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Y14 and hUpf3b Form an NMD-Activating Complex

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Summary

Messenger RNAs with premature translation termination codons (PTCs) are degraded by nonsense-mediated mRNA decay (NMD). In mammals, PTCs are discriminated from physiological stop codons by a process thought to involve the splicing-dependent deposition of an exon junction complex (EJC), EJC-mediated recruitment of Upf3, and Upf2 binding to the N terminus of Upf3. Here, we identify a conserved domain of hUpf3b that mediates an interaction with the EJC protein Y14. Tethered function analysis shows that the Y14/hUpf3b interaction is essential for NMD, while surprisingly the interaction between hUpf3b and hUpf2 is not. Nonetheless, hUpf2 is necessary for NMD mediated by tethered Y14. RNAi-induced knockdown and Y14 repletion of siRNA-treated cells implicates Y14 in the degradation of β-globin NS39 mRNA and demonstrates that Y14 is required for NMD induced by tethered hUpf3b. These results uncover a direct role of Y14 in NMD and suggest an unexpected hierarchy in the assembly of NMD complexes.

Introduction

In eukaryotic cells, a combination of surveillance and proofreading mechanisms ensure that transcripts arising from mutated alleles or defective processing events are recognized and rapidly eliminated. One of these surveillance pathways, nonsense-mediated decay (NMD), targets mRNAs with premature translation termination codons (PTCs) for rapid degradation (Li and Wilkinson, 1998; Culbertson, 1999; Frischmeyer and Dietz, 1999; Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Maquat and Carmichael, 2001; Moore, 2002; Schell et al., 2002). In yeast, the three proteins Upf1, Upf2, and Upf3 are essential for NMD (He et al., 1993; Cui et al., 1995; Czaplinski et al., 1999). All Upf proteins are conserved in worms, flies, and mammals (Perlick et al., 1996; Applequist et al., 1997; Page et al., 1999; Lykke-Andersen et al., 2000; Mendell et al., 2000; Serin et al., 2001). The mammalian Upf3 proteins are encoded by two genes, Upf3a and Upf3b (Lykke-Andersen et al., 2000; Serin et al., 2001). In human cells, the degradation of nonsense-containing mRNAs can be inhibited either by the expression of dominant-negative mutants (hUpf1), by RNAi (hUpf1 and hUpf2), or with antisense strategies (hUpf2) (Sun et al., 1998; Mendell et al., 2002; Wang et al., 2002). Furthermore, tethering of hUpf1, hUpf2, and hUpf3a/hUpf3b to the 3′ untranslated region (UTR) of a β-globin mRNA causes degradation of the mRNA (Lykke-Andersen et al., 2000). These data imply important functions of the hUpf proteins in NMD.

In mammals, the discrimination of premature from normal termination codons depends on their position within the transcript. In general, termination codons that are located more than 50–55 nt upstream of the 3′-most exon-exon junction are interpreted as premature by the cellular surveillance machinery, and the mRNAs are degraded (Thermann et al., 1998; Zhang et al., 1998). In contrast, PTC-bearing intronless mRNAs are normally not degraded by NMD (Maquat and Li, 2001; Neu-Yilik et al., 2001; Brocke et al., 2002), reflecting the importance of splicing in mammalian NMD. In addition to splicing, NMD requires translation: interference with translation initiation, elongation, or termination inactivates NMD (Belgrader et al., 1993; Carter et al., 1995; Thermann et al., 1998) and causes stabilization of PTC-containing mRNAs.

The position of introns is thought to be conveyed to the translational apparatus in the cytoplasm via shuttling RNA binding proteins. These are initially deposited as subunits of a large complex ~20–24 nt upstream of exon-exon junctions as a consequence of splicing and serve to provide positional RNA landmarks (Kataoka et al., 2000; Le Hir et al., 2000a, 2000b, 2001b). Subsequent to mRNA binding, EJCs function as binding platforms for mRNA export and NMD factors, respectively (Zhou et al., 2000; Kim et al., 2001a; Le Hir et al., 2001b; Lykke-Andersen et al., 2001). Of the Upf factors, nuclear hUpf3 is thought to be the first protein that interacts with the EJC (Lykke-Andersen et al., 2000, 2001; Kim et al., 2001a; Serin et al., 2001), followed by hUpf2, which is perinuclear and can bind to the N terminus of hUpf3a and hUpf3b (Lykke-Andersen et al., 2000; Mendell et al., 2000; Serin et al., 2001). The EJC protein RNPS1 has been implicated as a potential link between the EJC and the hUpf factors (Lykke-Andersen et al., 2001). RNPS1 can be coimmunoprecipitated with hUpf1, hUpf2, and hUpf3a/3b, and tethering of RNPS1 via the MS2-coat protein to the 3′UTR of a β-globin mRNA induces RNA degradation in a fashion similar to tethered hUpf proteins.

The EJC protein Y14 is stably associated with mRNAs both in the nucleus and in the cytoplasm (Kim et al., 2001b; Le Hir et al., 2001b; Reichert et al., 2002). Y14 is mostly found in close association with Magoh, the human homolog of *Drosophila* mago nashi (Kataoka et al., 2001; Le Hir et al., 2001a). Interestingly, Y14 accompanies mRNAs into polysomes, where it is removed by the translating ribosome (Dostie and Dreyfuss, 2002). This feature of Y14 would be fully consistent with a function as a marker for NMD (Kim et al., 2001a, 2001b). Y14 can also be immunoprecipitated with the hUpf3 proteins

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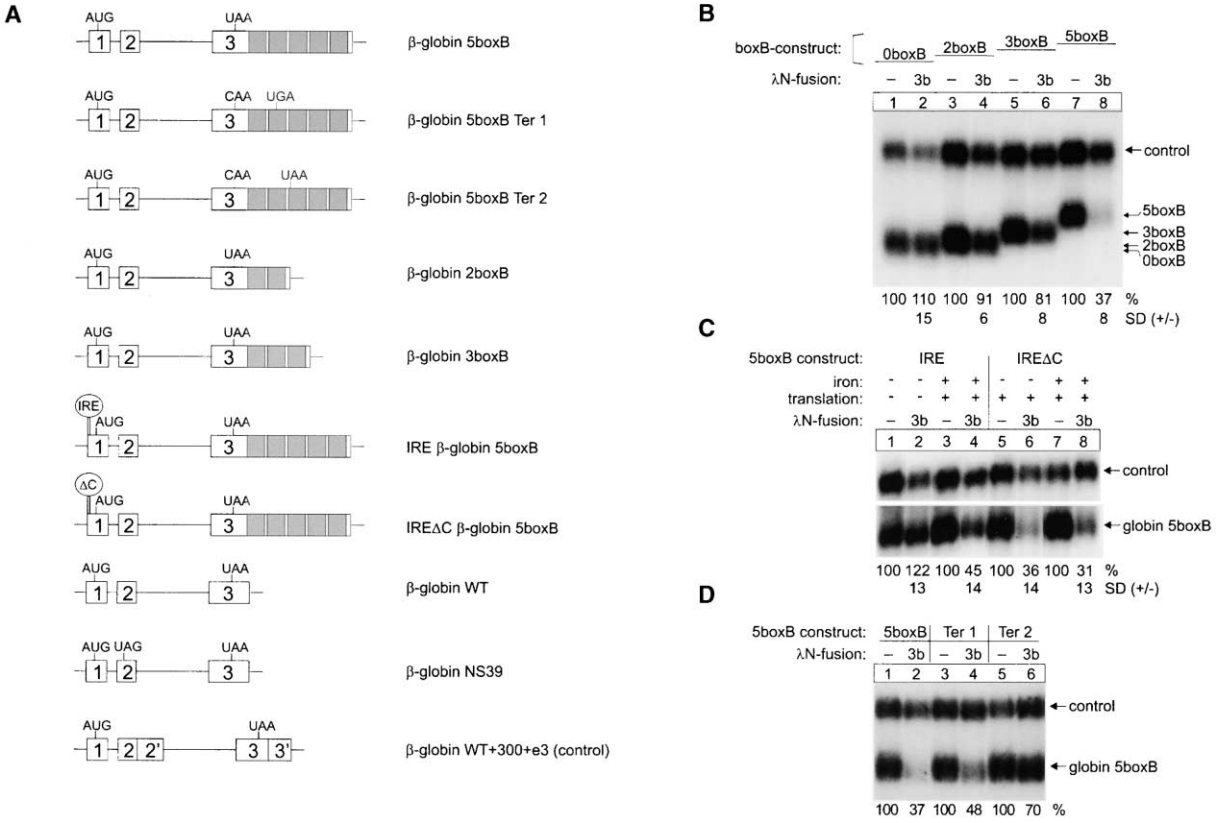

Figure 1. Functional Tethering of hUpf3b

(A) β-globin reporter constructs with 0, 2, 3, or 5 boxB sites (shaded) in the 3′UTR. In β-globin 5boxB Ter 1 and β-globin 5boxB Ter 2, the physiological termination codon (UAA) of the β-globin open reading frame has been mutated (CAA). Consequently, translation terminates at the indicated downstream positions within the five boxB sequences. IRE β-globin 5boxB or IREΔC β-globin 5boxB contain a functional or a nonfunctional IRE in the 5′UTR, respectively. β-globin wt and NS39 contain human β-globin genes with or without the NS39 mutation. β-globin wt+300+e3 expresses β-globin mRNA with extended second and third exons.

(B) Northern blot of functional hUpf3b tethering to the boxB sites in the β-globin 3′UTR. Northern blots of total cytoplasmic RNA of transfected cells were probed with a β-globin-specific radiolabeled antisense cRNA. Constructs with 0, 2, 3, 5 boxB sites in the 3′UTR were coexpressed with the transfection control and λN-hUpf3b (lanes 2, 4, 6, and 8) or λN-peptide (lanes 1, 3, 5, and 7). Mean values with standard deviations (SD) are indicated.

(C) Effect of functional hUpf3b tethering is translation dependent. Cells were cotransfected with 5boxB constructs with a functional IRE (lanes 1–4) or a nonfunctional IRE (IREΔC, lanes 5–8) in the 5′UTR and λN (lanes 1, 3, 5, and 7) or λN-hUpf3b (lanes 2, 4, 6, and 8). Translation of the IRE constructs was specifically regulated by iron depletion or repletion.

(D) Effects of functional hUpf3b tethering are posttranslational. 5boxB constructs with either the physiological termination codon (lanes 1 and 2) or with termination codons at two different downstream positions (lanes 3 and 4 Ter 1, lanes 5 and 6 Ter 2) were coexpressed with the control and λN (lanes 1, 3, and 5) or λN-hUpf3b (lanes 2, 4, and 6). Mean values were calculated from two independent experiments that varied by <3%.

(Kim et al., 2001a), but little is known about the molecular nature of this interaction. Tethering of a Y14-MS2-coat fusion protein to the 3′UTR of a reporter mRNA yielded a modest NMD response compared to tethered RNPS1 (Lykke-Andersen et al., 2001).

Here, we address the role of hUpf3b as a bridging molecule between the EJC and the Upf factors. We provide direct evidence for a role of Y14 in NMD and show that Y14 interacts with hUpf3b through the C terminus of hUpf3b. Surprisingly, this C-terminal interaction domain is critical for hUpf3b to actively promote NMD in tethered function analysis, while the interaction of hUpf2 with the N terminus of hUpf3b is not.

Results

Identification of Amino Acids 421–434 as a Functional NMD-Domain of hUpf3b

Tethering as a fusion polypeptide with the MS2 coat protein to the 3′ UTR of a reporter mRNA has been used to demonstrate the role of hUpf3b in NMD (Lykke-Andersen et al., 2000). To utilize a small RNA binding peptide as an alternative to the larger MS2 coat protein, we chose the λN-peptide (Mogridge et al., 1995). It binds to its cognate RNA binding site, termed boxB (Legault et al., 1998), with high affinity and can be used to efficiently tether λN-fusion proteins to an mRNA that contains

boxB sequences (De Gregorio et al., 1999, 2001). The small size of the λN RNA binding peptide (22 amino acids compared to >100 of MS2 coat) offers a potential advantage, because it may interfere less with the function of its fusion partner.

We generated reporter constructs based on the human β-globin gene which contain 2, 3, or 5 boxB sites in the 3′UTR (Figure 1A) at a position where an intron induces NMD of a transcript with a normal open reading frame (Thermann et al., 1998). Binding of NMD-proteins at this position would therefore be expected to recapitulate 3′splicing-induced NMD. The coexpression of λN-tagged hUpf3b together with a β-globin reporter-mRNA containing five boxB sites in the 3′UTR leads to a marked and specific reduction of the reporter mRNA levels (Figure 1B, lanes 7 and 8). This effect depends on the presence and on the number of boxB sites in the 3′UTR of the reporter mRNA (Figure 1B). Comparable expression levels of the λN-hUpf3b effector protein were controlled by immunoblot analysis of all samples (data not shown).

We next confirmed that NMD by tethered hUpf3b can be downregulated by specifically inhibiting translation via the IRE/IRP system. Expression levels of the boxB containing reporter mRNA with a functional IRE (IRE β-globin 5boxB; Figure 1A) are low under conditions of enabled translation (Figure 1C, lanes 3 and 4), whereas under conditions of disabled translation in iron-depleted cells, the mRNA levels are increased (Figure 1C, lanes 1 and 2). The specificity of these responses was further controlled by using the nonfunctional ΔC variant of the IRE that does not confer iron-dependent regulation of translation (IREΔC β-globin 5boxB, Figure 1A). NMD of the ΔC reporter mRNA variant is constitutively activated (Figure 1C, lanes 5–8). Furthermore, when we shift the termination codon of the reporter construct to more 3′ positions (Figure 1A, constructs β-globin 5boxB Ter 1 and Ter 2) and thus enable translating ribosomes to proceed into the 5boxB sites, tethered hUpf3b no longer activates efficient NMD (Figure 1D). This indicates that translation or a translation-like process precedes the recognition of tethered hUpf3b. Thus, our experimental system recapitulates translation-dependent bona fide NMD. These results also confirm previous reports that established hUpf3b as an important protein in human NMD (Lykke-Andersen et al., 2000, 2001; Kim et al., 2001a).

We next analyzed the functional domain architecture of hUpf3b and generated a set of λN-hUpf3b deletion mutants to identify NMD-relevant regions (Figure 2A). It has previously been shown that the hUpf2 interaction site (amino acids 49–143) resides in the extended conserved region between amino acids 49 and 279 that also contains an RRM-like domain (Serin et al., 2001). The interaction of hUpf3b with hUpf2 has been postulated to convey the discrimination signal from the EJC to hUpf1 (Lykke-Andersen et al., 2000, 2001). Surprisingly, deletions of the hUpf2-interaction site (Δ49–143) and other sequences of the RRM-like domain in the N-terminal part of hUpf3b do not abrogate the NMD function of tethered hUpf3b (Figure 2C, lanes 4–7), although the NMD activity of mutant Δ117–279 is slightly but reproducibly reduced (Figure 2C, lane 5). This result indicates that other regions of hUpf3b are important for NMD. Homology analyses identified a previously unrecognized stretch of 14 amino acids (position 421–434 in hUpf3b) that is highly conserved between hUpf3a and hUpf3b as well as Upf3 homologs of other multicellular eukaryotes, but not yeast (Figure 2B). This region might be relevant for splicing-dependent metazoan NMD but dispensable for the splicing-independent NMD of yeast. Deletion of these conserved amino acids (Δ421–434) results in a dramatic loss-of-function of λN-hUpf3b (Figure 2C, lane 9), although the wild-type (wt) protein and all deletion mutants are comparably expressed (Figure 2D). Only the Δ1–48 protein was expressed at a lower level (Figure 2D, lane 8). Nevertheless, hUpf3b Δ1–48 efficiently activates NMD of the reporter mRNA. We confirmed that the reporter mRNAs were translated with similar efficiencies regardless of the type of hUpf3b mutant tethered to their 3′UTRs. The abundance of the reporter β-globin proteins directly corresponds to the levels of the respective reporter mRNAs (compare Figures 2C and 2E; note that the β-globin band is partially obscured by an unspecific background band with slightly slower migration).

The role of the 421–434 domain was further dissected with point mutations (Figure 3A). Six out of seven of these point mutations (R417A, R419A, K421A, R423A, P424A, and Y429F) significantly reduce NMD (Figure 3B). The R423A mutation is particularly striking in that it causes a quantitatively similar loss-of-function as the deletion mutant Δ421–434 (Figure 3B, compare lane 6 with lane 10). Again, comparable protein expression levels of all point mutants and the deletion mutant Δ421–434 were confirmed by immunoblot analysis (Figure 3C). These results identify amino acids 421–434 as a functionally important domain of hUpf3b that is required to activate NMD. Within this region, the arginine at position 423 is a critical residue for NMD activity of tethered hUpf3b.

The 421–434 Domain of hUpf3b Mediates Interaction with a Y14-Containing Complex

We next aimed to identify potential NMD proteins that interact with the 421–434 domain. Considering previous evidence for an interaction between Y14 and hUpf3b (Kim et al., 2001a), we directly tested the interaction of wt and mutant hUpf3b with hUpf2 and Y14 by coimmunoprecipitation of FLAG-tagged hUpf3b wt, Δ421–434, R423A, and Δ49–143 (deletion of the hUpf2 interaction site) proteins that were expressed in HeLa cells. FLAG-hUpf3b wt interacts with both endogenous hUpf2 and Y14 (Figure 4A, lane 2), consistent with earlier data (Lykke-Andersen et al., 2000; Kim et al., 2001a). The mutant hUpf3b with the deleted hUpf2 interaction domain (Δ49–143) coprecipitates Y14 but not hUpf2 (Figure 4A, lane 5), indicating that Y14 and hUpf2 interact with independent regions of hUpf3b. In contrast, the NMD-inactive deletion (Δ421–434) and the point mutant R423A coprecipitate hUpf2 but not Y14 (Figure 4A, lanes 3 and 4). To exclude that the observed lack of interaction of the Δ421–434 and R423A mutants with Y14 is caused by mislocalization of the mutant proteins, subcellular localization was investigated by N-terminal GFP fusions

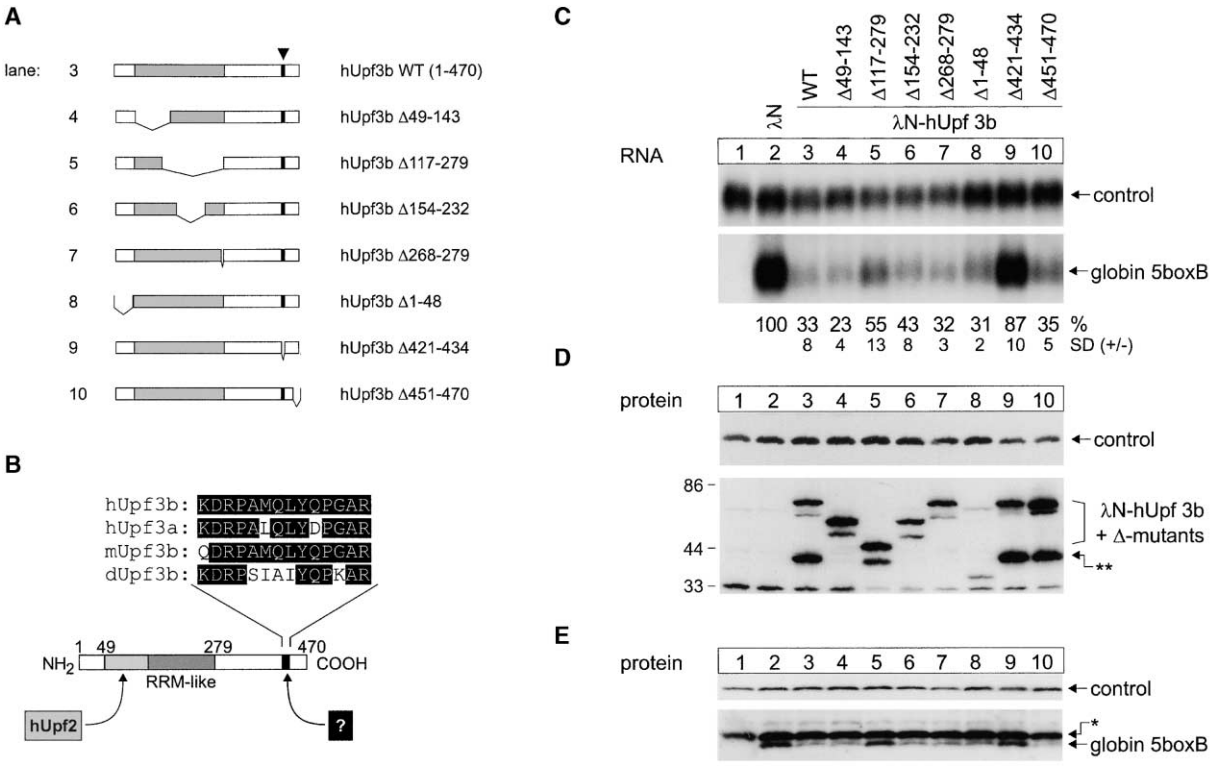

Figure 2. The C-Terminal 421–434 Domain Is Crucial for hUpf3b NMD Activity

(A) hUpf3b deletion mutants. The region that is conserved between Upf3 proteins of different species, including the hUpf2 interaction site and the RRM-like domain, is shaded. An additional sequence in the C-terminal part of hUpf3b that is highly conserved between the two human hUpf3 proteins and murine and *D. melanogaster* Upf3b homologs is displayed by a black box and marked by a black triangle.

(B) Phylogenetic conservation of 14 amino acids of the C-terminal Upf3b region. hUpf3b is shown with the conserved RRM-like domain (dark shade) and the hUpf2 interaction domain (light shade) and the highly homologous 14 amino acids region (black).

(C) Northern blot analysis of hUpf3b deletion mutants. Cells were cotransfected with λN-hUpf3b plasmids with either the wt sequence (lane 2) or with the deletion mutants (lanes 3–10) together with the control and the 5boxB reporter plasmids. Expression of the λN-peptide without hUpf3b served as a negative control (lane 1). Total cytoplasmic RNA was analyzed as described in Figure 1B.

(D) Immunoblot of hUpf3b wt and deletion mutants. The blot was probed with a λN-specific antibody (bottom panel), the transfection efficiency and loading were controlled by stripping and reprobing the membrane with a β-globin (control) specific antibody (upper panel). A prominent degradation product of hUpf3b that likely arises from proteolytic cleavage in the C-terminal region is marked (**). The diminished cytoplasmic expression of hUpf3b Δ1–48 (lane 7) probably results from an interference of the deletion with a predicted nuclear export sequence located directly downstream of the deleted sequence.

(E) Immunoblot to control for translation efficiency of the 5boxB reporter mRNA. Expression levels of the translation products of the control and the 5boxB reporter mRNA were determined with a β-globin-specific antibody. An unspecific background band with significant crossreactivity in this experiment is marked (*). The RNA and proteins analyzed in (C) through (E) were extracted from the same batch of transfected cells.

of hUpf3b wt, Δ49–143, Δ421–434, and R423A. This analysis revealed a comparable cellular distribution (nuclear accumulation) of all four proteins (see Supplemental Figure S1 at http://www.molecule.org/cgi/content/full/11/4/939/DC1). These data strongly suggest that tethered hUpf3b requires the interaction with Y14 or a Y14-containing complex, but not with hUpf2.

Tethered Y14 Activates NMD

If the interaction of Y14 with hUpf3b is important for NMD, Y14 tethering to an appropriate position should activate NMD. Nevertheless, the effect of Y14 was only marginal in an experimental system employing MS2 coat fusion proteins (Lykke-Andersen et al., 2001). In contrast, tethering of a λN-Y14 fusion protein to the β-globin 5boxB reporter causes a dramatic reduction of reporter mRNA abundance (Figure 4B, lanes 7 and 8). Remarkably, tethered Y14 exerts an even stronger effect than tethered hUpf3b. Analogous to the hUpf3b data, the λN-Y14 dependent reduction of reporter mRNA expression levels is amplified by increasing the number of boxB sites in the 3′UTR (Figure 4B, lanes 3–8), whereas an mRNA without boxB sequences in the 3′UTR is not affected by coexpressed λN-Y14 (Figure 4B, lanes 1 and 2). 3′ shifting of the termination codon shows that the 5boxB mRNA is stabilized when translation termination is repositioned downstream (Figure 4C), which demonstrates that efficient NMD requires translation termination to occur upstream of all λN-Y14 binding sites. Using the IRE/IRP-system (see above), we confirmed the dependence of reporter mRNA downregulation on its cytoplasmic translation (data not shown). Together, these

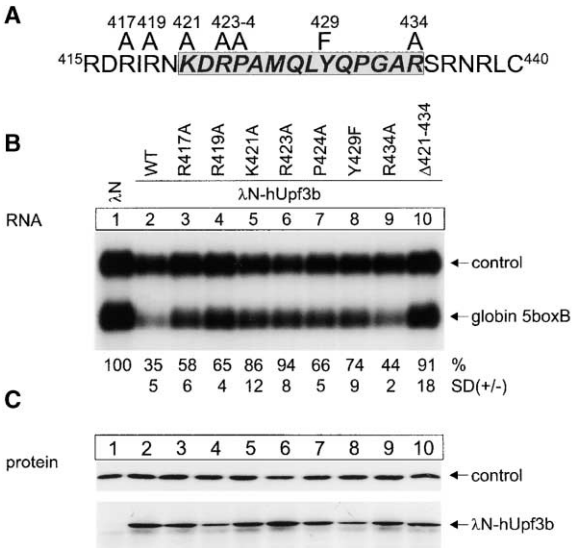

Figure 3. Point Mutations of the 421–434 Domain Interfere with hUpf3b NMD Activity

(A) Amino acid sequence and point mutants of λN-hUpf3b. (B) Northern blot of hUpf3b point mutants in a functional tethering assay (see Figure 2C). (C) Immunoblot of the λN-hUpf3b point mutants (see Figure 2D).

data show that the reduced reporter mRNA expression mediated by tethered Y14 fulfils the criteria of NMD, implicating Y14 as a bona fide NMD protein.

The C-Terminal 150 Amino Acid Fragment of hUpf3b Acts as a Dominant-Negative Inhibitor of Tethered hUpf3b but Is Not Sufficient to Autonomously Induce NMD

We next analyzed whether the C-terminal domain of hUpf3b is also sufficient to cause NMD when tethered to the 3′UTR of the reporter mRNA. The expression of λN-tagged C-terminal hUpf3b fragments with a length of 70, 100, 150, and 200 amino acids (including amino acids 421–434) did not significantly reduce the abundance of the 5boxB reporter mRNA (data not shown). Thus, the C terminus of hUpf3b is not sufficient to induce NMD in the λN-boxB tethering system. As Y14 binds to the C terminus of hUpf3b, we analyzed whether coexpressed untagged C-terminal hUpf3b fragments interfere with the function of tethered hUpf3b. A fragment containing the C-terminal 150 amino acids including the 421–434 region of hUpf3b reduces the efficiency of hUpf3b-induced NMD (Figure 5A, lanes 2–4), suggesting a dominant-negative effect as it might be expected when competition occurs for the binding of a critical protein that interacts with the C terminus of hUpf3b. Similar results were obtained with the C-terminal 200 amino

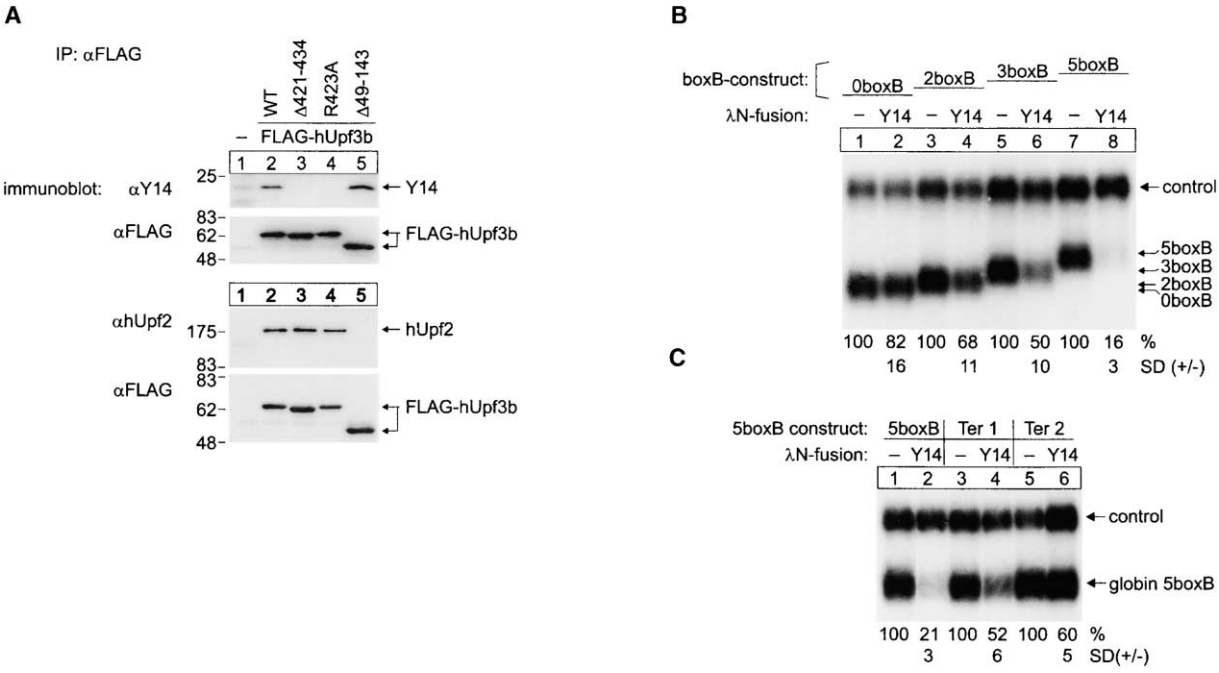

Figure 4. Y14 Acts as an NMD Protein and Binds to the hUpf3b 421–434 Domain

(A) Coimmunoprecipitation analysis of FLAG-hUpf3b with Y14 and hUpf2. Cells were either untransfected (lane 1) or transfected with FLAG-tagged hUpf3b wt (lane 2), Δ421–434 (lane 3), R423A (lane 4), and Δ49–143 (lane 5). Membranes were probed with either Y14 (upper panel) or hUpf2 (bottom panel) specific antibodies. The blots were reprobed with anti-FLAG antibody to control for immunoprecipitation efficiency and equal loading. The blots shown are representative of three independent experiments.

(B) Northern blot of functional Y14 tethering to the 5boxB sites in the β-globin 3′UTR. Cotransfection experiments with λN-Y14 and reporter plasmids (see Figure 1B).

(C) Effects of Y14 are posttranslational. Cells were cotransfected with λN-Y14 and reporter plasmids (see Figures 1A and 1D).

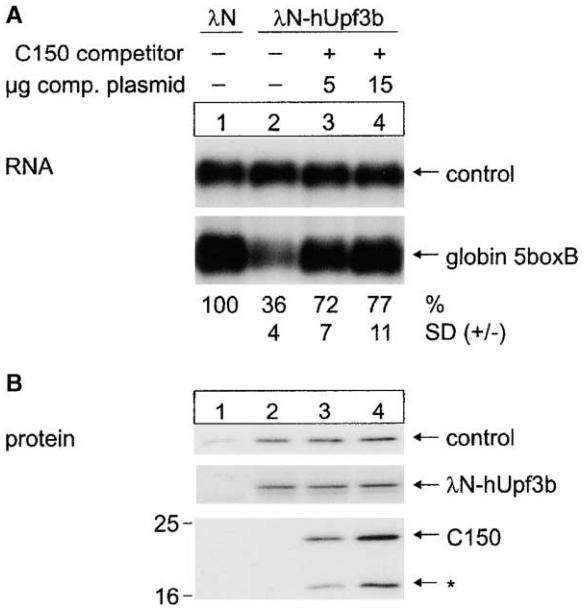

Figure 5. The C-Terminal Fragment of hUpf3b Inhibits hUpf3b-Induced NMD

(A) Northern blot of cells that were cotransfected with λN-hUpf3b, the control, and the 5boxB reporter constructs. λN served as a negative control (lane 1). Increasing amounts (5 or 15 μg) of a 150 amino acid C-terminal competitor fragment (C150) were added to the λN-hUpf3b transfections. The parental vector was included in transfections to adjust the total amount of plasmid DNA.

(B) Western blot analysis of effector proteins. Cytoplasmic extracts from cells used in (A) were analyzed with a λN-specific antibody to visualize λN-hUpf3b (middle panel), or with a hUpf3b-specific antibody to visualize the C150 competitor polypeptide (bottom panel). The additional anti-hUpf3b-reacting band (*) is likely to arise from proteolytic cleavage (see Figure 2D). Transfection efficiency and equal loading were controlled by probing with a β-globin (control)-specific antibody (top panel).

acids of hUpf3b (data not shown). The expression of both the λN-tagged hUpf3b and the untagged C150 fragment were controlled by immunoblot analyses (Figure 5B). Together, the data show that the C-terminal domain of hUpf3b acts as a dominant-negative inhibitor of λN-hUpf3b-induced NMD, underscoring that a critical NMD-function of hUpf3b resides in its C-terminal 150 amino acids.

Knockdown of Y14 Interferes with Nonsense- and hUpf3b-Activated NMD

The specific role of Y14 in NMD was directly analyzed by depletion of the endogenous Y14 protein by RNAi. Immunoblot analyses show the specific reduction of Y14 in the cells transfected with two different, nonoverlapping Y14-specific siRNAs (Figure 6B, middle panel, lanes 5–8), whereas the expression of the λN-hUpf3b fusion protein is not affected by this treatment (Figure 6B, bottom panel). This specific knockdown of Y14 in cells cotransfected with λN-hUpf3b and the reporter mRNA impairs the activity of the NMD pathway (Figure 6A, lanes 5–8), whereas control cells treated with luciferase (Luc) siRNA (Figure 6A, lanes 3 and 4) display comparable NMD efficiencies to untreated cells (Figure 6A, lanes 1 and 2). Thus, hUpf3b-activated NMD depends on Y14. We next tested whether Y14-activated NMD requires the "downstream" NMD factors hUpf1 and hUpf2. Indeed, RNAi against hUpf1 and hUpf2 inhibits λN-Y14 NMD by approximately 3-fold (reporter mRNA abundance of 41% versus 14%, Figure 6C, lanes 3 and 4) and 2.4-fold (34% versus 14%, Figure 6C, lanes 5 and 6), respectively. The control Luc siRNA does not affect λN-Y14 NMD (Figure 6C, lanes 1 and 2). The expression levels of hUpf1 and hUpf2 proteins were analyzed by immunoblot (Figure 6D, middle and bottom panels). RNAi against hUpf1 results in an efficient depletion of the protein (Figure 6D, lanes 3 and 4). Human Upf2 protein levels are also consistently reduced, albeit less efficiently (Figure 6D, lanes 5 and 6). We conclude from these analyses that λN-Y14 NMD depends on hUpf1 and hUpf2, suggesting that these two proteins are required for later steps of NMD.

To test whether the reduction of endogenous Y14 affects a natural NMD substrate, a nonsense (NS) mutated β-globin mRNA was used. β-globin wt or NS39 mRNAs were transiently expressed in HeLa cells that were either left untreated or treated with Luc siRNA (negative control), a hUpf1-specific siRNA (positive control), or two different Y14-specific siRNAs. In untreated (Figure 7A, lanes 1 and 2) or Luc siRNA-treated (Figure 7A, lanes 3 and 4) cells, the NS39 β-globin mRNA is reduced to 12% of wt mRNA levels, which is comparable to previous results (Thermann et al., 1998). Transfection of the hUpf1 siRNA stabilizes β-globin NS39 mRNA compared to Luc siRNA-treated cells 3.8-fold (Figure 7A, lanes 9 and 10). The analysis of Y14 siRNA-treated cells is confounded by a growth arrest and reduced cell viability that was more pronounced than the growth defect observed for hUpf1-depleted cells. This effect of Y14 RNAi is likely to reflect the reported requirement for Y14 in normal cell growth and survival (Le Hir et al., 2001a). However, in cells that display a partial depletion of Y14 by RNAi, the NS39 β-globin mRNA is consistently and reproducibly stabilized, albeit to a lesser extent than in hUpf1 siRNA-treated cells (2.0- and 1.8-fold increase; Figure 7A, lanes 5–8). The specific knockdown of the respective proteins was confirmed by immunoblot analysis of cell extracts (Figure 7B).

It has been previously shown that allele-specific sequence differences can be used to restitute the expression of genes that have been knocked down by RNAi with an exogenous transfected gene (Mendell et al., 2002). As a further step to establish the role of Y14 in NMD, we employed this strategy by using a Y14 expression vector carrying silent mutations at the siRNA target sequences which allows the replacement of endogenous Y14 with FLAG-tagged functional Y14. Immunoblot analysis of cells treated with Y14 siRNA shows that the RNAi-insensitive FLAG-Y14 (referred to as $Y14^{(ins)}$) is not affected by the siRNA transfection and is expressed at levels comparable to endogenous Y14 (Figure 7C, compare lanes 1 and 3). The FLAG-$Y14^{(ins)}$ fully restores the degradation of the accumulated NS39 mRNA in cells transfected with Y14 siRNA (Figure 7C, lanes 11 and 12), although a comparable cellular growth impairment was observed in all samples that were pretransfected with the Y14 targeting siRNA. Interestingly, the expression of the NS39 mRNA in cells expressing the FLAG-$Y14^{(ins)}$ (Figure 7C, lanes 3, 4, 7, 8, 11, 12) is lower than in

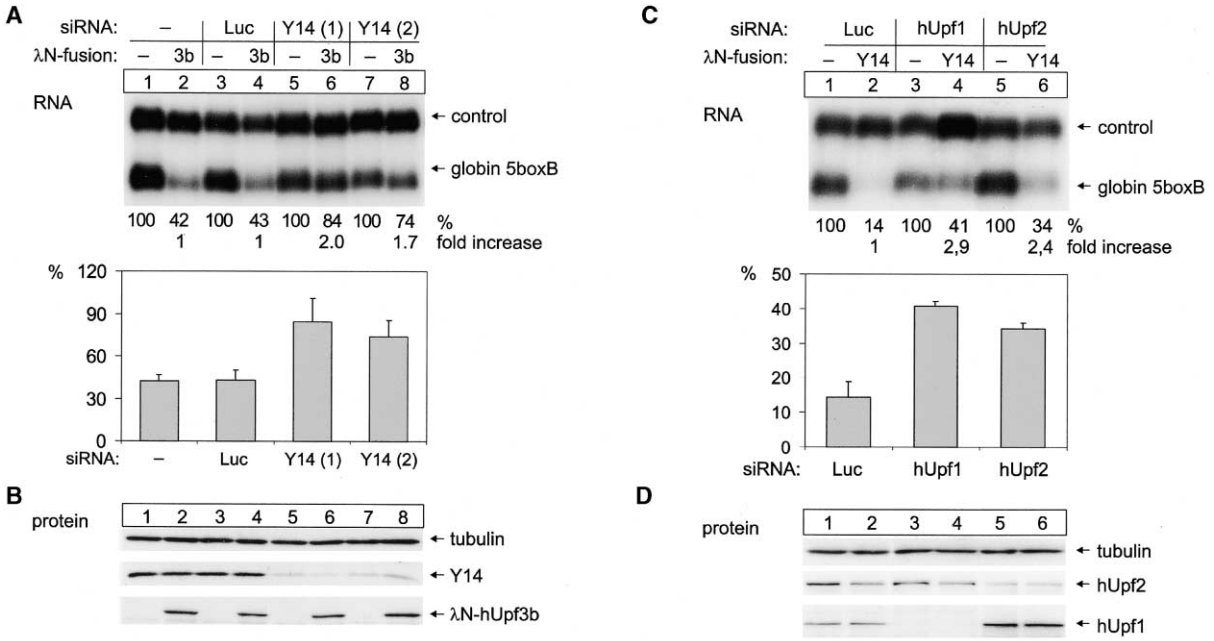

Figure 6. RNAi Inhibits NMD of hUpf3b and Y14 Tethered mRNA

(A) Y14 RNAi upregulates NMD substrate expression in a hUpf3b tethering assay. Cells were treated with two different siRNAs directed against Y14 to specifically reduce the expression of endogenous Y14 (Y14 [1], lanes 5 and 6; Y14 [2], lanes 7 and 8). Untreated cells (lanes 1 and 2) and cells treated with Luc siRNAs (lanes 3 and 4) served as controls. After siRNA treatment, cells were cotransfected with λN (lanes 1, 3, 5, and 7) or λN-hUpf3b (lanes 2, 4, 6, and 8) together with the control and the 5boxB reporter constructs. Reporter mRNA expression was determined on Northern blots of total cytoplasmic RNA.

(B) Y14 siRNAs specifically reduce Y14 protein expression. Extracts from the cells analyzed in (A) were used for immunoblot analyses. λN-hUpf3b fusion protein and endogenous Y14 expression were determined with λN- and Y14-specific antibodies. The membrane was reprobed with a tubulin-specific antibody to control for equal loading.

(C) NMD activity of tethered Y14 depends on hUpf1 and hUpf2. Cells were treated with either Luc (lanes 1 and 2), hUpf1 (lanes 3 and 4), or hUpf2 (lanes 5 and 6) siRNAs. Cells were then transfected with λN (lanes 1, 3, and 5) or λN-Y14 (lanes 2, 4, and 6) together with the control and the 5boxB reporter constructs.

(D) hUpf1 and hUpf2 abundance in transfected cell extracts. Immunoblotting with either hUpf1 (bottom panel)- or hUpf2-specific antibodies (middle panel). The membrane was reprobed with a tubulin-specific antibody (top panel).

cells that were mock transfected with the empty vector (Figure 7C, lanes 1, 2, 5, 6, 9, 10). This result indicates that the transgenic Y14 also stimulates NMD in cells not depleted of their endogenous Y14.

Thus, Y14 is an important component of the NMD pathway both of a functionally tethered mRNA and of a natural NMD substrate.

Discussion

The human NMD machinery is able to distinguish proper and improper translation termination codons within an mRNA. To achieve this discrimination, the spatial relationship between exon-exon junctions and the translation termination codon is interpreted by NMD factors. Consequently, a termination codon that is located more than ~50 nt upstream of the 3′-most exon-exon junction will cause the degradation of the respective mRNA (Thermann et al., 1998; Zhang et al., 1998). Components of the splicing-dependent EJC are thought to mark the position of the spliced introns for cytoplasmic NMD factors and be recognized by a posttermination surveillance complex (Le Hir et al., 2000a, 2000b; Lykke-Andersen et al., 2001; Kim et al., 2001a).

We analyzed the NMD function of several deletion mutants of hUpf3b, because hUpf3b has been postulated to represent the first NMD-specific factor that is recruited by the EJC after splicing in the nucleus (Lykke-Andersen et al., 2000, 2001; Kim et al., 2001a). According to the current model of NMD, the interaction between hUpf3b and hUpf2 mediates the recognition of the EJC by the cytoplasmic NMD proteins hUpf1 and hUpf2. Thus, deletion of the hUpf2 binding site within the N terminus of hUpf3b was expected to render this mutant hUpf3b protein NMD-inactive in a tethering system. Surprisingly, the deletion of the hUpf2 binding domain did not inactivate the hUpf3b NMD function. This unexpected result established that the hUpf2 binding domain of tethered hUpf3b (consisting of 95 amino acids from position 49–143) is dispensable for the activation of NMD and suggested that another region of hUpf3b confers its NMD activity. Why the direct interaction of hUpf3b with hUpf2 is not required for its NMD activity remains to be elucidated. While multiple scenarios can be envisaged, it is possible that the hUpf2 interacting region of hUpf3b is only dispensable when hUpf3b is directly tethered to the mRNA. This can be tested by replacing endogenous hUpf3b by the deletion mutant via RNAi. If

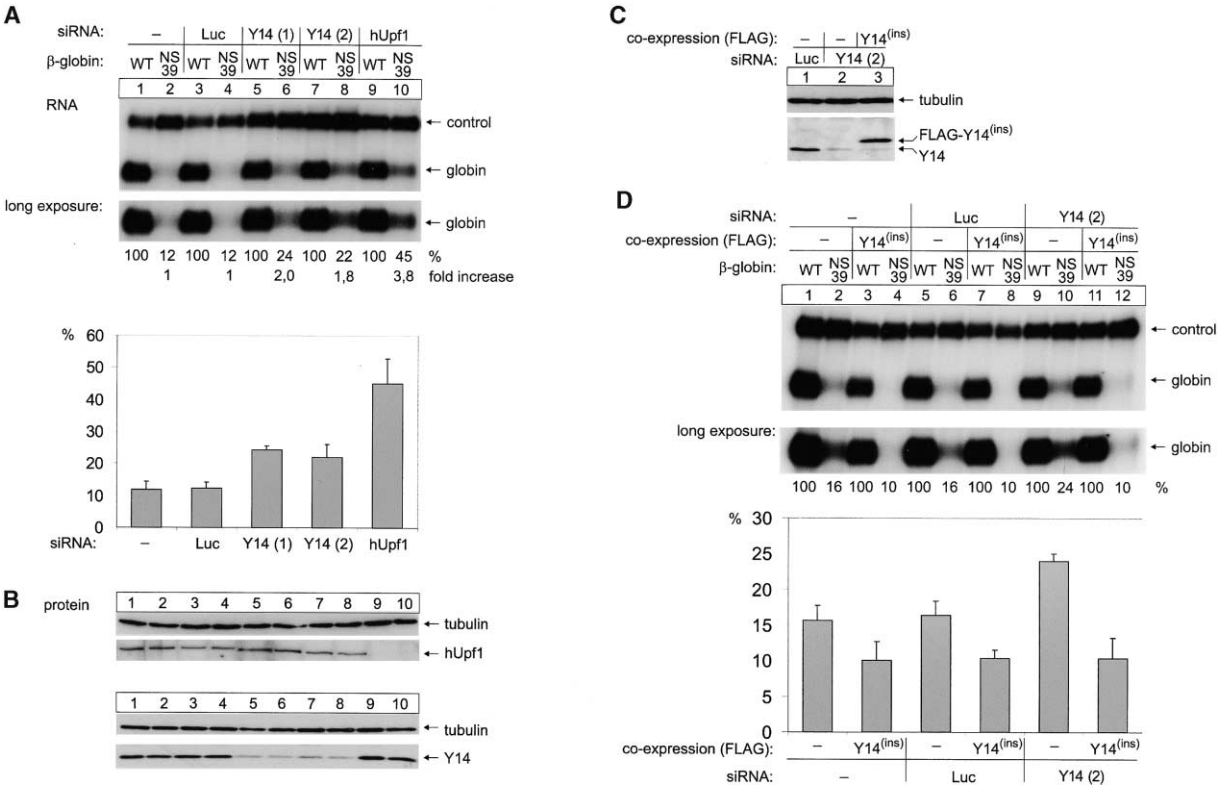

Figure 7. Y14 RNAi Inhibits NMD of a Natural Substrate

(A) Y14 and hUpf1 RNAi upregulate the expression of an NS-mutated β-globin mRNA. Cells were treated with two different Y14 siRNAs (Y14 [1], lanes 5 and 6; Y14 [2], lanes 7 and 8) and a hUpf1 siRNA (lanes 9 and 10). Untreated cells (lanes 1 and 2) and cells treated with Luc siRNA (lanes 3 and 4) served as controls. Cells were then transfected with plasmids expressing either the β-globin wt mRNA (wt, lanes 1, 3, 5, 7, and 9) or a β-globin NS39 mRNA (lanes 2, 4, 6, 8, and 10) together with the control. Bottom panel: longer exposure of the bands corresponding to β-globin mRNA to facilitate the assessment of NS39 expression levels.

(B) RNAi specifically downregulates Y14 and hUpf1. Extracts from the cells described in (A) were used for immunoblot analysis with hUpf1- and Y14-specific antibodies. Equal loading of all lanes was controlled by reprobing the membrane with a tubulin-specific antibody.

(C) Expression of FLAG-Y14$^{(ins)}$ in Y14 siRNA pre-treated cells. Cells were transfected with Y14 (2) (lanes 2 and 3) or the Luc siRNAs (lane 1). 28 hr later, cells were transfected with either the empty FLAG (lanes 1 and 2) or the FLAG-Y14$^{(ins)}$ vector (lane 3). 44 hr afterwards, extracts were prepared and immunoblotted using a Y14-specific antibody (bottom panel). The membrane was reprobed with a tubulin antibody (top panel).

(D) Restitution of endogenous Y14 with transfected FLAG-Y14$^{(ins)}$. Northern blot of total cytoplasmic RNA of cells transfected with either Luc (lanes 5–8) or Y14 (2) siRNA (lanes 9–12). Untreated cells (lanes 1–4) served as negative controls. 28 hr later, the cells were transfected with wt (lanes 1, 3, 5, 7, 9, and 11) or NS39 containing (lanes 2, 4, 6, 8, 10, and 12) β-globin expression plasmids. Empty FLAG (lanes 1, 2, 5, 6, 9, and 10) or FLAG-Y14$^{(ins)}$ vectors (lanes 3, 4, 7, 8, 11, and 12) were cotransfected. A longer exposure of the band corresponding to β-globin mRNA is shown (bottom panel).

true, the hUpf2/hUpf3b interaction may exert a stabilizing effect during the remodeling of NMD complexes which is dispensable when hUpf3b is tethered.

A previously uncharacterized region of 14 amino acids near the C terminus of hUpf3b was found to be functionally important and potentially forms a bridge between the EJC and the NMD machinery. This region is highly conserved between the two human Upf3 proteins (hUpf3a and 3b) and hUpf3b homologs of other higher eukaryotes, but not present in yeast Upf3. Deletion and single point mutations of this small region cause an impressive loss-of-function of tethered hUpf3b. It directly or indirectly binds the EJC protein Y14 independently of hUpf2, implicating Y14 as an NMD protein. The specific role of Y14 as an NMD factor was confirmed in a tethering assay, which contrasts quantitatively but not qualitatively with previous tethering studies that employed the MS2 coat system (Lykke-Andersen et al., 2001).

The function of Y14 in the human NMD pathway, that may possibly also involve its heterodimerization partner Magoh (Kataoka et al., 2001; Le Hir et al., 2001a), was further analyzed using a combination of RNAi and the λN-tethering system. Depletion of Y14 abolishes NMD activated by tethered hUpf3b and also affects the downregulation of nonsense-mutated β-globin mRNA as a natural NMD substrate. It is intriguing that other components of the EJC such as RNPS1 can also activate NMD in a functional tethering assay (Lykke-Andersen et al., 2001), which suggests that more than one protein of the EJC may cooperate in the triggering of the NMD-pathway. This may afford different levels of regulation of NMD or reflect the assembly of multisubunit NMD-complexes that can be recruited by more than one protein.

It is now well established that Y14 represents one of the core components of the EJC and binds to the mRNA at an early step of spliceosome assembly (Reichert et

al., 2002). With a linear model of NMD-activating steps in mind, it was therefore surprising to find that the function of hUpf3b depends on Y14. This observation may possibly be explained by hUpf3b recruiting Y14 to the EJC as a very early step of NMD. However, we do not think this is likely, because Y14 but not hUpf3b has been demonstrated to be recruited to the EJC early in the splicing process (Reichert et al., 2002). Furthermore, the C-terminal hUpf3b fragments including the Y14 binding site were not sufficient to activate NMD. Therefore, we propose that hUpf3b requires a second, Y14-dependent signal to become active in NMD. According to this model, Y14 binds to the mRNA at an early step of EJC recruitment. The hUpf3b protein enters the complex at a later step and is likely to activate the NMD pathway. It remains to be elucidated if hUpf3b requires further EJC components in addition to Y14 to become active. Alternatively, NMD could also be triggered by the interaction of Y14 with other redundant or cooperating proteins such as hUpf3a and RNPS1, which may lead to the activation of distinct pathways of degradation.

In conclusion, our data reveal a surprising complexity and hierarchy of interactions of NMD- and EJC proteins that enable mRNA surveillance. The dynamics and composition of the EJC may influence the NMD efficiency and the localization of degradation of distinct NMD substrates (nucleus-associated versus cytoplasmic NMD; Belgrader et al., 1994; Kugler et al., 1995; Moriarty et al., 1998; Stephenson and Maquat, 1996; Thermann et al., 1998; Sun et al., 2000). Furthermore, it may also contribute to cell type-specific NMD differences (Aoufouchi et al., 1996; Carter et al., 1996; Li and Wilkinson, 1998). Finally, the complex organization of RNA-protein interactions may enable regulation of NMD, a currently unexplored field.

Experimental Procedures

Plasmids

The λN-peptide was PCR amplified from pλ-4G (De Gregorio et al., 1999) and inserted between NheI and XhoI sites of pCI-neo (Promega) to give pCI-λN. pCI-λNhUpf3b and pCI-λNY14 were created by inserting the cDNAs of hUpf3b and Y14 into the XhoI/XbaI or XhoI/NotI sites of pCI-λN. hUpf3b and Y14 cDNAs were amplified from HeLa cell cDNA. The sequences of all oligonucleotides that were used for cloning and mutagenesis are available upon request. All deletions and point mutants were introduced into pCI-λNhUpf3b by site-directed mutagenesis.

β-globin wt and NS39 were described previously (Thermann et al., 1998). An elongated human β-globin gene (wt+300+e3) served as a control for transfection efficiency in all experiments. This was created by the insertion of β-globin exon 3 sequences into the SalI restriction site of β-globin wt+300 (Neu-Yilik et al., 2001).

β-globin 2boxB, β-globin 3boxB, and β-globin 5boxB were created by successive insertions of PCR-amplified boxB sequences into the ApaI and XhoI sites of wt-SP-Ter (Thermann et al., 1998). The ApaI site was subsequently mutated to avoid interference by base-pairing with the boxB stem. β-globin 5boxB vectors with an IRE or IREΔC in the 5'UTR were constructed by substituting the EcoRI/XbaI fragment of wt-IRE or wt-IREΔC (Thermann et al., 1998) by an EcoRI/XbaI fragment of β-globin 5boxB. In β-globin 5boxB Ter 1 and Ter 2, the downstream termination codons were generated by point mutating the physiological termination codon and by frameshift mutagenesis of downstream sequences.

The plasmid pCI-neo-FLAG was created by inserting the FLAG-sequence into the NheI/XhoI sites of pCI-neo. Wild-type and mutants of hUpf3b were inserted between XhoI and NotI sites of pCI-neo-FLAG from the corresponding λN-hUpf3b expression vectors. The RNAi-insensitive FLAG-Y14$^{(ins)}$ was constructed by site-directed mutagenesis of a FLAG-Y14 plasmid.

Cell Culture and Transfections

HeLa cells were grown in DMEM and transfected by calciumphosphate precipitation with standard methods. For tethering experiments with hUpf3b and Y14, cells were cotransfected in 10 cm dishes with 10 μg of β-globin 5boxB, 4 μg of the control (wt+300+e3), 2 μg of a GFP-expression vector, and 25 μg of a plasmid expressing the λN-fusion protein. The competition experiments (Figure 5) were carried out by transfecting 8 μg of the 5boxB construct, 3 μg of the control construct, 2 μg of a GFP-expression vector, 16 μg of the λN-expression plasmid (λN, λN-hUpf3b or λN-Y14), and 5 or 15 μg of the C150 expression plasmids. For immunoprecipitation studies, HeLa cells were transfected with 15 μg of FLAG-expression vectors in 10 cm dishes. Translation of IRE-containing mRNAs was specifically regulated by supplementing the culture medium with either 100 μM deferoxamine mesilate 24 hr posttransfection to block translation, or 100 μM heme arginate 36 hr posttransfection to enable translation.

Transient transfections of siRNA were done with Oligofectamine (Invitrogen) according to the manufacturer's recommendations. A detailed protocol for siRNA transfections is included in the supplemental data (http://www/.molecule.org/cgi/content/full/11/4/939/DC1).

RNA Analysis

Total cytoplasmic RNA was isolated from the supernatant of homogenized cells as previously described (Gehring et al., 2001). For the siRNA experiments, a slightly modified lysis buffer (10 mM Tris-Cl [pH 7.5], 8 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% sodium-deoxycholate, and 5 mM vanadyl-ribosyl-complex) was used. When subsequent protein analysis was required, the protease-inhibitors *complete* (Roche Applied Science) and PMSF were included in the lysis buffer. Northern blot analysis was performed with 1.5–5 μg of total cytoplasmic RNA according to standard protocols.

Signal Quantification

Radioactive signals were quantified by phosphoimaging in a FLA-3000 fluorescent image analyzer (Raytest, Fujifilm). The indicated expression levels were calculated after correction for transfection efficiency. Mean values and standard deviations (SD) of all experiments shown were calculated from at least three independent experiments.

Immunoprecipitation and Protein Analysis

Immunoblot analysis was performed using 10–30 μg of cytoplasmic extracts for SDS-polyacrylamide gel electrophoresis. Subsequently, proteins were transferred to PVDF-membranes using a semi-dry electroblotting system. Membranes were blocked either with 5% non-fat skimmed milk in TBS-Tween (0.1%) for hUpf1-, hUpf2-, hUpf3b-, β-globin-, FLAG-, and tubulin-antibodies, or 10% FCS in TBS-Tween for λN- and Y14 antibodies.

FLAG-hUpf3b complexes were immunoprecipitated from RNaseA-treated HeLa cell lysates (20 μg/ml, 15 min on ice) with M2 anti-FLAG agarose (Sigma) at 4°C for 1 hr in lysis buffer (50 mM Tris [pH 7.2], 150 mM NaCl, 1 mM EDTA, 0.5 mM PMSF, 0.5% Triton X-100, + *complete* protease inhibitor). Beads were washed four times in lysis buffer without protease inhibitors. Precipitated complexes were eluted with SDS-sample buffer and analyzed by Western blotting.

λN-fusion proteins were detected using an anti-λN antibody (De Gregorio et al., 1999).

HRP-conjugated anti-mouse or anti-rabbit antibodies (Sigma) were used as secondary antibodies at 1:5,000 or 1:10,000 dilutions. Blots were developed with ECL or ECLplus reagent (Amersham).

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