4boxB reporter resulted in a three- to four-fold higher abundance of the reporter mRNA than the same construct following co-transfection of  $\lambda$ NV5-Y14 and MS2 without PABPC1 (Figure 1B, compare lanes 4 and 6). This effect is position dependent because PABPC1 tethering 3' instead of 5' of the EJC protein does not affect reporter mRNA abundance (Figure 1B, lane 5). Similar results were obtained when tethered RNPS1 or MAGOH was used instead of tethered Y14 (Supplementary Figure 1). These data show that PABPC1 can overcome the effect of tethered EJC proteins when positioned closer to the termination codon.

Both the yeast and mammalian poly(A)-binding proteins interact with eRF3 (Hoshino *et al*, 1999; Cosson *et al*, 2002a,b). In yeast, this interaction stimulates translation termination and the release of the nascent polypeptide from the ribosome. It is noteworthy that the domain of eRF3 that is directly bound by PABPC1 corresponds to that bound by UPF1 (see below). These data suggest a possible functional



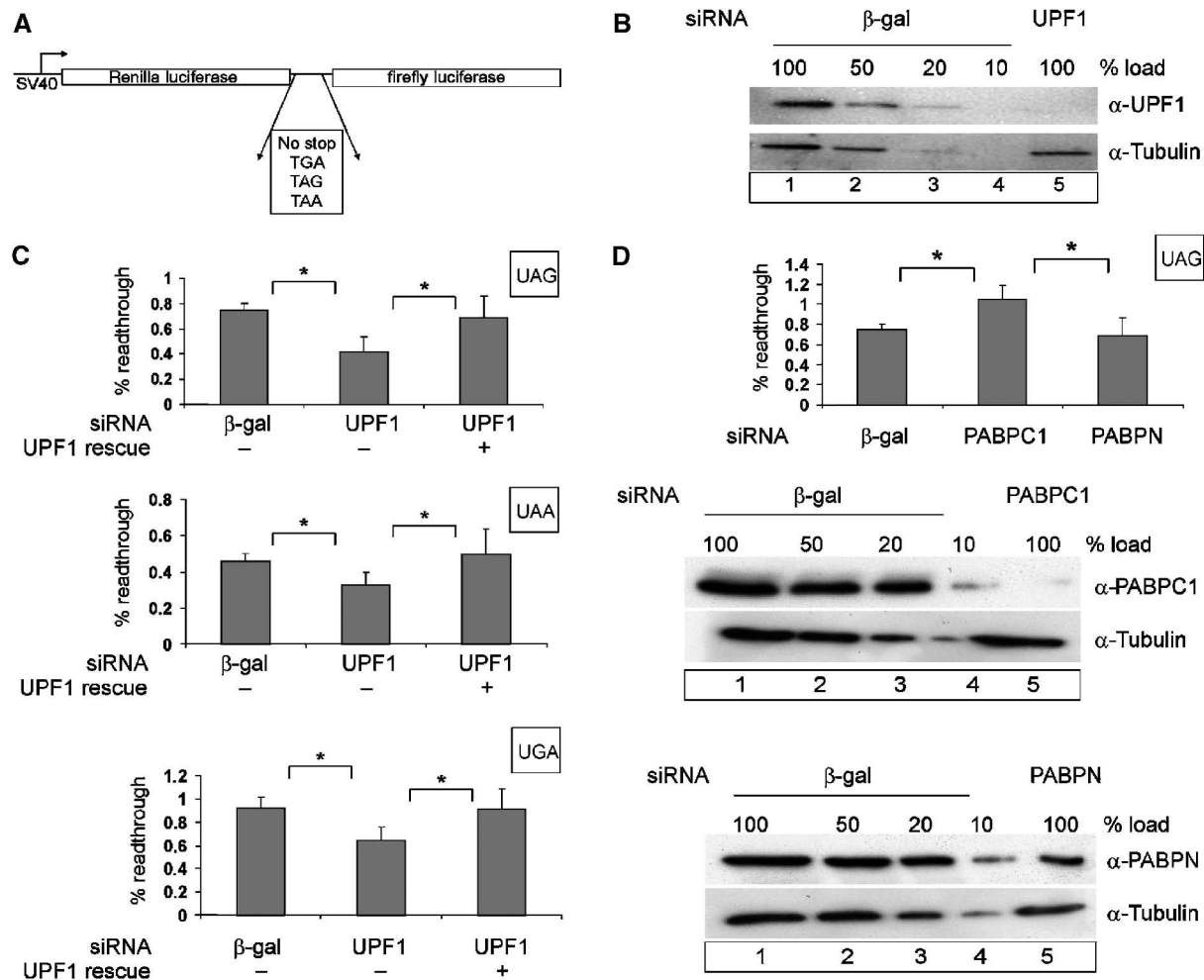
**Figure 1** Antagonistic relationship of PABPC1 and the EJC in stop codon definition. (A) Schematic representation of the 4boxB/4MS2 and 4MS2/4boxB reporters. White and black boxes represent exons and introns of the  $\beta$ -globin gene, respectively. BoxB and MS2 sites are shown as white boxes or black loops, respectively. (B) Tethering of PABPC1 and the EJC protein Y14. HeLa cells were transfected with the indicated 4boxB/4MS2 or 4MS2/4boxB constructs and the plasmids expressing the MS2-PABPC1 and  $\lambda$ NV5-Y14 fusion proteins as indicated. Total RNA samples were analysed by northern blotting. The levels of the 4boxB/4MS2 and the 4MS2/4boxB reporter mRNAs were normalized to the control  $\beta$ -globin reporter (ctrl). For each reporter, mRNA levels of the reporter without any tethered factor (MS2 protein and  $\lambda$ N peptide alone) were set as 1.0 (lanes 1 and 2). Values were calculated from three independent experiments, error bars represent standard deviations.

inter-relationship between these three proteins in NMD, which we explored by further analyses in mammalian cells.

### **UPF1 and PABPC1 modulate the efficiency of translation termination**

To test the role of mammalian UPF1 in translation termination, we used a readthrough assay in a dual luciferase reporter (Grentzmann *et al*, 1998; Howard *et al*, 2001). This reporter is composed of two in-frame open reading frames (ORFs) encoding *Renilla* and firefly luciferase, respectively, which are interrupted by a stop codon (Figure 2A). The 5' *Renilla* luciferase ORF is constitutively translated and serves as an internal standard, against which the expression of the 3' firefly luciferase ORF is used as a quantitative measure of readthrough by the elongating ribosome. The ratio between firefly luciferase and *Renilla* luciferase activities thus reflects the efficiency of translation termination. The design of this reporter ensures that potentially nonspecific effects on RNA synthesis, RNA stability or translation are internally controlled.

The ratio of *Renilla*/firefly luciferase activity in HeLa cells transfected with the reporter lacking an intervening stop codon and hence encoding a fusion protein was defined as 100% readthrough. Cells co-transfected with reporters



**Figure 2** Effect of UPF1 and PABPC1 on translational readthrough and termination. (A) Schematic representation of the dual luciferase reporters used for measuring translation termination readthrough. (B, C) UPF1 knockdown decreases the level of readthrough at all three stop codons. Protein lysates from HeLa cells that were transfected with siRNAs against  $\beta$ -galactosidase ( $\beta$ -gal) (B, lanes 1–4) or UPF1 (B, lane 5) were immunoblotted with an anti-UPF1 antibody. A dilution series corresponding to 50, 20 and 10% of the protein amount that was used in lane 1 (20  $\mu$ g) was loaded to assess the efficiency of UPF1 depletion. Reprobing with a tubulin-specific antibody was performed as a loading control. At 48 h after siRNA depletion, HeLa cells were transfected with the dual luciferase construct (p2luc-stop). The percentage of readthrough at the three different stop codons is shown (C). The results obtained with the construct without a stop codon were set as 100% readthrough. To complement the UPF1 depletion, cells were transfected with 0.2  $\mu$ g of siRNA-resistant FLAG-UPF1<sup>R</sup>. The values were calculated from six independent depletion experiments, error bars represent standard deviations. \*Statistical significance at  $P < 0.05$ . (D) PABPC1 knockdown increases the level of readthrough at a UAG stop codon. Depletions of PABPC1 and nuclear PABP were achieved by siRNA treatment as described in (B). HeLa cells were transfected with the dual luciferase construct p2luc-TAG or with the construct without an interspersed stop codon p2luc-if 48 hours after siRNA treatment. The percentage of readthrough at the UAG stop codon was calculated as described in (C). \*Statistical significance at  $P < 0.05$ .

bearing any of the three stop codons and a  $\beta$ -galactosidase siRNA display readthrough activities of  $0.45 \pm 0.07\%$  for UAA,  $0.75 \pm 0.05\%$  for UAG and  $0.95 \pm 0.06\%$  for UGA (Figure 2C). In cells that were co-transfected with the same reporters but with UPF1-specific siRNA and in which UPF1 levels were depleted to  $< 10\%$  (Figure 2B), readthrough decreased 1.5- to 2-fold ( $P < 0.05$ ) (Figure 2C). The specificity of this effect was controlled by re-establishing termination efficiency with UPF1 constructs that carry a functionally silent mutation of the sequences that were targeted in the endogenous transcript by the siRNA (Gehring *et al*, 2005; Figure 2C).

Because of the role of the poly(A)-binding protein in translation termination (Cosson *et al*, 2002b), and because

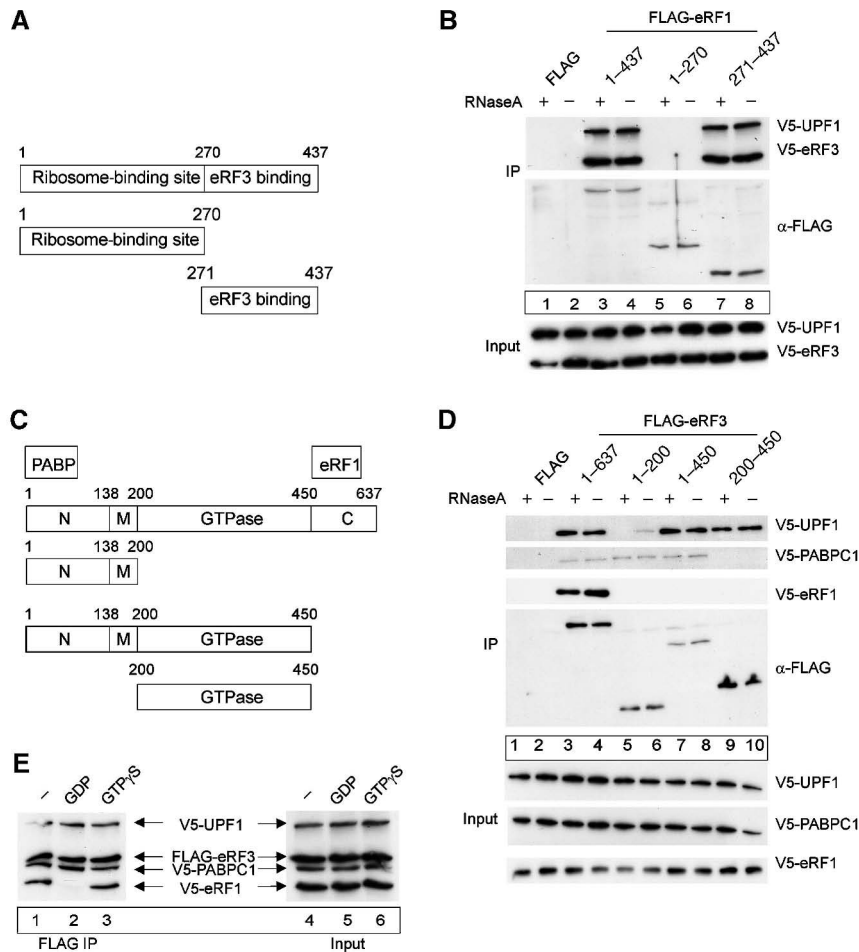
of the interference of this protein with NMD in yeast (Amrani *et al*, 2004), *Drosophila* cells (Behm-Ansmant *et al*, 2007a) and in our functional tethering assays (Figure 1B), we next tested the effect of PABPC1 depletion on translation termination in our reporter system. Interestingly, PABPC1 depletion significantly increases readthrough 1.5-fold ( $\pm 0.12$ ), in contrast to the depletion of the control, nuclear PABP (PABPN), that lacks the interaction domain with eRF3 (Figure 2D). These data indicate an antagonistic relationship between UPF1 and PABPC1, where UPF1 interferes with and PABPC1 stimulates the efficiency of translation termination. Potentially, the antagonistic effects of these proteins in NMD may relate to their respective roles in translation termination.

**Determinants of the interactions between UPF1, eRF1, eRF3 and PABPC1**

A role of UPF1 in termination-coupled NMD has been suggested by the identification of the SURF complex consisting of UPF1, its kinase SMG1 and the release factors eRF1 and eRF3 (Kashima *et al*, 2006; Supplementary Figure 2). Our results described above also implicate PABPC1, and we next characterized the biochemical interactions between these proteins.

Human eRF1 is composed of two functionally distinct domains (Figure 3A) (Frolova *et al*, 1994, 2000). The N-terminal part mediates binding to the ribosome and is fully competent as a release factor in translation termination. The C-terminal third of the polypeptide is responsible for binding

eRF3 and protein phosphatase 2A (PP2A) (Andjelkovic *et al*, 1996; Merkulova *et al*, 1999; Frolova *et al*, 2000; Song *et al*, 2000). To identify the region of eRF1 that binds UPF1, we co-transfected HeLa cells with plasmids expressing V5-tagged UPF1 and FLAG-tagged full-length eRF1, or the eRF1 C- and N-terminal domains, respectively. As a positive control, V5-eRF3 was also co-transfected. Following immunoprecipitation of eRF1 with anti-FLAG antibodies, the interaction with UPF1 and eRF3 was analysed by western blotting with anti-V5 antibodies. As predicted, full-length eRF1 and its C-terminal domain (eRF1 271–437) efficiently bind eRF3 (Figure 3B, lanes 3 and 4 and 7 and 8). Of note, UPF1 binds to the C-terminal part of eRF1 (lanes 7 and 8), whereas the N-terminal domain extending from residues 1–270 does



**Figure 3** Definition of biochemical interactions between UPF1, release factors and PABPC1. (A, B) UPF1 and eRF3 interact with the C-terminal part of eRF1. (A) Schematic representation of the domain structure of human release factor eRF1. The ribosome- and the eRF3-binding domain are depicted. (B) Co-immunoprecipitation experiments with eRF1 fragments. HeLa cells were co-transfected with an empty FLAG plasmid (lanes 1 and 2), with FLAG-eRF1 (lanes 3 and 4) or the indicated FLAG-tagged fragments of eRF1 (lanes 5–8) together with a plasmid for V5-UPF1 and V5-eRF3. Immunoprecipitations were carried out in the presence or absence of RNaseA. V5-tagged proteins were detected by immunoblotting with an anti-V5 antibody. Lysate (5%) used for the immunoprecipitations was loaded in the input lanes. (C, D) Interaction of eRF3 with eRF1, UPF1 and PABPC1. (C) Schematic representation of the domain structure of the human release factor eRF3. N-terminal (N; PABPC1 binding), middle (M), GTPase- and C-terminal (C; eRF3 binding) parts of eRF3 are indicated. (D) Co-immunoprecipitation experiments with eRF3 fragments. HeLa cells were transfected with an empty FLAG plasmid (lanes 1 and 2), with FLAG-eRF3 (lanes 3 and 4) or the indicated FLAG-tagged fragments of eRF3 (lanes 5–10) together with a plasmid for V5-UPF1, V5-PABPC1 and V5-eRF1. Immunoprecipitations were carried out as described in (B). V5-tagged proteins were detected by immunoblotting with an anti-V5 antibody. Lysate (5%) used for the immunoprecipitations was loaded in the input lanes. (E) UPF1 interacts with eRF3 in the presence of GDP or GTP. Co-immunoprecipitation experiments with eRF3 in the presence of GTP or GDP. HeLa cells were transfected with the plasmid FLAG-eRF3 (lanes 1–6) and with plasmids V5-UPF1, V5-PABPC1 and V5-eRF1, respectively. Immunoprecipitation was carried out in the presence of RNaseA and the guanine nucleotides (1 μM) as indicated (lanes 2 and 3). V5-tagged proteins were detected by immunoblotting with an anti-V5 antibody as described above. Lysate (2.5%) used for the immunoprecipitations was loaded in the input lanes (lanes 4–6).

not detectably bind UPF1 (lanes 5 and 6). These results indicate that eRF1 either binds UPF1 and eRF3 with the same domain or interacts with UPF1 through eRF3, which in both cases suggests an intricate relationship of these three proteins in translation termination (Figure 7).

As a next step, we characterized which of the functional domains of human eRF3 participate in this interaction. Human eRF3a (referred to as eRF3 throughout the text) consists of three domains (Figure 3C), an N-terminal domain extending from residues 1–138, which interacts with PABPC1 (Cosson *et al*, 2002b), a positively charged middle domain extending from residues 139–200 and a large C-terminal GTPase domain extending from residues 201–637, which has been shown to be essential for translation termination (Alkalaeva *et al*, 2006). This GTPase domain can be further subdivided into two parts with an N-terminal portion containing four GTP-binding motifs (G1–G4; 200–450) and a C-terminal part that interacts with eRF1 (Stansfield *et al*, 1995; Zhouravleva *et al*, 1995; Frolova *et al*, 1996, 1998; Jakobsen *et al*, 2001). Accordingly, we co-transfected different FLAG-tagged eRF3 variants with V5-UPF1, V5-eRF1 and V5-PABPC1 and immunoprecipitated on FLAG antibody beads in the presence or absence of RNase A (Figure 3D). PABPC1 and eRF1 show efficient binding to the expected N- and C-terminal domains of eRF3, respectively, serving as positive controls for the reaction (lanes 3–8 and lanes 3 and 4). UPF1 binds to the GTPase domain of eRF3 (Figure 3D, lanes 9 and 10) both in the presence and absence of RNA, whereas it shows weak binding to the N terminus of eRF3 in the absence of RNaseA (Figure 3D, compare lanes 5 and 6).

Considering that UPF1 binds to the GTPase domain of eRF3, we next analysed if the interaction is influenced by whether eRF3 is in the GTP- or the GDP-bound states. eRF3 stimulates termination in a GTP-dependent manner, and eRF1 together with the ribosome exerts an effect as a composite GTPase-activating protein towards eRF3 (Frolova *et al*, 1996). The eRF1:eRF3:GTP:Mg<sup>2+</sup> complex binds to the pre-termination ribosome and, following hydrolysis of GTP, induces efficient peptide release from the ribosome (Alkalaeva *et al*, 2006; Haurlyuk *et al*, 2006; Mitkevich *et al*, 2006). We performed immunoprecipitation analysis between FLAG-eRF3 and V5-UPF1 either in the presence of GTP $\gamma$ S, a non-hydrolysable GTP analogue, or in the presence of GDP. The binding of eRF1 to eRF3 served as a positive control because eRF1 binds eRF3 under non-equilibrium conditions such as an immunoprecipitation only in the presence of GTP, that is, before peptide release (Pisareva *et al*, 2006). We also monitored PABPC1 binding to eRF3 because PABPC1 interacts with the N-terminal domain of eRF3 and not with the GTPase domain as eRF1 and UPF1 do (see Figure 3D). As expected, the eRF1:eRF3 interaction occurs in the presence of GTP (Figure 3E, lanes 2 and 3), whereas UPF1 binds to both eRF3:GDP and a complex containing eRF3:GTP (such as eRF1:eRF3:GTP (Figure 3E, lanes 2 and 3). These data indicate that the interaction of UPF1 with the release factors can occur prior to GTP hydrolysis and peptide release, and that it can be maintained post-terminationally (Figure 7).

#### **eRF3 binds to the cysteine–histidine-rich domain of UPF1**

We next analysed which domain of UPF1 interacts with eRF3. UPF1 is a multifunctional protein that has RNA binding,

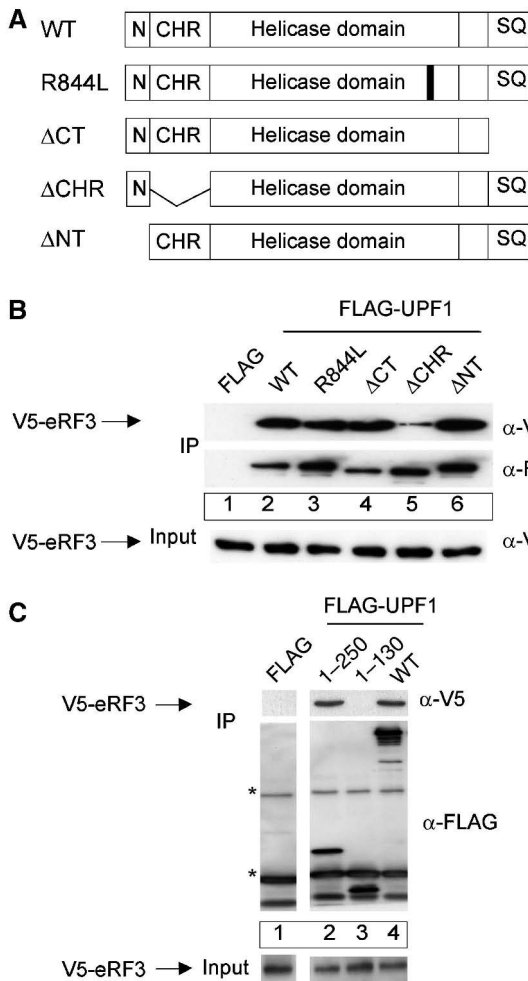
ATPase and RNA helicase properties and is structurally organized into at least two domains. One of these domains is a cysteine- and histidine-rich region (Cys–His-rich region, CHR, amino acids 123–213) near its N-terminus, which includes three zinc-finger motifs (Kadlec *et al*, 2006). The other domain, referred to as the core domain, includes six conserved helicase motifs that are common to members of the helicase I superfamily (Altamura *et al*, 1992; Czaplinski *et al*, 1995; Weng *et al*, 1996a). This two-domain structure is flanked by amino-acid sequences, which are important for SMG1-dependent phosphorylation (Yamashita *et al*, 2001, 2005; Ohnishi *et al*, 2003).

We mapped the regions of UPF1 that are important for the interaction with eRF3 by immunoprecipitation analyses with FLAG-UPF1 mutants (Figure 4A). These mutants included the R844L dominant-negative that inactivates the helicase domain (Sun *et al*, 1998), a C-terminal deletion mutant of residues 1074–1118 ( $\Delta$ CT), an N-terminal deletion mutant of residues 1–40 ( $\Delta$ NT) and a mutant with a deletion of residues 130–250 including the Cys–His-rich region ( $\Delta$ CHR). The  $\Delta$ CHR mutant displays reduced binding to eRF3 compared with wild type (Figure 4B, compare lanes 2 and 5), whereas the other deletion mutants and the R844L mutant show similar binding as the WT protein (Figure 4B, compare lane 2 with lanes 3, 4 and 6). We confirmed that the UPF1 CHR domain represents the *bona fide* eRF3-binding site by immunoprecipitation analysis with V5-tagged eRF3 and FLAG-tagged UPF1 fragments (Figure 4C). The UPF1 fragment containing residues 1–250 including the CHR (residues 123–213) shows a similar interaction with eRF3 as UPF1 wild type does (Figure 4C, compare lanes 2 and 4), whereas the UPF1 fragment including residues 1–130, thus excluding the CHR, does not display eRF3 binding (Figure 4C, lane 3). The protein–protein contact between UPF1 and eRF3 is thus established by binding between the CHR of UPF1 and the C-terminal GTPase domain of eRF3 (Figure 7).

#### **Phosphorylation of UPF1 and NMD can be alternatively supported by UPF2 or UPF3b**

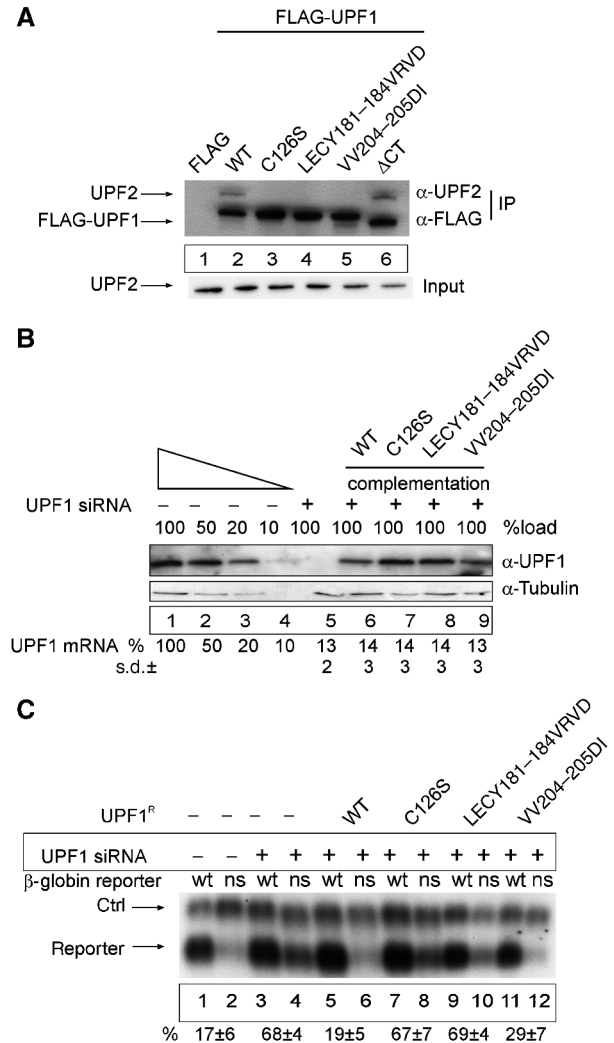
The CHR of UPF1, which we have shown to be critical for eRF3 binding, has previously been demonstrated to be important also for the interaction of UPF1 with UPF2 (Applequist *et al*, 1997; Kadlec *et al*, 2006) and to modulate translation termination and stop codon readthrough in yeast (Weng *et al*, 1996b, 2001). This domain thus represents a critical region of UPF1 that establishes its contact with both the translation termination apparatus and the EJC. We analysed this domain of UPF1 in more detail by mutagenesis screening of conserved residues in this region. From a pool of UPF1 mutants, we selected three variants that completely abolished the interaction with UPF2 (Figure 5A). The affected residues (C126, LECY181–184 and VV204–205) are located in those parts of the protein that have been shown in structural analyses to directly contact UPF2 (Kadlec *et al*, 2006) and to be critical for bridging UPF1 to the EJC (Kashima *et al*, 2006).

We functionally tested the NMD activity of these mutants in HeLa cells that had been treated with siRNA against endogenous UPF1 (or  $\beta$ -galactosidase siRNA as a negative control). UPF1 RNAi was efficient and depleted the endogenous protein to <10% (Figure 5B, lanes 1–5). Subsequently, these cells were co-transfected with a  $\beta$ -globin NMD reporter carrying either the wild-type sequence or a nonsense



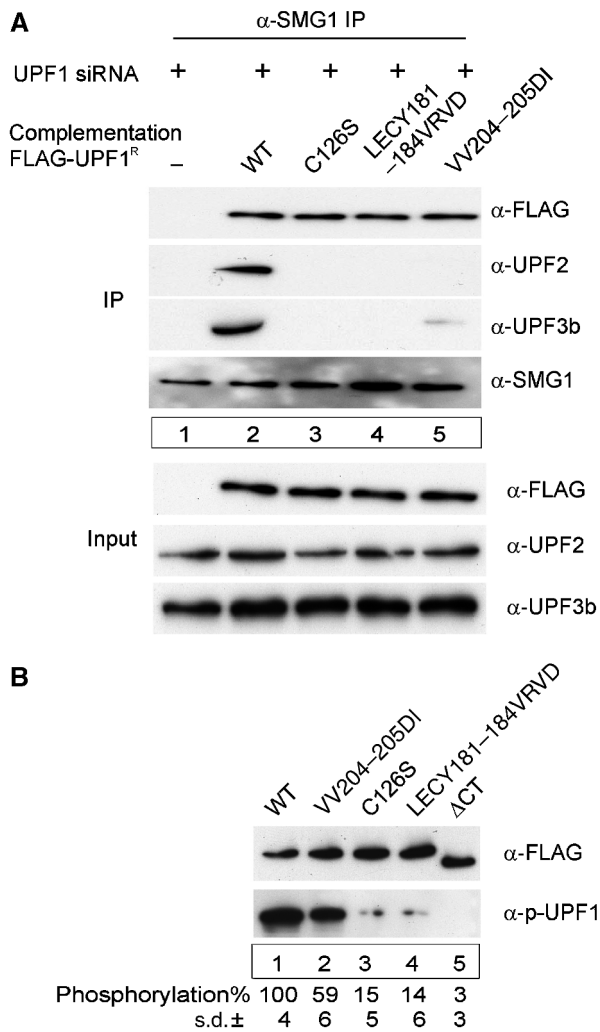
**Figure 4** eRF3 binds to the cysteine–histidine-rich region (CHR) of UPF1. (A) Schematic representation of the domain structure of human UPF1. Abbreviations used: WT—wild-type UPF1; R844L—dominant-negative point mutant of the helicase domain; ΔCT—mutant lacking the C-terminal amino acids 1074–1118; ΔCHR—mutant lacking amino acids 130–250 including the CHR; ΔNT—mutant lacking the N-terminal amino acids 1–40 (N); SQ—serine/threonine-glutamine-rich ((S/T)-Q-rich) motifs. (B) Co-immunoprecipitation experiments with UPF1 mutants. HeLa cells were transfected with an empty FLAG plasmid (lane 1), with wild-type FLAG-UPF1 (lane 2), the point mutant FLAG-R844L (lane 3) or the indicated FLAG-tagged fragments of UPF1 (lanes 4–6) together with a V5-eRF3 plasmid. Immunoprecipitations were carried out as described in Figure 3B in the presence of RNaseA. V5-eRF3 was detected by immunoblotting with an anti-V5 antibody. The input lane shows 4% of the lysate used per assay. (C) Co-immunoprecipitation experiments with the N-terminal fragment of UPF1. HeLa cells were transfected with an empty FLAG plasmid (lane 1), with FLAG-UPF1 (lane 4) or the indicated FLAG-tagged fragments of UPF1 (lanes 2 and 3) together with a V5-eRF3 plasmid. Immunoprecipitation and the detection of V5-eRF3 were carried out as described in (B). \*Immunoglobulin heavy and light chains. Lysate (5%) used for the immunoprecipitations was loaded in the input lane.

mutation at position 39 (NS39), and with one of the UPF1 variants that were engineered to be resistant to the UPF1-specific siRNA (UPF1<sup>R</sup>; Figure 5B, lanes 6–9). The NS39 reporter is expressed at a level of 17±6% of wild-type mRNA in cells treated with β-galactosidase-specific siRNA (Figure 5C, lanes 1 and 2) Upon depletion of UPF1, the expression of the NS39 mRNA increases four-fold (Figure 5C, lanes 3 and 4). As



**Figure 5** An UPF1 mutant that triggers NMD but fails to interact with UPF2. (A) Mutational analysis of the UPF2-binding site within the UPF1 CHR region. HeLa cells were transfected with empty FLAG plasmid (lane 1), wild-type FLAG-UPF1 (lane 2) or with the indicated FLAG-tagged mutants of UPF1 (lanes 3–6). Cell extract preparation, immunoprecipitation procedure and immunoblotting were carried out as described in Figure 3B in the presence of RNaseA. The UPF2 protein was detected by immunoblotting with an anti-UPF2 antibody. Lysate (5%) used for the immunoprecipitations was loaded in the input lane. (B, C) Functional analysis of UPF1 CHR mutants. (B) Functional complementation of UPF1 depletion. Immunoblotting analysis of lysates obtained from UPF1-depleted and UPF1-complemented cells was performed as described in Figure 2B and as described previously (Gehring *et al*, 2005). For the complementation of UPF1-depleted cells, cells were transfected with 0.2 μg FLAG-UPF1<sup>R</sup>. UPF1 mRNA levels were determined by qRT-PCR using primers that are specific for the endogenous mRNA and do not amplify the plasmid-derived UPF1 mRNA. The values were calculated from four independent experiments with standard deviations (±s.d.). (C) Northern blot analysis of RNA isolated from HeLa cells transfected with siRNA against β-galactosidase (lanes 1–2) or UPF1 (lanes 3–12). At 48 h after siRNA transfections, the cells were co-transfected with plasmids for the transfection efficiency control (ctrl), the NMD reporters (reporter; wt—wild-type β-globin, ns—NS39 β-globin mutant) and plasmids expressing the depicted FLAG-UPF1<sup>R</sup> variants. The values were calculated from three independent experiments. Error bars represent s.d.

predicted, co-transfected WT-UPF1<sup>R</sup> completely rescues NMD of NS39 (Figure 5C, lanes 5 and 6). The UPF1<sup>R</sup> mutants C126S and LECY181–184VRVD fail to rescue NMD activity



**Figure 6** SMG1-dependent phosphorylation of UPF1 correlates with binding of UPF2 or UPF3b. (A) SMG1 precipitates UPF1 mutants. Complementation of the UPF1 depletion with the indicated Flag-UPF1<sup>R</sup> variants was carried out as described in Figure 5B. An anti-SMG1 antibody was used to immunoprecipitate SMG1-complexes. Anti-FLAG, anti-UPF2, anti-UPF3b and anti-SMG1 antibodies were used to visualize the respective proteins in the immunoprecipitates. Lysate (10%) used for the immunoprecipitations was loaded in the input lanes. (B) Mutations in the Cys-Hisrich region of UPF1 affect its phosphorylation. HeLa cells were transfected with plasmids expressing the indicated FLAG-UPF1 mutants. After 48 h, cells were lysed and UPF1 mutants were immunoprecipitated with anti-FLAG agarose beads in the presence of phosphatase inhibitors. Immunoprecipitates were extensively washed, bound proteins were eluted and equal amounts of the eluted proteins were separated by SDS-PAGE. Immunoblotting was performed with an anti-phospho-UPF1 antibody ( $\alpha$ -p-UPF1) or a FLAG antibody. The bands were quantified using an Odyssey Infrared Imaging System (LI-COR Biosciences).

(Figure 5C, lanes 7–10), which is consistent with the important role of UPF1:UPF2 binding in NMD (Kashima *et al*, 2006). Surprisingly, the UPF1<sup>R</sup> mutant VV204–205DI does rescue NMD activity (Figure 5C, lanes 11 and 12), although similar to the other two mutants it was confirmed not to detectably interact with UPF2 (Figure 5A, lanes 3–5). This result shows that NMD can be triggered even by an UPF1 mutant that does not display UPF2-binding capacity and hence that other features of UPF1 may suffice for the NMD activity of this mutant.

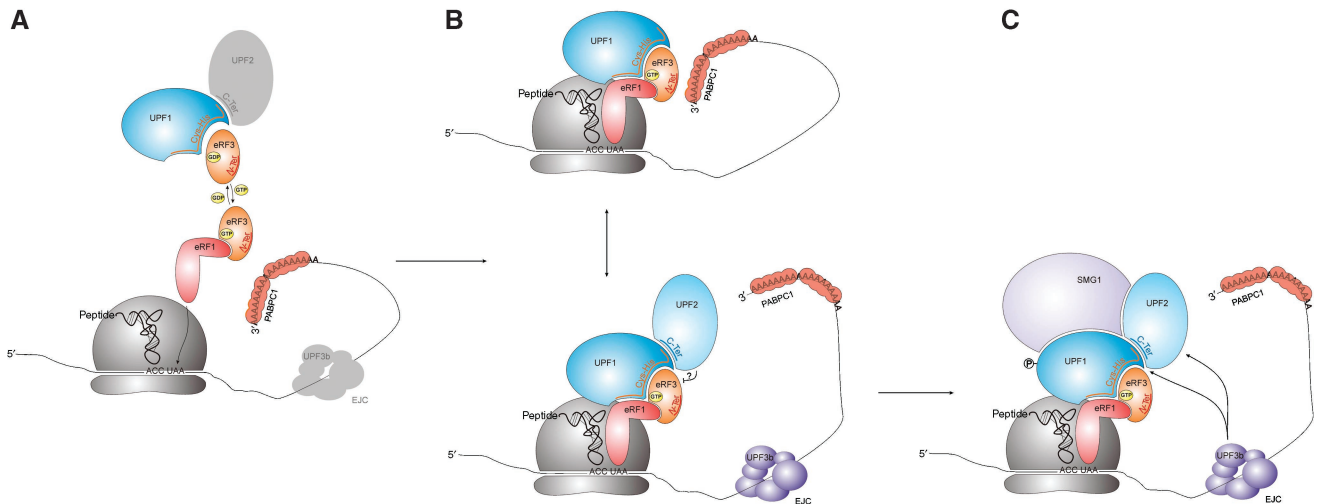
We next explored which feature of UPF1 could complement the UPF2-binding incompetent mutant. The phosphorylation of UPF1 by SMG1 is a key event of the NMD pathway (Page *et al*, 1999; Pal *et al*, 2001; Ohnishi *et al*, 2003; Kashima *et al*, 2006). Therefore, we tested the capacity of the UPF1 mutants to be phosphorylated. We transfected HeLa cells with FLAG-tagged mutants of UPF1, and immunoprecipitated these mutants in the presence of phosphatase inhibitors. The immunoprecipitates were eluted and aliquots immunoblotted either with a phospho-specific UPF1 antibody (Yamashita *et al*, 2001), or a FLAG antibody that was used as an internal control for protein loading (Figure 6B). As expected, the C126S, the LECY181–184VRVD and the  $\Delta$ CT mutants display strongly reduced phosphorylation when compared with WT-UPF1 (Figure 6B, compare lanes 3–5 and 1). Importantly, the VV204–205DI mutant is phosphorylated almost normally (Figure 6B, lane 2), which indicates that UPF2 is not absolutely required for UPF1 phosphorylation and NMD activity.

Because SMG1 is the UPF1 kinase and is contained within the functionally critical SURF complex (Kashima *et al*, 2006), we next tested whether the UPF1 mutations affect SMG1 binding. We treated cells with UPF1-specific siRNAs and complemented with FLAG-tagged UPF1<sup>R</sup> mutants in a similar way as described in Figure 5B. We then immunoprecipitated endogenous SMG1 with specific antibodies and assayed for cofactor binding with FLAG-, UPF2- and UPF3b-specific antibodies (Figure 6A). SMG1 antibodies efficiently pull down both wild-type and mutant UPF1 (anti-FLAG, Figure 6A, lanes 2–5). Further immunoblotting analyses of the precipitates demonstrate that the WT-UPF1:SMG1 complex efficiently precipitates both UPF2 and UPF3b (Figure 6A, lane 2), whereas the SMG1 complex with the UPF1 mutants C126S and LECY181–184VRVD interacts with neither UPF2 nor UPF3b (Figure 6A, lanes 3–4). Of note, the SMG1:UPF1-VV204–205DI complex fails to bind UPF2, but interacts with UPF3b albeit somewhat less efficiently than WT-UPF1 (Figure 6A, lane 5). These results confirm that phosphorylation of UPF1 is a critical step of the NMD pathway. Significantly, they support the notion that UPF1 phosphorylation and NMD can be mediated by an interaction with UPF2 or with UPF3b. These data thus also provide a biochemical and mechanistic correlate of previously proposed alternative NMD pathways (Gehring *et al*, 2005; Chan *et al*, 2007), and for the NMD activity of patients with inactivating UPF3b mutations (Tarpey *et al*, 2007).

## Discussion

Nonsense-mediated mRNA decay is triggered by translation termination at premature stop codons, which induces the assembly of a 3' mRNP that leads to the rapid degradation of the transcript. Upf1 proteins have a central function in NMD in all organisms that have been studied. Yeast and human Upf1 proteins are recruited to prematurely terminating ribosomes through interactions with the release factors (Czaplinski *et al*, 1998; Kashima *et al*, 2006), and the NMD activity of UPF1 is regulated by phosphorylation and dephosphorylation (Page *et al*, 1999; Chiu *et al*, 2003; Ohnishi *et al*, 2003; Fukuhara *et al*, 2005).

In yeast, translation termination efficiency differs at natural and premature stop codons; whereas termination at natural stop codons is efficient and quick, termination at



**Figure 7** An integrated model for mammalian NMD pathways. Schematic diagram of the structural and the functional interaction networks between release factors, UPF proteins, PABPC1, SMG1 and the EJC. Note that the diagram is not time-resolved, and hence some depicted interactions may not occur simultaneously and/or be competitive. **(A)** The Cys-His-rich domain of UPF1 (Cys-His) binds to the C terminus of UPF2 (see also Kadlec *et al*, 2006) and to the GTPase domain of eRF3. In the presence of GTP (reflecting the pre-termination state), the C-terminal end of eRF1 binds to the C-terminal end of eRF3, whereas this interaction does not occur in the presence of GDP (reflecting the post-termination state). The C terminus of eRF1 may directly bind to UPF1 or this interaction may be bridged by eRF3. **(B)** The C terminus of PABPC1 binds to the N terminus of eRF3 (N-Ter). During translation termination, UPF1 monitors the correct position of the termination codon. An interaction between PABPC1 and eRF3 'certifies' a normal termination event (top panel), whereas a downstream EJC is indicating premature termination (bottom panel). (?)—a possible competition between UPF2 and eRF3 for UPF1 binding. **(C)** In case of premature termination, the EJC triggers the SMG1-dependent UPF1 phosphorylation through UPF2 or UPF3b, which leads to NMD.

premature stop codons is inefficient and slow (Amrani *et al*, 2004, 2006). This inefficiency of termination at a premature termination codon has been explained by the failure to assemble an appropriate 3' mRNP and instead the assembly of an inappropriate 3' mRNP termed *faux* 3'-UTR. Poly(A)-binding protein appears to be an important architectural component of an appropriate 3' mRNP because repositioning of the protein to sufficient proximity to a premature termination codon redefines it to being normal, leading to efficient translation termination and NMD suppression in *Saccharomyces cerevisiae* (Amrani *et al*, 2004) and in *Drosophila melanogaster* cells (Behm-Ansmant *et al*, 2007a, b). In contrast to yeast and fly cells, an EJC downstream of a stop codon provides critical positional information for mammalian NMD to discriminate a premature termination codon from a natural stop codon (Carter *et al*, 1996; Thermann *et al*, 1998; Le Hir *et al*, 2000). The EJC is thought to interact with the mRNP recruited to the vicinity of the termination codon, referred to as SURF complex and to induce phosphorylation of UPF1 (Kashima *et al*, 2006).

Our results indicate that PABPC1 stimulates (Figure 2D) and UPF1 inhibits (Figure 2C) efficient translation termination in human cells. Furthermore, PABPC1 inhibits (Figure 1B and Supplementary Figure 1) and UPF1 stimulates NMD (Applequist *et al*, 1997). These results correlate with *in vitro* toeprint experiments in yeast extracts, which show that intermediates of termination complexes are sufficiently stable to be detectable only in the presence of a premature termination codon and of Upf1p (Amrani *et al*, 2004). Although depletion of Upf1p in yeast has been shown to cause nonsense suppression *in vivo* and amino-acid substitutions within the highly conserved CHR domain of Upf1p have previously been shown to affect nonsense suppression and NMD (Weng *et al*, 1996a, b), other data have challenged this

view (Bidou *et al*, 2000; Harger and Dinman, 2004), and the effect of Upf1p on translation termination may be context dependent (Keeling *et al*, 2004). Significantly, human UPF1 is not only homologous to yeast Upf1p but also to another yeast helicase with a CHR domain named Mtt1p. Similar to human UPF1, Mtt1p binds to the N-terminus and to the GTPase domains of eRF3 and can inhibit translation termination (Czaplinski *et al*, 2000). In the course of evolution, human UPF1 may thus have acquired characteristics of both yeast Upf1p and Mtt1p.

In analogy to the *faux* 3'-UTR model in yeast, we propose that the juxtaposition of PABPC1 and a stop codon can overcome the NMD-stimulating effect of a 3' EJC (Figure 1). However, the NMD-inducing effect of the EJC-UPF1 interaction is dominant over the effect of PABPC1, if the EJC is placed closer to the stop codon than PABPC1 (Figure 1B). This dominance is maintained even when PABPC1 is located in the immediate 3' neighbourhood of the EJC, showing that absolute linear distance of PABPC1 to the stop codon *per se* is not a sufficient determinant of a proper 3' mRNP. These results provide a basis to suggest that human NMD has evolutionarily retained components of the NMD machinery of lower eukaryotes.

The biochemical correlate of the functionally antagonistic effect of UPF1 on translation termination is reflected by eRF1 binding to UPF1 and to eRF3 with the same domain (Figure 3B). These data thus suggest that the presence of UPF1 may interfere with functionally effective eRF1:eRF3 binding and, consequently, efficient translation termination; this hypothesis remains to be tested. Furthermore, UPF1 binds to the GTPase domain of eRF3, which has previously been implicated in termination-coupled processes and is considered to function as a molecular switch connecting GTP hydrolysis to constitutive mRNA decay and to NMD



through shortening of poly(A) tails (Hosoda *et al*, 2003; Kobayashi *et al*, 2004). Finally, the data reported here demonstrate that the ability of Upf1 proteins to interact with both the pre-termination eRF1:eRF3:GTP complex and the post-termination eRF3:GDP-containing complex, is conserved between human UPF1 and yeast Upf1p (Kobayashi *et al*, 2004). We further suggest that the inhibitory effect of UPF1 on termination may result in a delay of the termination process with inefficient disassembly of the terminating ribosome and release of the nascent peptide. Thus, in case of premature termination there is sufficient time for the EJC to interact with UPF1, induce UPF1 phosphorylation by SMG1 and rapid RNA degradation. By contrast, in case of normal termination and without an EJC being present, PABPC1 interacts with the termination complex, which stimulates termination efficiency and results in the dissociation of the release factors and UPF1 from the mRNA, thus maintaining its stability (Figure 7).

Our data also shed new light on the question of how the EJC can interact with UPF1 to become phosphorylated and to trigger NMD. A number of previous studies indicated that the EJC consisting of the proteins eIF4AIII, RNPS1, BTZ, Y14 and MAGOH interacts with UPF3b and UPF2, which in turn bind to and induce phosphorylation of UPF1 (Lykke-Andersen *et al*, 2000, 2001; Kim *et al*, 2001; Gehring *et al*, 2003; Kashima *et al*, 2006; Kunz *et al*, 2006). Such a simple linear model of NMD has been challenged by the finding of both UPF2- and UPF3b-independent NMD and by the identification of patients with inactivating UPF3b mutations and normal NMD function (Gehring *et al*, 2005; Chan *et al*, 2007; Tarpey *et al*, 2007). Our data now identify a mutant of UPF1 (VV204–205DI) that can be phosphorylated (Figure 6B) and is NMD active in spite of its inability to interact detectably with UPF2 (Figure 5A–C). Interestingly, the VV204–205 motif is not conserved in Upf1 proteins of some organisms such as *Encephalitozoon cuniculi*, *Entamoeba histolytica* and *Giardia lamblia*. Significantly, the genomes of these organisms do not contain UPF2 homologous genes, suggesting that Upf1 proteins in these organisms function in an UPF2-independent manner (Kadlec *et al*, 2006). Importantly, the VV204–205DI mutant is capable to interact with UPF3b (Figure 6A) establishing an UPF2-independent interaction of UPF1 with the EJC.

These data thus provide direct evidence that SMG1-dependent phosphorylation of UPF1 can be stimulated independently by UPF2 or UPF3b, and unveil a potential biochemical correlate for previously identified alternative NMD pathways that can function independently of either UPF2 or UPF3b. Finally, our integrated NMD model (Figure 7) could also help explain cases of splicing-independent mammalian NMD (Buhler *et al*, 2006), if PABPC1-stimulated efficient termination is blocked and/or UPF1 phosphorylation triggered by other features of the mRNA or factors binding to it.

## Materials and methods

### Plasmid constructs

$\beta$ -Globin 4boxB/4MS2 and 4MS2/4boxB were constructed by introducing sequences for the 4MS2 or 4boxB into the 3'-UTR of 4boxB- $\beta$ -globin or 4MS2- $\beta$ -globin, respectively. pCI-neo-MS2, pCI-neo- $\lambda$ NV5, pCI-FLAG, pCI-V5, wt300 + e3, 4boxB- and 4MS2- $\beta$ -globin, and expression vectors for UPF1, Y14, MAGOH, RNPS1 and UPF2 were described previously (Danckwardt *et al*, 2002; Gehring

*et al*, 2003, 2005; Schell *et al*, 2003). Plasmid pET15b-eRF3a expressing 6His-eRF3a was kind gifts of LY Frolova (Jakobsen *et al*, 2001). eRF1 and PABPC1 cDNAs were PCR-amplified from HeLa cDNA, eRF3a was amplified from pET15b-eRF3a and used for subcloning to pCI-neo-FLAG, pCI-neo-V5, pCI-neo-MS2 and pCI-neo- $\lambda$ NV5. Deletion mutants of PABPC1, eRF3, eRF1 and UPF1 were created using appropriate PCR primer pairs (Supplementary Table 1). Site-specific mutants of UPF1 were generated by site-directed mutagenesis (GeneTailor, Invitrogen). The plasmid p2luc-if was a generous gift of JF Atkins (Howard *et al*, 2001). The plasmids p2luc-TAA, p2luc-TAG and p2luc-TGA were produced by site-directed mutagenesis of p2luc-if plasmid.

### Cell culture, plasmid and siRNA transfections

HeLa cells were grown and transfected with DNA or siRNA as described (Gehring *et al*, 2003, 2005). For tethering experiments, 2  $\mu$ g of 4MS2/4boxB or 4boxB/4MS2 reporter, 1  $\mu$ g of control WT globin, 0.2  $\mu$ g of GFP reporter and 1  $\mu$ g of respective pCI-neo-MS2 or pCI-neo- $\lambda$ NV5 reporter were used. For transfection of  $\beta$ -globin WT or NS39, we used 1  $\mu$ g of pCI-neo-WT or pCI-neo-NS39 reporter, 0.5  $\mu$ g of the control plasmid wt300 + e3, and 0.1  $\mu$ g of the GFP-expressing plasmid. Transfections for IPs were carried out with 2  $\mu$ g of the FLAG expression vector and 0.5–3  $\mu$ g of V5 expression plasmids together with 0.5  $\mu$ g of GFP vector.

siRNA transfection and UPF1-complementation assays were carried out as described (Gehring *et al*, 2005).

### Dual luciferase assay

HeLa cells transfected with 2  $\mu$ g p2luc-TAA, p2luc-TAG or p2luc-TGA were collected 48 h after transfection, lysed with passive lysis buffer (Promega) and luciferase activity was determined using the Dual Luciferase reporter assay (Promega) as described in Howard *et al* (2001).

### RNA extraction and analysis

Total cytoplasmic RNA was analysed by northern blotting and qRT-PCR as described (Gehring *et al*, 2003). Quantification of signals was carried out in FLA-3000 fluorescent analyser. Percentages and standard deviations were calculated as described in Kunz *et al* (2006).

### Protein extraction, western blotting and immunoprecipitations

Complexes of FLAG-tagged proteins were immunoprecipitated as described (Gehring *et al*, 2003). Unless otherwise stated, cells were lysed for IP reactions in lysis buffer (20 mM Tris-HCl, pH 7.2, 150 mM KCl, 2 mM MgCl<sub>2</sub>, 0.5% NP-40 and protease cocktail inhibitor 'complete' (Roche Diagnostics)). The lysis buffer for immunoprecipitation with eRFs was supplemented with 10 mM MgCl<sub>2</sub>.

Anti-SMG1 (Cell Signalling) antibodies were used for immunoprecipitations using Protein A/G Plus-Agarose (Santa Cruz Biotechnology) according to the manufacturer's recommendations. The 14 000 g supernatants (S14), used as cytosolic fractions, were pre-cleared by incubation with the Protein A/G Plus-Agarose beads pre-blocked with BSA. Subsequently, samples were incubated overnight with mouse anti-SMG1 at 4°C. The pre-blocked beads were then added to the samples for 2–3 h at 4°C. Afterwards, beads were washed three times with washing buffer (20 mM Tris-HCl, pH 7.2, 200 mM KCl, 2 mM MgCl<sub>2</sub>). The immunoprecipitates were eluted with 1  $\times$  SDS-PAGE loading buffer.

For immunoblotting analysis following siRNA depletion, 20  $\mu$ g of cytoplasmic lysates was subjected to SDS-PAGE (Gehring *et al*, 2005).

For quantification of UPF1 phosphorylation levels, FLAG-UPF1 mutants were immunoprecipitated as described above. The lysis buffer was supplemented with a phosphatase inhibitor cocktail ('PhosSTOP'; Roche Diagnostics). An anti-phospho-UPF1 antibody was used for detection of phosphorylated UPF1 (Kashima *et al*, 2006), FLAG antibodies were used as a control for protein loading. Infrared IRDye-labeled secondary antibodies were used for the detection of bands using an Odyssey Infrared Imaging System (LI-COR Biosciences).

### Supplementary data

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

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