

# An alternative branch of the nonsense-mediated decay pathway

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**The T-cell receptor (TCR) locus undergoes programmed rearrangements that frequently generate premature termination codons (PTCs). The PTC-bearing transcripts derived from such nonproductively rearranged genes are dramatically downregulated by the nonsense-mediated decay (NMD) pathway. Here, we show that depletion of the NMD factor UPF3b does not impair TCR $\beta$  NMD, thereby distinguishing it from classical NMD. Depletion of the related factor UPF3a, by itself or in combination with UPF3b, also has no effect on TCR $\beta$  NMD. Mapping experiments revealed the identity of TCR $\beta$  sequences that elicit a switch to UPF3b dependence. This regulation is not a peculiarity of TCR $\beta$ , as we identified many wild-type genes, including one essential for NMD, that transcribe NMD-targeted mRNAs whose downregulation is little or not affected by UPF3a and UPF3b depletion. We propose that we have uncovered an alternative branch of the NMD pathway that not only degrades aberrant mRNAs but also regulates normal mRNAs, including one that participates in a negative feedback loop controlling the magnitude of NMD.**

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## Introduction

Nonsense-mediated decay (NMD) degrades aberrant mRNAs containing premature termination codons (PTCs). By curtailing the survival of PTC-containing mRNAs, NMD reduces the synthesis of truncated proteins, some of which have dominant-negative or deleterious gain-of-function effects (Jacobson and Peltz, 1996; Neu-Yilik *et al.*, 2004; Maquat, 2004; Wilkinson, 2005; Chang *et al.*, 2007). Hence, NMD is widely thought to mitigate the adverse phenotypic consequences of many naturally occurring nonsense and frame-

shift mutations (Frischmeyer and Dietz, 1999; Holbrook *et al.*, 2004). NMD is crucial for degrading not only mRNAs from mutant genes but also mRNAs transcribed from a subset of normal genes, including those with upstream reading frames (uORFs) and those made out of frame as a result of alternative splicing (Lelivelt and Culbertson, 1999; He *et al.*, 2003; Mendell *et al.*, 2004; Mitrovich and Anderson, 2005; Rehwinkel *et al.*, 2006).

In mammalian cells, the degradation of transcripts harboring PTCs depends on both translation (Belgrader *et al.*, 1993; Li *et al.*, 1997; Zhang and Maquat, 1997; Wang *et al.*, 2002c) and pre-mRNA splicing (Carter *et al.*, 1996; Thermann *et al.*, 1998; Zhang *et al.*, 1998). The translation apparatus recognizes the stop codon, and the splicing machinery deposits a dynamic assembly of proteins necessary for NMD (Le Hir *et al.*, 2000; Lejeune *et al.*, 2002). This assembly, known as the exon junction complex (EJC), elicits NMD only when it is deposited downstream of a stop codon. This was initially suggested by the discovery that a spliceable intron must follow a stop codon to elicit NMD (Carter *et al.*, 1996; Thermann *et al.*, 1998). Later, both gain-of-function and loss-of-function experiments demonstrated the role of particular EJC factors in NMD (Gehring *et al.*, 2003; Palacios *et al.*, 2004; Gehring *et al.*, 2005; Kim *et al.*, 2005).

Our laboratory has focused its efforts on the molecular mechanism that degrades PTC-containing mRNAs transcribed from the T-cell receptor- $\beta$  (TCR $\beta$ ) gene locus. TCR $\beta$  transcripts frequently contain PTCs, as the TCR $\beta$  gene locus undergoes error-prone programmed DNA rearrangements during lymphocyte development (Li and Wilkinson, 1998; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999; Bruce and Wilkinson, 2003; Gudikote and Wilkinson, 2006; Chang *et al.*, 2007). The extremely frequent acquisition of PTCs at the TCR $\beta$  locus may have generated intense selection pressure over evolutionary time to efficiently eliminate TCR $\beta$  transcripts harboring PTCs. In agreement with this hypothesis, we previously showed that TCR $\beta$  transcripts harboring PTCs undergo much more dramatic downregulation (to ~1–5% of normal levels) than do transcripts from the non-rearranging genes that have been examined (to ~10–30% of normal levels) (Carter *et al.*, 1995; Li and Wilkinson, 1998; Gudikote and Wilkinson, 2002). This robust downregulatory response is not mediated by factors specific to T cells, nor is it elicited specifically by the TCR $\beta$  promoter; rather, it is elicited by specific sequences present in TCR $\beta$  transcripts (Gudikote and Wilkinson, 2002).

In addition to having a robust downregulatory response, TCR $\beta$  transcripts are uniquely downregulated by PTCs in at least two other ways. First, TCR $\beta$  mRNAs exhibit polar regulation such that PTCs closer to the 5' end of the penultimate exon elicit greater downregulation than do those near the 3' end (Wang *et al.*, 2002a). The only other mammalian transcripts known to exhibit polar regulation are those transcribed from the related gene locus, I $\mu$ , which, like the TCR $\beta$  locus, undergoes programmed rearrangements (Buhler *et al.*,

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2004; Gudikote and Wilkinson, 2006). Second, in contrast to most known transcripts, TCR $\beta$  transcripts are downregulated by PTCs closer than 50 nucleotides (nt) from the final exon-exon junction (Carter *et al*, 1996; Wang *et al*, 2002a). As with polar regulation, the only other transcripts known to deviate from this '-50 boundary' rule (Maquat, 2004) are I $\mu$ m transcripts (Buhler *et al*, 2004). The -50 boundary rule is widely thought to reflect the likelihood that the ribosome would have displaced the EJC by the time the translation apparatus recognizes a stop codon closer than the -50-nt position (Maquat, 2004; Wilkinson, 2005). Because TCR $\beta$  transcripts can be downregulated by PTCs much closer than the -50-nt position, this raises the possibility that an entity other than the classical EJC can serve as the second signal for TCR $\beta$  NMD.

In support of this idea, we demonstrate in this study that TCR $\beta$  NMD is unperturbed when the EJC proteins UPF3a and UPF3b (also called Upf3 and Upf3x, respectively) are severely reduced in levels by RNA interference (RNAi). These two related nuclear-cytoplasmic shuttling proteins are encoded by distinct genes on two different human chromosomes (Kim *et al*, 2001; Serin *et al*, 2001). They correspond to a single gene that, when mutated, prevents NMD in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* and strongly reduces NMD when knocked down by RNAi in cultured *Drosophila melanogaster* cells (Leeds *et al*, 1991, 1992; Pulak and Anderson, 1993; Gatfield *et al*, 2003; Rehwinkel *et al*, 2005). In mammals, there are two lines of evidence supporting a role for UPF3b in NMD. First, tethering UPF3b (as a MS2 fusion protein) downstream of the stop codon in human  $\beta$ -globin mRNA elicits mRNA decay (Lykke-Andersen *et al*, 2000). Second, transient transfection of a small interfering RNA (siRNA) specific for UPF3b inhibits  $\beta$ -globin NMD (Kim *et al*, 2005). In contrast, the role of UPF3a in NMD is not clear, as UPF3a only modestly elicits NMD when tethered downstream of a stop codon (Lykke-Andersen *et al*, 2000), and the functional consequences of its depletion have not been reported.

To address the role of UPF3a and UPF3b in TCR $\beta$  NMD, we generated cell clones that constitutively express low levels of UPF3a, UPF3b, or both. Using these cell clones in combination with transiently transfected vector-based RNAi plasmids to further knock down UPF3a and/or UPF3b, we discovered that TCR $\beta$  NMD is unperturbed by the depletion of UPF3a, UPF3b, or both. Mapping experiments identified sequences in TCR $\beta$  required for its unique regulatory properties. Microarray and real-time analyses revealed transcripts from many wild-type genes that share with TCR $\beta$  transcripts the ability to undergo NMD in the face of severely depleted UPF3a and UPF3b levels. Many of these transcripts have NMD features and are downregulated by the NMD factors UPF1 and eIF4AIII, indicating that they are bona fide NMD substrates. Our results suggest that at least two NMD pathways with different substrate specificities exist: one that depends on the UPF3 factors and another that is independent of these factors.

## Results

### **Establishment of HeLa cell clones stably depleted of UPF3b**

To examine the role of UPF3b in NMD, we stably depleted UPF3b using a vector-based short-hairpin RNA (shRNA)

approach. HeLa cells were transfected with a UPF3b shRNA expression vector that we developed (see Materials and methods), followed by antibiotic selection for cell clones that had stably integrated the plasmid. From a total of 93 clones, two were identified (B31 and B83) that expressed the most reduced levels of UPF3b mRNA, as shown by ribonuclease (RNase) protection analysis (Figure 1A). These cell clones also had reduced UPF3b protein levels, as demonstrated by Western blot analysis of B31 and B83 cell lysates compared with serial dilutions of control cell lysates (Figure 1B). For control cells, we used both parental HeLa cells and a HeLa cell clone stably transfected with a shRNA vector targeting firefly luciferase (Luc). When transiently transfected with a Luc expression vector, this Luc cell clone had  $\geq 90\%$  reduced Luc levels compared with parental HeLa cells (data not shown).

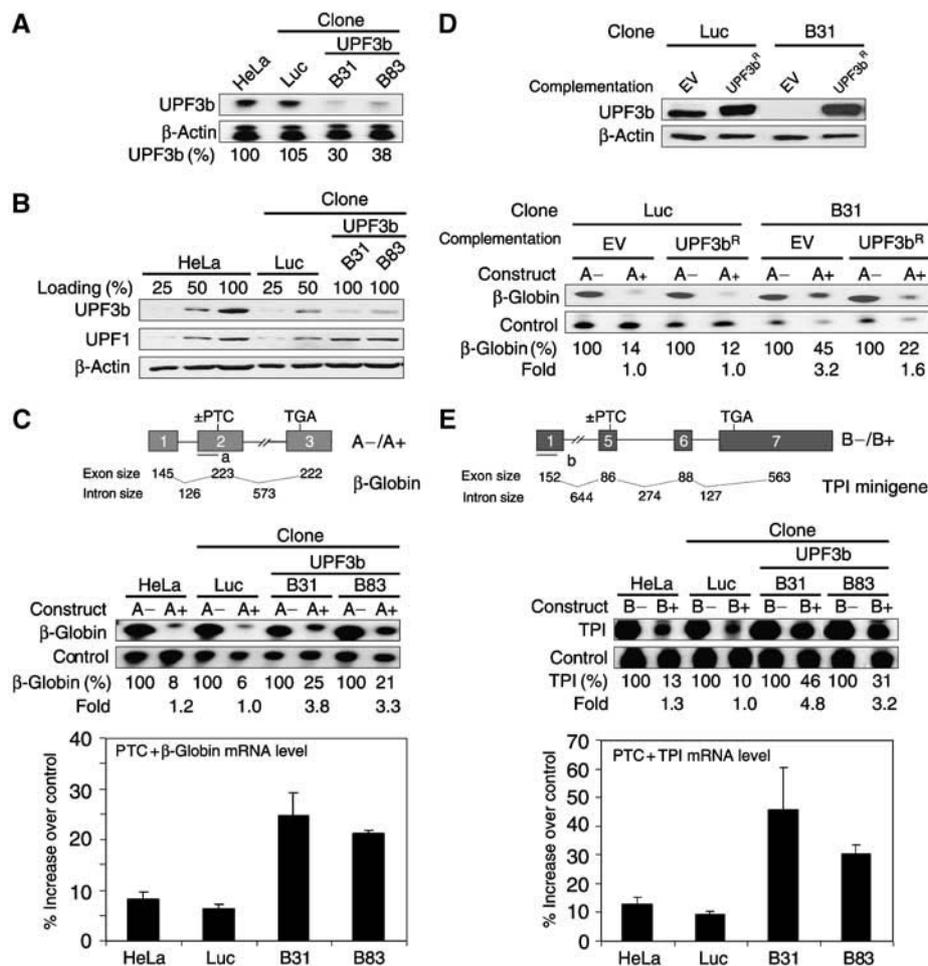
Both UPF3b-depleted cell clones had significantly reduced doubling time compared with both the control Luc cell clone and untransfected HeLa cells (Supplementary Figure S1). The modest reduction in UPF3b levels ( $\sim 30\%$  of normal) in these cell clones indicated that UPF3b is limiting in these cells and that even a modest reduction is sufficient to elicit physiological effects. We therefore suspected that it would not be possible to grow cell clones with much lower levels of UPF3b.

To determine whether the UPF3b-depleted cell clones had a defect in NMD, we transiently transfected them with PTC-bearing (PTC+) and PTC-lacking (PTC-) versions of the  $\beta$ -globin gene (Figure 1C).  $\beta$ -globin mRNA levels were determined by RNase protection analysis using a cotransfected gene as an internal control to correct for differences in transfection efficiency and loading. For each cell line, the steady-state level of mature mRNA from the PTC- construct was set to 100%, and the PTC+ mRNA level was determined relative to that. This analysis demonstrated that the B31 and B83 cell clones expressed elevated levels of PTC+  $\beta$ -globin transcripts compared with the Luc cell clone and the parental HeLa cell line, indicating that NMD was impaired in the former. The degree of upregulation ( $>3$ -fold) was comparable to that reported by other groups that depleted NMD factors using transiently transfected siRNAs (Gehring *et al*, 2003; Palacios *et al*, 2004; Kim *et al*, 2005; Usuki *et al*, 2006; Wittmann *et al*, 2006). To control for potential off-target effects of RNAi, we transfected an shRNA-resistant UPF3b expression vector and found that it restored NMD (Figure 1D).

As an independent test of whether NMD was reversed in the B31 and B83 cell clones, we transfected PTC+ and PTC- versions of a triose phosphate isomerase (TPI) minigene (Figure 1E) that we previously showed to be an NMD substrate (Gudikote *et al*, 2005). As with the  $\beta$ -globin transcripts, TPI transcripts exhibited reversed NMD ( $>3$ -fold upregulation of PTC+ TPI mRNA) in the B31 and B83 cell clones (Figure 1E). We conclude that the B31 and B83 cell clones have sufficiently low levels of UPF3b to exhibit impaired NMD.

### **Normal TCR $\beta$ NMD in UPF3b-depleted cells**

TCR $\beta$  transcripts are downregulated by an NMD mechanism that has several atypical features (see Introduction; Gudikote and Wilkinson, 2002, 2006; Wang *et al*, 2002a; Gudikote *et al*, 2005). The generation of UPF3b-deficient cell clones gave us an opportunity to determine whether TCR $\beta$  NMD is also



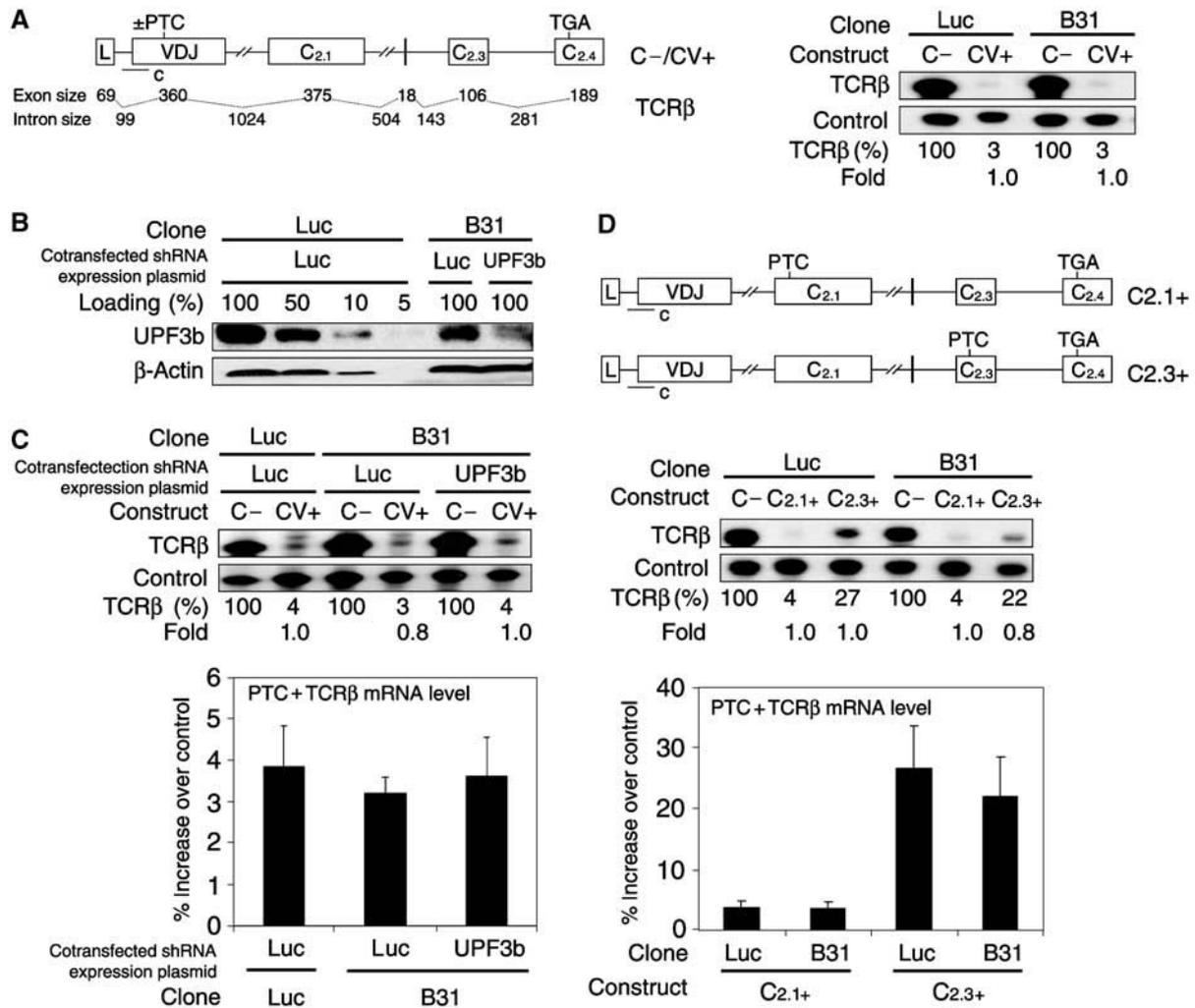
**Figure 1** NMD is impaired in HeLa cells stably expressing UPF3b shRNA. (A) RNase protection analysis (using probe f, complementary to UPF3b) of total cellular RNA from HeLa cell clones stably transfected with a UPF3b shRNA expression plasmid (B31 and B83) or a luciferase shRNA expression plasmid (Luc). The Luc clone and untransfected HeLa cells served as negative controls. The numbers below the blot are UPF3b mRNA relative levels normalized to  $\beta$ -actin mRNA levels. (B) Western blot analysis of the cells in panel A. To assess the efficiency of UPF3b shRNA knockdown, serial dilutions of the negative control lysates were loaded. For loading controls, the membrane was reprobed with  $\beta$ -actin and UPF1 antibodies. (C) RNase protection analysis (using probe a) of total cellular RNA isolated from the cells in panel A transiently transfected with PTC+ and PTC- versions of the  $\beta$ -globin construct shown (A+ and A-, respectively). The internal control is a cotransfected TCR $\beta$  construct (C-). The numbers below the blot are relative mRNA levels (PTC- is set to 100) and the fold difference in PTC-/PTC+ ratio relative to Luc control cells. (D) Functional complementation of UPF3b depletion. Upper: Western blot analysis of UPF3b depletion and complementation. For complementation of UPF3b depletion, the Luc and B31 clones were transfected with an empty expression vector (EV) or an RNAi-resistant UPF3b expression vector (UPF3b<sup>R</sup>). Lower: RNase protection analysis of total cellular RNA isolated from Luc and B31 clones cotransfected with A- or A+ constructs (as described in panel C) and EV or UPF3b<sup>R</sup>. (E) RNase protection analysis (using probe b) of total RNA isolated from the cells in panel A transiently transfected with PTC+ and PTC- versions of the TPI construct shown (B+ and B-, respectively). The values in panels C and E are the means of three independent transfection experiments. The results in panel D are representative of two independent experiments. Error bars indicate standard deviation.

atypical in its requirement for UPF3b. To test this, we transiently transfected PTC+ and PTC- TCR $\beta$  constructs (Figure 2A, left) into the B31 cell clone. Analysis of the expressed mRNAs revealed that PTC+ TCR $\beta$  transcripts were expressed at normal levels in these UPF3b-deficient cells (Figure 2A, right). Thus, unlike  $\beta$ -globin and TPI transcripts, TCR $\beta$  transcripts are downregulated by NMD independent of UPF3b depletion.

Given that TCR $\beta$  NMD is translation dependent (Carter *et al*, 1995, 1996), we next determined whether TCR $\beta$  translation is compromised in UPF3b-depleted cells. We examined this by transfecting a wild-type TCR $\beta$  construct (C- in Figure 2A) into the UPF3b-depleted B31 and B83 cell clones

and determining translation efficiency by calculating the TCR $\beta$  protein-to-mRNA ratio. We found that the B31 and B83 cell clones exhibited translation efficiencies that were indistinguishable from those of the Luc control cell clone (Supplementary Figure S2). We conclude that depletion of UPF3b affects neither TCR $\beta$  translation nor TCR $\beta$  NMD.

To more definitively test whether TCR $\beta$  NMD is impervious to reduced levels of UPF3b, we further reduced UPF3b levels by introducing more copies of the UPF3b shRNA construct into the UPF3b-depleted cell clone B31 by transient transfection. This 'super-RNAi' approach depleted UPF3b protein levels to 10% of normal (Supplementary Figure S3 and Figure 2B). Despite this further reduction in UPF3b level,



**Figure 2** TCRβ NMD is unperturbed by depletion of UPF3b. (A) RNase protection analysis (using probe c) of total cellular RNA isolated from the B31 and Luc cell clones transiently transfected with construct C- or CV+. (B) Western blot analysis (performed as in Figure 1B) of the UPF3b-depleted B31 cell clone transiently transfected with a UPF3b shRNA expression plasmid to further reduce the level of UPF3b. The Luc cell clone and Luc shRNA expression plasmid served as negative controls. (C) RNase protection analysis (using probe c) of total cellular RNA isolated from cells transiently transfected as in panel B, along with the indicated NMD reporter constructs. (D) RNase protection analysis (using probe c) of total cellular RNA isolated from the B31 and Luc cell clones transiently transfected (as in panel C) with the indicated constructs. The values in panel A are representatives of two independent experiments. The results in panels C and D are the means of these independent transfection experiments. Error bars indicate standard deviation.

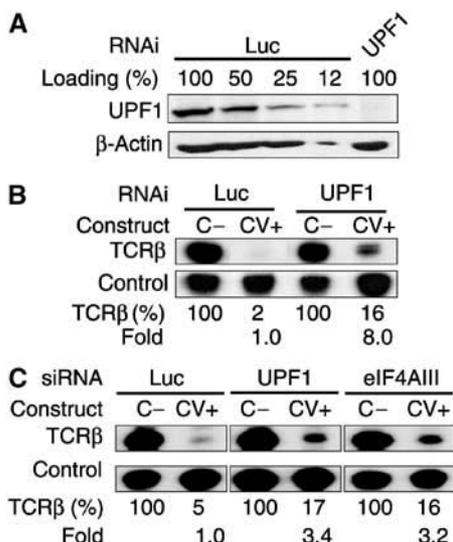
TCRβ NMD was still not affected, regardless of whether the PTC was in the VDJ (Figure 2C), C2.1, or C2.3 (Figure 2D) exons.

To determine whether the insensitivity of TCRβ NMD to UPF3 knockdown was the result of long-term culture under UPF3b-depleted conditions, we performed transient transfections with a synthetic siRNA targeting UPF3b. Consistent with what we found in cells stably depleted of UPF3b, transient transfection of the UPF3b siRNA reversed NMD of β-globin but not of TCRβ (Supplementary Figure S4). To determine the specificity of the insensitivity of TCRβ to UPF3b knockdown, we used a synthetic siRNA targeting another EJC component, eIF4AIII, which has been shown to function in TCRβ NMD (Ferraiuolo *et al*, 2004; Palacios *et al*, 2004; Shibuya *et al*, 2004; Shibuya *et al*, 2006). We found that knockdown of eIF4AIII reversed TCRβ NMD in our HeLa cells (Supplementary Figure S4).

We next examined whether TCRβ NMD depends on UPF1, which is thought to be a universal NMD factor required for

the decay of all transcripts harboring PTCs. UPF1 is first recruited to transcripts through its direct interaction with release factors when translation terminates (Czapinski *et al*, 1998). After its recruitment, UPF1 interacts with the essential NMD protein UPF2 and probably other factors recruited to the EJC after RNA splicing, ultimately leading to rapid RNA decay (Serin *et al*, 2001; Lykke-Andersen, 2002; Gehring *et al*, 2003; Kashima *et al*, 2006). We found that depletion of UPF1 by transient siRNA (Figure 3A) strongly reversed TCRβ NMD (Figure 3B). The eight-fold upregulation of PTC+ TCRβ mRNA elicited by depletion of UPF1 was comparable to or greater than the upregulation of PTC+ β-globin mRNA reported by others (Palacios *et al*, 2004; Kim *et al*, 2005).

Finally, we examined the possibility that long-term depletion of UPF3b selects for cells with an altered TCRβ NMD response that is independent of other NMD factors. To test this, we used transient siRNA to knock down the expression of UPF1 and eIF4AIII in cells stably depleted of UPF3b. We



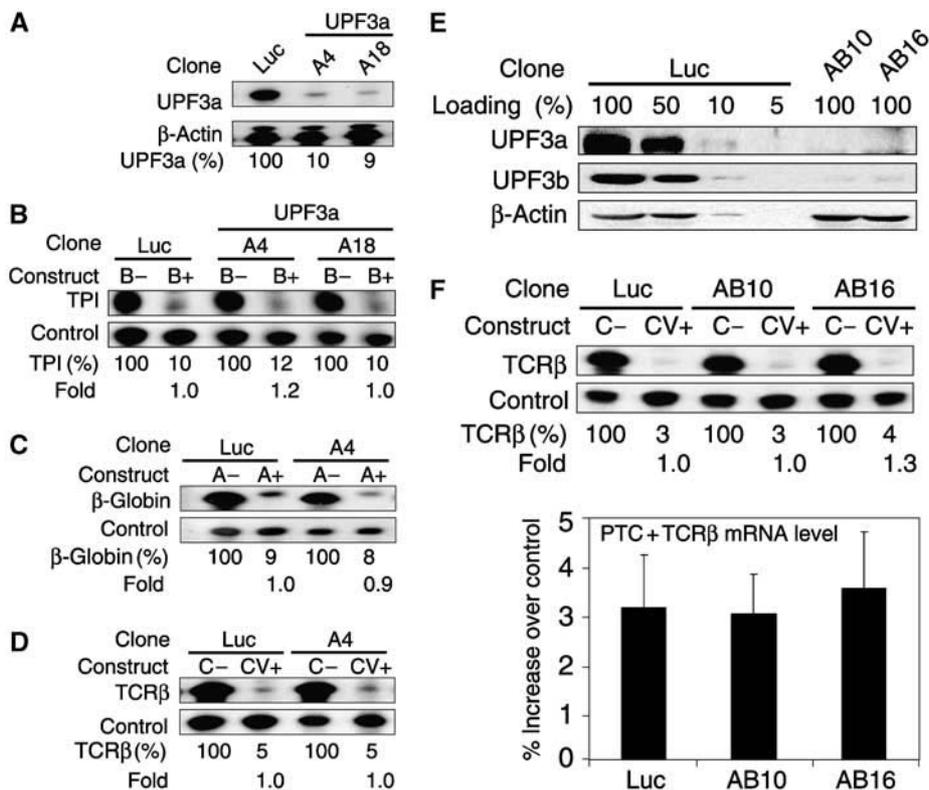
**Figure 3** TCRβ NMD in UPF3b-depleted cells requires UPF1 and eIF4AIII. (A) Western blot analysis of HeLa cells transiently transfected with siRNAs specific for UPF1 or luciferase (negative control). (B) RNase protection analysis (using probe c) of total RNA isolated from the cells in panel A transiently transfected with the TCRβ constructs C- and CV+ 48 h after siRNAs were transfected. (C) RNase protection analysis (using probe c) of total cellular RNA isolated from the B31 cell clone transiently transfected with constructs C- and CV+ 48 h after siRNAs were transfected. The results in panels B and C are representative of two independent experiments.

found that knockdown of either UPF1 or eIF4AIII in these cells reversed TCRβ NMD (Figure 3C) just as it did in HeLa cells with normal levels of UPF3b (Figure 3A and Supplementary Figure S4). Thus, cells grown for long periods of time with low amounts of UPF3b do not have an altered TCRβ NMD response, at least with respect to dependence on UPF1 and eIF4AIII.

We conclude that TCRβ NMD requires UPF1 and the EJC factor eIF4AIII, but is unaffected by depletion of the EJC factor UPF3b. Collectively, these data suggest that TCRβ transcripts are regulated by a unique, UPF3b-independent branch of the NMD pathway.

#### Depletion of both UPF3a and UPF3b has no effect on TCRβ NMD

We next determined whether the UPF3b-related factor UPF3a can be used in place of UPF3b to elicit TCRβ NMD. To address this, we stably transfected HeLa cell clones with an shRNA construct specific for UPF3a and identified cell clones that had reduced UPF3a levels. Supplementary Figure S5 shows the data from clones A4 and A18, both of which had only ≤15% of normal UPF3a levels but had a normal magnitude of TCRβ NMD (data not shown). We then used our 'super-RNAi' approach to further reduce UPF3a mRNA levels to ≤10% of normal (Figure 4A). This also had no effect on TCRβ NMD, nor did it affect TPI or β-globin NMD (Figure 4B–



**Figure 4** TCRβ NMD is unperturbed by depletion of both UPF3a and UPF3b. (A) RNase protection analysis (using probe g, complementary to UPF3a) of the UPF3a-deficient cell clones A4 and A18 (described in Supplementary Figure S5) transiently transfected with a UPF3a shRNA expression plasmid to further reduce the level of UPF3a. (B–D) RNase protection analysis of total cellular RNA isolated from cells transiently transfected as in panel A, along with the indicated NMD reporter constructs. (E) Western blot analysis of the UPF3a- and UPF3b-deficient HeLa cell clones AB10 and AB16 (described in Supplementary Figure S6) transiently transfected with UPF3a and UPF3b shRNA expression plasmids to further reduce UPF3a and UPF3b levels. (F) RNase protection analysis of total cellular RNA isolated from cell clones transiently transfected as in panel E, along with the indicated NMD reporter constructs. The results in panels B–D are representative of two independent experiments. The values in panel F are the means of three independent transfection experiments. Error bars indicate standard deviation.

D). We conclude that severe depletion of UPF3a has no effect on the NMD substrates we tested, suggesting that UPF3a is not required for their decay.

To determine whether UPF3a could compensate for UPF3b, we generated HeLa cell clones depleted of both UPF3a and UPF3b by stably transfecting HeLa cells with two shRNA vectors, one specific for UPF3a and the other for UPF3b. We identified two cell clones, AB10 and AB16, both of which had substantially reduced levels of both UPF3a and UPF3b mRNA and protein (~20% of normal; Supplementary Figure S6). Despite the low levels of UPF3a and UPF3b, the AB10 and AB16 cell clones had normal TCR $\beta$  NMD (data not shown) even when they were also transiently transfected with the UPF3a and UPF3b shRNA plasmids to further reduce UPF3a and UPF3b levels to  $\leq$ 10% of normal (Figure 4E and F). We conclude that drastic reduction of both UPF3a and UPF3b has no effect on TCR $\beta$  NMD. Thus, TCR $\beta$  transcripts harboring PTCs are downregulated by an NMD mechanism that either requires exceedingly low levels of these two related EJC factors or is completely independent of both. In either case, TCR $\beta$  mRNA is clearly different from standard NMD substrates ( $\beta$ -globin and TPI mRNA) in its requirement for the EJC factor UPF3b.

#### **The TCR $\beta$ VDJ exon and adjacent intron sequences mediate insensitivity to UPF3b depletion**

To identify sequences required for the atypical TCR $\beta$  NMD response, we made various deletions in the TCR $\beta$  gene. We found that deletion of the VDJ exon and adjacent intron sequences converted TCR $\beta$  into a UPF3b-dependent NMD substrate (Figure 5A). Insertion of this sequence into the TPI minigene described in Figure 1E conferred UPF3b independence (Figure 5B). These data indicated that at least one *cis* element exists in this region that drives UPF3b-independent NMD.

In an attempt to map the element, we made smaller deletions. Deletion of the 3' half of the VDJ exon and downstream TCR $\beta$  intron sequences did not cause a switch to UPF3b-dependent NMD (Figure 5C), nor did various deletions in the 5' half of the VDJ exon (data not shown). These mapping studies suggested that the 5' half of VDJ and its upstream flanking intron are the minimal region required to confer UPF3b independence. It also ruled out the existence of a single non-redundant element in the VDJ exon or downstream intron.

To assess whether multiple redundant *cis* elements exist in the VDJ exon, we replaced it with an exon from the TPI gene. This also had no effect on UPF3b dependence (Figure 5D), ruling out the possibility that the VDJ exon has an obligate role in this response. Similarly, replacing the upstream and downstream TCR $\beta$  introns with  $\beta$ -globin introns had no effect (Figure 5E). Taken together, these mapping experiments imply that the region encompassing the VDJ exon and adjacent introns has multiple redundant elements essential for UPF3b-independent NMD.

#### **Identification of wild-type transcripts regulated by the alternative branch of the NMD pathway**

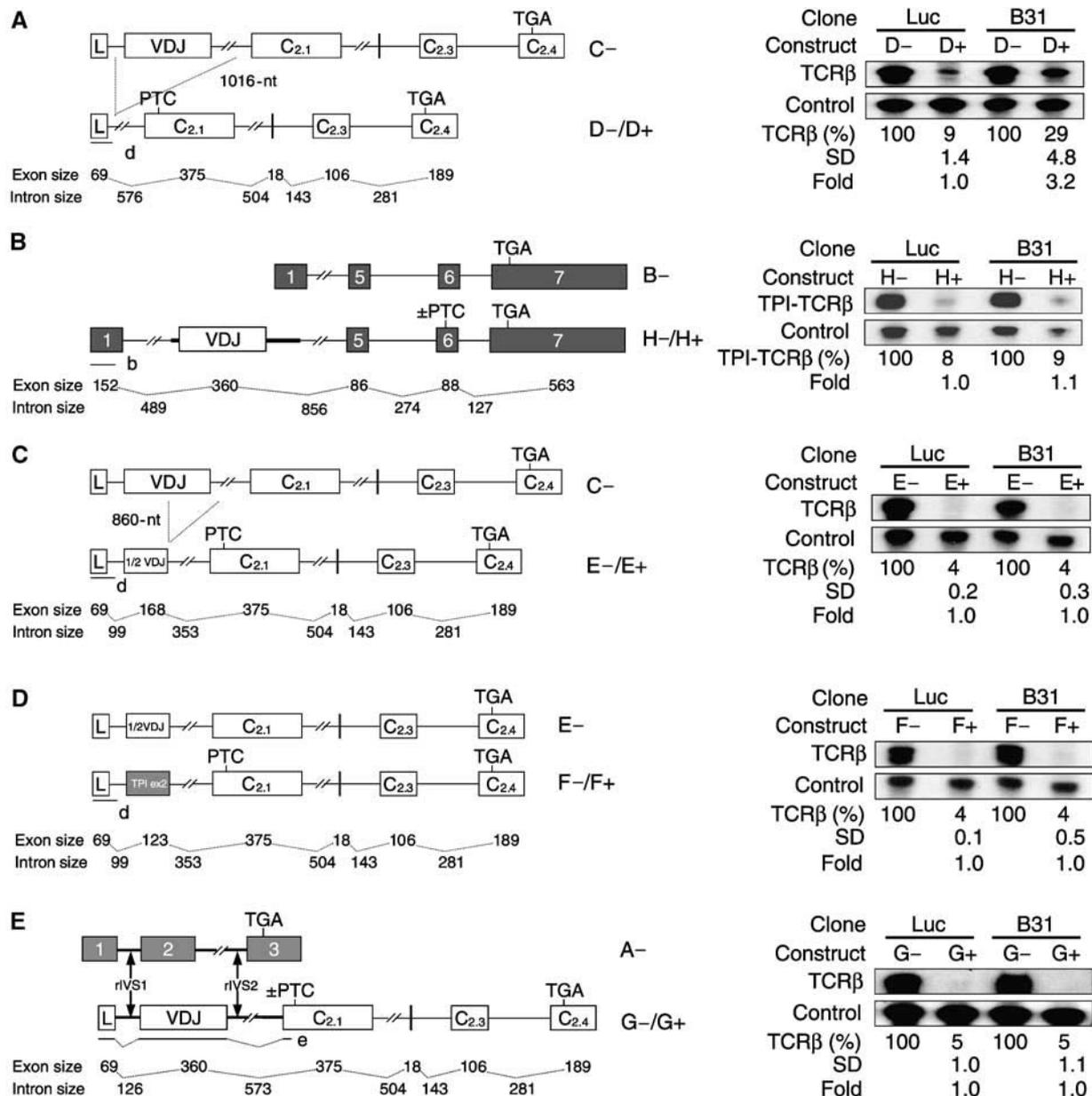
To assess whether the UPF3b-insensitive NMD pathway acts specifically on TCR $\beta$  transcripts or has a more general role, we performed microarray analysis on UPF3b-depleted, UPF1-depleted, and control HeLa cells. This analysis revealed that

817 transcripts (~4% of the ~21 700 transcripts expressed) were upregulated by at least two-fold in response to depletion of UPF1, whereas 396 transcripts (~2%) were upregulated by at least two-fold in response to depletion of UPF3b ( $P < 0.01$ ; Supplementary Table I lists the transcripts upregulated by  $\geq 3$ -fold in response to UPF3b depletion). We found that many of the transcripts regulated by UPF1 (348 of 817) were not significantly regulated by UPF3b, making them candidate substrates for the alternative branch of the NMD pathway (Supplementary Table II lists those in this category that were upregulated by  $\geq 4$ -fold in response to depletion of UPF1). Many of these transcripts encode proteins with common functions, including metabolism, signal transduction, transcription, and translation. Our microarray analysis identified 61 transcripts upregulated by  $\geq 2$ -fold after depletion of either UPF1 or UPF3b ( $P < 0.01$ ). These transcripts are candidates to be regulated by the classical branch of the NMD pathway.

At least one-third of both classes of transcripts had NMD-inducing features, including frameshift-inducing alternative splicing, the presence of a stop codon >50-nt upstream of the final exon-exon junction, or a uORF that would introduce a stop codon upstream of the main reading frame. The actual proportion of direct targets may be significantly higher than one-third, as the Ensembl and NCBI Entrez Gene databases we used to identify PTC-bearing alternative spliced transcripts only identify a small proportion of alternatively spliced human transcripts. PTC-bearing alternatively spliced transcripts are likely to be common substrates of NMD, as greater than one-third of human genes transcribe pre-mRNAs that undergo alternative splicing (Lewis *et al*, 2003).

To validate the authenticity of our microarray analysis, we performed real-time PCR analysis on randomly selected transcripts with NMD features that were indicated by the microarray analysis to be downregulated by UPF1 but not UPF3b. We found that 10 of the 10 transcripts that we tested in this category were upregulated after UPF1 depletion (most by three-fold or more), but were little or not affected by UPF3b depletion (Figure 6A and B, dark bars). Included among these transcripts was *SMG5*, which encodes an NMD factor (Anders *et al*, 2003; Chiu *et al*, 2003; Gatfield *et al*, 2003). We also used real-time PCR to determine whether our microarray analysis correctly identified transcripts regulated by the classical branch of the NMD pathway (regulated by both UPF1 and UPF3b). We found that four of four transcripts identified by microarray analysis as being in this category were indeed regulated in this manner (Figure 6A and B, light bars).

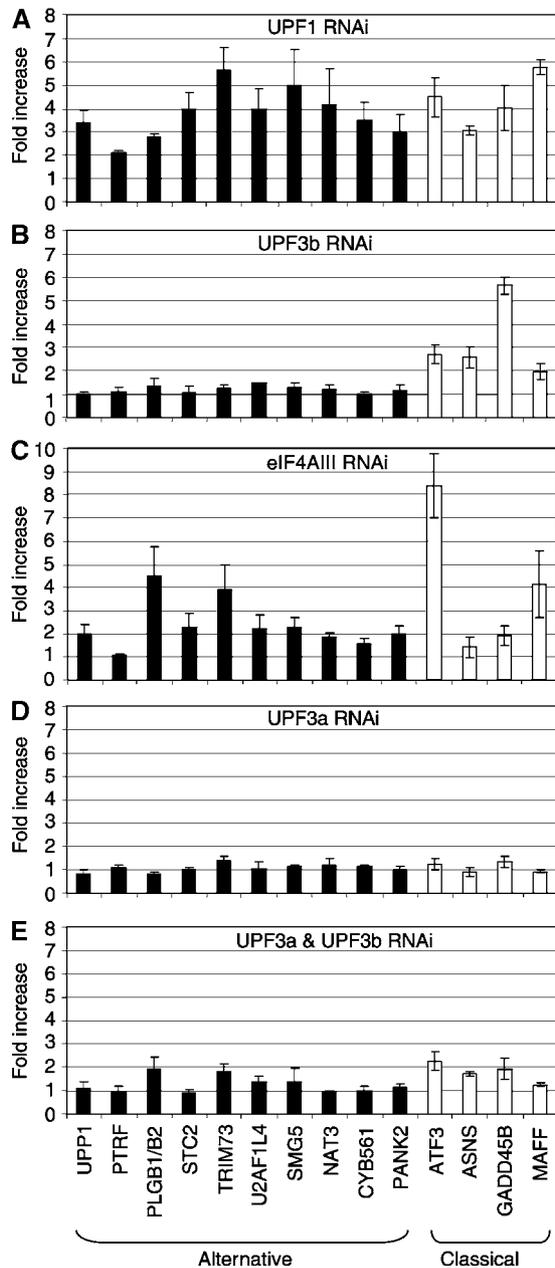
To further assess whether these endogenously regulated transcripts are truly NMD substrates, we performed real-time PCR analysis on cells depleted of the EJC core factor eIF4AIII. We found that nine of ten transcripts in the alternative branch (Figure 6C, dark bars) and three of four transcripts in the classical branch (Figure 6C, light bars) were significantly regulated by eIF4AIII, strongly suggesting that most of these transcripts are bona fide NMD substrates. The only exceptions were *ASNS* and *PTRF*. Although it is possible that these two transcripts are not regulated by NMD, we suspect that they are, as they both have NMD features and both were previously shown to be stabilized when UPF1 is knocked down by RNAi (Mendell *et al*, 2004). These transcripts may only require low levels of eIF4AIII to be degraded by NMD, or they may use an eIF4AIII-independent NMD pathway.



**Figure 5** NMD of TCRβ is rendered UPF3b dependent by deletion of the VDJ exon and adjacent intron sequences. (A–E) RNAse protection analysis of total cellular RNA isolated from the UPF3b-deficient B31 cell clone transiently transfected with the UPF3b shRNA expression plasmid to further reduce UPF3b levels (as described in Figure 2B). The Luc cell clone served as the negative control, as described in Figure 1A. All cells were also transiently transfected with the TCRβ constructs indicated in each panel. Construct D lacks the VDJ exon and part of the flanking introns. Construct E lacks the 3' half of the VDJ exon and the 5' portion of the downstream intron. Construct F has TPI exon 2 in place of the VDJ exon. Construct G has intron 1 (rIVS1) and intron 2 (rIVS2) from rabbit β-globin (indicated in bold) in place of TCRβ IVS1 and IVS2, respectively. Construct H is a TCRβ TPI-chimera construct that has the TCRβ VDJ exon and flanking intron sequences. Expression of each construct was quantified as in Figure 1C using the probes indicated in each panel. The values below each blot are the mean mRNA levels ± standard deviation and fold change in PTC–/PTC+ ratio, determined as in Figure 1C from three independent transfection experiments, except that the blot in panel B is representative of two independent experiments.

Lastly, we examined the role of UPF3a in the regulation of these endogenous transcripts. We found that their level was little or not affected by depletion of UPF3a (Figure 6D). To assess whether the role of UPF3a is redundant with that of UPF3b, we analyzed cells depleted of both UPF3a and UPF3b. We found that eight of the ten transcripts in the alternative branch (Figure 6E, dark bars) were little or not affected by depletion of UPF3a and UPF3b, indicating that they behave like TCRβ transcripts. The two exceptions, TRIM73 and

PLGB1/B2, were modestly upregulated in UPF3a/UPF3b-depleted cells ( $P < 0.01$ ). Because these two transcripts were not significantly upregulated in cells depleted of UPF3a or UPF3b alone, UPF3a and UPF3b appear to act redundantly on these transcripts. However, we cannot rule out the possibility that they act non-redundantly, as the TRIM73 and PLGB1/B2 transcripts might be slightly elevated in cells depleted of UPF3a or UPF3b alone (Figure 6B and D, dark bars). As a control, we examined transcripts in the classical branch,



**Figure 6** Endogenous NMD targets of the classical and alternative branches of the NMD pathway. (A–E) Real-time PCR analysis of the abundance of endogenous transcripts in cells depleted of UPF3b, UPF3a, both UPF3a and UPF3b, UPF1, or eIF4AIII (UPF1, UPF3a, UPF3b, and eIF4AIII levels were reduced to ~15, ~10, ~10, and ~20%, respectively; see Materials and methods for further details). The values shown are the average fold ( $n \geq 3$ ) increase relative to negative control Luc cells. Expression levels were normalized to that of the housekeeping transcript L19. Error bars indicate standard deviation.

which by definition are upregulated after depletion of UPF3b alone (Figure 6B, light bars). As expected, these transcripts were also upregulated in cells depleted of both UPF3a and UPF3b (Figure 6E, light bars).

## Discussion

Our data lead us to propose the existence of an alternative branch of the NMD RNA surveillance pathway that does not

depend on the well-studied NMD factor UPF3b and the related but less-studied factor UPF3a. A key line of evidence for this alternative branch is our discovery that depletion of UPF3a or UPF3b does not perturb the dramatic downregulatory response of TCR $\beta$  transcripts to PTCs (Figures 2 and 4). In other respects, the mechanism responsible for the downregulation of TCR $\beta$  transcripts has characteristics typical of NMD: (i) it depends on other known NMD factors, including UPF1 and eIF4AIII (Figure 3 and Supplementary Figure S4); (ii) it is elicited specifically by nonsense codons, not missense or silent codons; (iii) it depends on a start ATG in a Kozak consensus context; (iv) it can be initiated by an internal ribosome entry site; (v) it is inhibited by a translation-blocking stem-loop; and (vi) it is inhibited by suppressor tRNAs (Carter *et al*, 1995, 1996; Li *et al*, 1997; Wang *et al*, 2002b, c). The notion that we have identified an alternative branch of the NMD pathway independent of UPF3b is further strengthened by our microarray analysis, which uncovered 348 natural mRNAs regulated by UPF1 but regulated very little or not at all by UPF3b. Real-time analysis of a subset of these transcripts demonstrated that most of them are also regulated by the EJC/NMD factor eIF4AIII, providing further evidence that they are bona fide NMD substrates (Figure 6).

We obtained several lines of evidence suggesting that the alternative branch of the NMD pathway is independent of UPF3b. First, cells stably depleted of UPF3b, which had an impaired  $\beta$ -globin NMD response (Figure 1C) and a growth defect (Supplementary Figure S1), had a normal TCR $\beta$  NMD response (Figure 2A). Second, cells transiently depleted of UPF3b displayed an identical differential response (Supplementary Figure S4). Third, even cells massively depleted of UPF3b through both stable and transient transfection of the UPF3b RNAi vector had no defect in TCR $\beta$  NMD (Figure 2C and D). Fourth, cells that received both stable and transient UPF3b RNAi vector treatment also exhibited no change in the levels of a set of UPF1-dependent NMD substrates that we identified by microarray analysis (Figure 6 and Supplementary Table II). This lack of responsiveness was unique, as these UPF3b-depleted cells had a strong defect in classical NMD, as the NMD response of  $\beta$ -globin and TPI transcripts was strongly impaired (Figure 1C and E). Furthermore, microarray analysis revealed many UPF1-dependent NMD substrates that were strongly affected in these UPF3b-depleted cells (Supplementary Table I and Figure 6).

Although these many lines of evidence support the notion that we have identified a branch of the NMD pathway that is independent of UPF3b, the technical limitations of the RNAi approach do not allow us rule out the possibility that trace levels of UPF3b have some role. In theory, this issue could be addressed by using homologous recombination to ablate the UPF3b gene, but we suspect that this is not feasible, as a null mutation in another NMD gene, *Upf1* (*Rent1*), prevents the survival of embryonic fibroblast cells and is embryonic lethal in mice (Medghalchi *et al*, 2001). Indeed, we found that even a three-fold reduction in UPF3b levels elicited growth defects in HeLa cells (Supplementary Figure S1).

We found that SMG5 mRNA is a likely substrate of the alternative NMD pathway, as it was strongly upregulated after depletion of UPF1 or eIF4AIII but not UPF3a or UPF3b (Figure 6). Our finding that SMG5 is regulated by UPF1 confirms an earlier study showing that knockdown of UPF1 upregulates the level of SMG5 mRNA (Mendell *et al*, 2004).

Because SMG5 is an NMD factor, we propose that it participates with the alternative branch of the NMD pathway in a negative-feedback circuit that buffers the magnitude of NMD. Such a feedback network may be conserved, as SMG5 mRNA has also been shown to be downregulated by NMD in cultured *D. melanogaster* cells (Rehwinkel *et al*, 2005). It remains to be determined whether SMG5 transcripts are directly regulated by NMD and why it is regulated by a pathway impervious to UPF3a and UPF3b depletion.

Recently, Gehring *et al* (2005) reported evidence for a UPF2-independent branch of the NMD pathway. It is unlikely that this UPF2-independent branch of the NMD pathway is the same as the branch that acts on TCR $\beta$  transcripts, as TCR $\beta$  NMD requires UPF2 (Mendell *et al*, 2002; Wang *et al*, 2002c). Gehring *et al* also reported the existence of a UPF2-dependent pathway that appeared to be independent of EJC core factors, including eIF4AIII (Gehring *et al*, 2005). This is also a distinct pathway from the one we describe here, as TCR $\beta$  NMD depends on eIF4AIII (Supplementary Figure S4; Ferraiuolo *et al*, 2004; Palacios *et al*, 2004; Shibuya *et al*, 2004, 2006). Another branch of the NMD pathway may be one that is completely independent of the EJC, as some groups have reported that some PTC-bearing mammalian transcripts are degraded by NMD despite not having an exon–exon junction downstream of the PTC (Buhler *et al*, 2006; Chang *et al*, 2007).

Future studies will be required to determine why different mammalian transcripts have different NMD cofactor requirements. One possibility is that the composition of the EJC deposited on different transcripts and exon–exon junctions varies. Another possibility is that NMD cofactor requirements are dictated by post-EJC deposition events, such as the transcript's intracellular location when the PTC is recognized or when mRNA decay is initiated. Our mapping experiments indicated that a region encompassing the TCR $\beta$  VDJ exon and adjacent intron sequences is responsible for UPF3b-independent NMD (Figure 5A and B). However, no single *cis* element in this region is responsible, as deletion or substitution of the VDJ exon and adjacent introns did not elicit UPF3b dependence (Figure 5C–E). We suggest that good candidates for redundant elements within this region are those that promote RNA splicing. This follows from the fact that RNA splicing is responsible for EJC deposition and that RNA splicing efficiency is often controlled by multiple redundant *cis* elements. Furthermore, efficient splicing is responsible for another unique feature of TCR $\beta$  transcripts: their robust downregulation in response to PTCs (Gudikote *et al*, 2005). TCR $\beta$  transcripts normally have efficient splicing signals; mutations that weaken TCR $\beta$  splicing reduce the magnitude of TCR $\beta$  mRNA downregulation in response to PTCs. Conversely, improvement of the splicing signals in a poor NMD substrate such as TPI mRNA greatly stimulates its downregulation in response to PTCs (Gudikote *et al*, 2005). It will be important in the future to determine whether splicing efficiency also dictates whether a transcript is degraded by a UPF3-dependent or UPF3-independent mechanism.

Several issues regarding alternative branches of the NMD pathway remain. Why do these alternative input branches exist? Did they acquire distinct mRNA substrate specificities using different EJC cofactors because of evolutionary selective forces acting on NMD? Alternatively, did selective forces act on other functions of EJC factors, such as mRNA localiza-

tion, translation termination, and translation initiation (Palacios *et al*, 2004; Wilkinson, 2005)? What is the relationship between mammalian NMD pathways and the NMD pathways present in other organisms? One possible answer to the latter question is that lower organisms use an NMD mechanism similar to the UPF3b-independent mammalian pathway described here. Consistent with this notion, both TCR $\beta$  NMD and *S. cerevisiae* NMD are polar, whereas most other mammalian transcripts are not regulated in this manner (see Introduction; Cao and Parker, 2003; Gudikote and Wilkinson, 2006). Clearly, we are only just beginning to understand the evolution and function of NMD RNA surveillance mechanisms and how they discriminate different mRNA targets using different cofactors.

## Materials and methods

### Plasmids

shRNA expression vectors targeting firefly luciferase (G-488), UPF3a (G-490), and UPF3b (G-480) were generated by subcloning PCR products that contained both the target sequence and the mouse U6 promoter into the pcDNA3.1/Zeo or pcDNA5.0/neo vectors (Invitrogen), as described (Sui *et al*, 2002) ([http://mcmanslab.ucsf.edu/protocols/PCR\\_Strategy.pdf](http://mcmanslab.ucsf.edu/protocols/PCR_Strategy.pdf)). The firefly luciferase, UPF3a, and UPF3b target sequences introduced were 5'-GTGCGCTGCTGGTCGCAAC-3' (Miyagishi and Taira, 2002), 5'-GAGCTCATTGCTAGAAGAA-3', 5'-GGTGGTAATGCGAAGATTA-3', respectively. Constructs A- (G-544), A+ (G-545), B- (G-414), B+ (G-415), C- ( $\beta$ -290), CV+ ( $\beta$ -595), C2.1+ ( $\beta$ -1025), C2.3+ ( $\beta$ -696), D- ( $\beta$ -780), D+ ( $\beta$ -1026), E- ( $\beta$ -913), E+ ( $\beta$ -1036), F- ( $\beta$ -974), F+ ( $\beta$ -1038), and J- (G-435) were previously described (Gudikote and Wilkinson, 2002; Wang *et al*, 2002a; Chen and Shyu, 2003; Gudikote *et al*, 2005). Constructs G- ( $\beta$ -1116) and G+ ( $\beta$ -1117) were generated by substituting introns IVS1 and IVS2 in constructs C- and C2.1+ with construct A- introns IVS1 and IVS2, respectively.

### Cell culture, transfection, and RNAi

HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. To generate cell clones stably expressing shRNAs, HeLa cells cultured to ~50% confluency in 60-mm plates were transfected with 1  $\mu$ g of shRNA expression plasmid using Lipofectamine (Invitrogen). Clones resistant to antibiotic were selected using 400  $\mu$ g/ml zeocin for cells transfected with G-480, G-488, and G-480/G-490 and 700  $\mu$ g/ml G418 for cells transfected with G-490.

To further reduce the expression of UPF3a, UPF3b, or both, 1  $\mu$ g of shRNA expression plasmids was transiently cotransfected with NMD reporter plasmids into cell clones stably expressing the corresponding shRNA plasmids. To deplete UPF1, eIF4AIII, or UPF3b, HeLa cells were transiently transfected with a UPF1-, eIF4AIII-, or UPF3b-specific siRNA (Ambion) for 48 h as previously described (Mendell *et al*, 2004; Palacios *et al*, 2004; Kim *et al*, 2005). A firefly luciferase-specific siRNA (same sequence as in the shRNA expression vector; Ambion) was used as a negative control.

To assess NMD, NMD reporter plasmids (700 ng) were transiently transfected into UPF3a- or UPF3b-depleted cells or siRNA-treated cells along with human  $\beta$ -globin (construct J-; 30 ng) (Gudikote *et al*, 2005) or TCR $\beta$  (construct C-; 100 ng) as a transfection efficiency control, using Lipofectamine. Cells were harvested 42–48 h after transient transfection.

### RNA and protein analysis

Total RNA was isolated as described (Li *et al*, 1997; Carter *et al*, 1996) or using the Ultraspec RNA isolation kit (Biotecx). RNase protection analysis was carried out on 10  $\mu$ g of total cellular RNA as described previously (Gudikote *et al*, 2005). Riboprobe a was transcribed from a *Xba*I/*Bam*HI construct A+ fragment. Riboprobes b, c, and d were described previously (Gudikote *et al*, 2005). Riboprobe e was transcribed from an TCR $\beta$  RT-PCR product from HeLa cells transfected with construct C-. Riboprobes f and g were

transcribed from UPF3b and UPF3a cDNA templates, respectively (Kunz *et al*, 2006). Riboprobe h was transcribed from an endogenous eIF4AIII RT-PCR product from HeLa cells.

Real-time PCR analysis was performed as previously described (MacLean *et al*, 2005). Microarray analysis was performed at the University of Iowa DNA Facility on total cellular RNA from the B31 and Luc cell clones transiently transfected with the UPF3b shRNA and Luc shRNA expression vectors, respectively, or on HeLa cells transiently transfected with the luciferase or UPF1 siRNAs (two independent samples each, generated as described in Figures 2B and 3A), using an Affymetrix human genome GeneChip U133 plus array. Transcripts deemed to have altered expression were those whose levels were significantly different in all possible comparisons ( $P < 0.01$ ). For Western blotting analysis, various amounts of total cell lysates were electrophoresed in SDS-polyacrylamide, transferred to Hybond ECL nitrocellulose (Amersham), and probed with

antibodies against UPF3a, UPF3b, and UPF1 (Lykke-Andersen *et al*, 2000) or  $\beta$ -actin (Sigma).

#### Supplementary data

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

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