Human Upf Proteins in NMD

Guramrit Singh and Jens Lykke-Andersen*

Abstract

The human Upf (hUpf) proteins work at the core of the nonsense-mediated mRNA decay (NMD) pathway. The three hUpf proteins, hUpf1, hUpf2 and hUpf3, form the hUpf complex, which is critical for the recognition and degradation of mRNAs containing premature termination codons (PTCs). The recognition of PTC-containing mRNAs by the hUpf complex in mammalian cells is promoted by the splicing dependent exon-junction complex (EJC), with which hUpf3 interacts. Following the recognition of PTCs, the hUpf complex is believed to disrupt mRNP structure to prevent further translation and trigger mRNA decay. Emerging evidence suggests that hSmg proteins involved in phosphorylation and dephosphorylation of hUpf1 may play a key role in delivering PTC-containing mRNAs to the mRNA decay machinery.

Introduction

In recent years the cellular machinery that identifies mRNAs with premature termination codons (PTCs) and subjects them to NMD has been characterized in several eukaryotes. The NMD machinery has the capability to discriminate PTC-containing mRNAs from normal mRNAs, and to inhibit translation and activate decay of the NMD target mRNAs. The three Upf (*Up-f*rameshift) proteins, Upf1, Upf2 and Upf3, work at the heart of this pathway in all organisms studied. The Upf proteins were first discovered in yeast,[1-3] and orthologs have subsequently been identified in other eukaryotes.[4] The conservation across species is highest for Upf1 and lower for Upf2 and Upf3.[4] However, despite this conservation, mechanistic differences may exist between various organisms in how PTCs are recognized by the NMD machinery.[5] In this chapter, we discuss the current understanding of the role of Upf proteins in the recognition of mRNAs that are targeted for NMD in humans, and how these proteins may shunt the mRNA from the translational pool to the mRNA decay machinery.

Evidence That Upf Proteins Are Involved in the Human NMD Pathway

It is now well established that human Upf (referred to as hUpf hereafter) proteins play an essential role in NMD. Initial evidence came from identification of the human ortholog to yeast Upf1p based on sequence similarity.[6,7] Several residues of yeast Upf1p that are essential for NMD were found to be conserved in hUpf1. An arginine-to-cysteine mutation at residue 844 was shown to create a dominant-negative form of hUpf1 that impairs NMD in human cells.[8] A similar mutation was earlier shown to inhibit NMD in yeast.[9] Subsequently, hUpf2 and hUpf3, the human orthologs to the other two essential yeast NMD proteins, were identified. The demonstration that they interact with hUpf1 implicated these two proteins in the human NMD pathway as well.[10-12]

*Corresponding Author: Jens Lykke-Andersen—Molecular, Cellular and Developmental Biology, 347 UCB, University of Colorado at Boulder, Boulder, Colorado 80309-0347, U.S.A. Email: jens.lykke-andersen@colorado.edu

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Further evidence that hUpf proteins function in NMD was obtained by showing that each hUpf protein induces the NMD-like decay of mRNAs to which they are artificially tethered.[12] This gain-of-function manipulation of hUpf proteins triggers mRNA decay only when the proteins are tethered downstream of a translation termination codon.[12] More recently, RNA interference-mediated depletion of hUpf proteins from human cells was shown to inhibit NMD, demonstrating that these proteins are essential for NMD.[13,14]

hUpf1 Is an RNA Helicase and a Phosphoprotein

Among the three core NMD proteins, Upf1 shows the highest sequence conservation.[4] The central region of hUpf1 is 58% identical to yeast Upf1p[6] and, like yeast Upf1p, it is an ATP-dependent RNA helicase.[15] The conserved central region consists of two putative cysteine-rich zinc-finger motifs and seven group I helicase motifs (Fig. 1). The role of the zinc-finger motifs in NMD is not known. The ATPase activity of the protein resides in helicase motifs Ia and II (Fig. 1), and is linked to the essential 5' to 3' helicase activity of the protein.[15] Mutated variants of hUpf1 that lack ATPase activity due to mutation of highly conserved aspartate and glutamate residues in helicase motif II show no double-stranded RNA unwinding activity[15] and are inactive in NMD in yeast.[16]

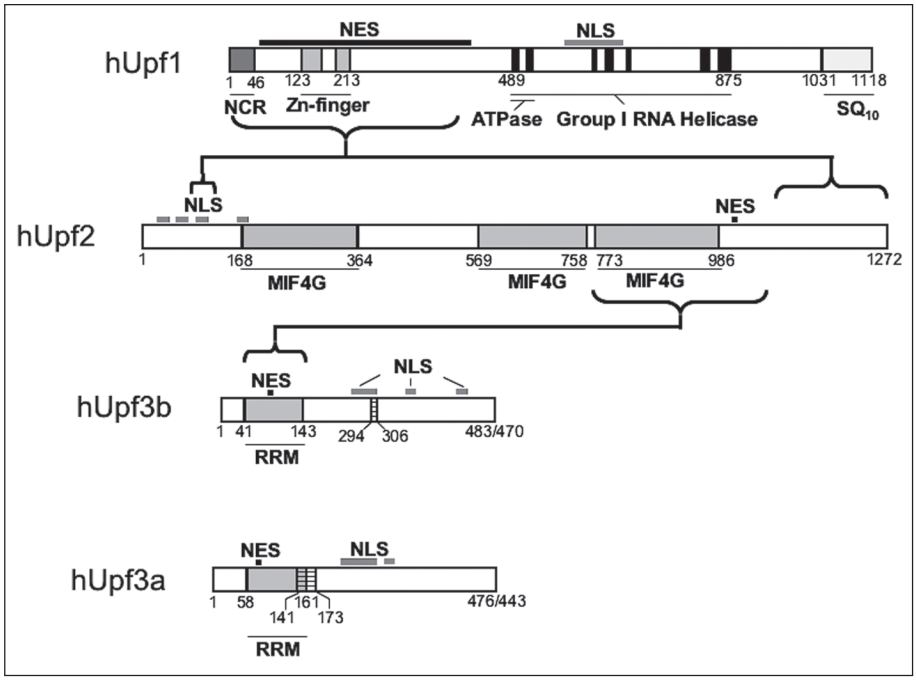

Figure 1. Schematic representations of human Upf proteins. Specific domains are shaded grey or black for each protein. A black bar on top of each representation indicates a putative nuclear export signal (NES) and a grey bar represents a putative nuclear localization signal (NLS). Regions of interactions between hUpf proteins are indicated by brackets with connecting lines. Amino-acids 141-173 corresponding to the alternatively spliced exon 4 of hUpf3a, and amino-acids 294-306 corresponding to the alternatively spliced exon 8 of hUpf3b are shown by striped rectangles. NCR: N-terminal conserved region, found in Upf1 proteins of all metazoans that have been analyzed; SQ_{10}: region containing ten SQ motifs; MIF4G: middle portion of eIF4G-like domain; RRM: RNA recognition motif.

The C-terminus of hUpf1 contains several serine/glutamine (SQ) and serine/glutamine/proline (SQP) repeats (Fig. 1). Multiple serines (S1073, S1078, S1096 and S1116) in SQ repeats are targets of phosphorylation by hSmg1, a phosphatidylinositol 3-kinase related protein kinase involved in NMD (see chapter by Yamashita et al).[17] The N-terminus of hUpf1 contains a proline-glycine rich region, and it is also rich in negatively charged residues. An N-terminal region of hUpf1 (amino acids 1-46), which is conserved among metazoans but is not found in yeast, has recently been shown to mediate interaction with Smg5, a protein that forms part of a complex responsible for dephosphorylating Upf1 in both humans and *C. elegans* (see chapters by Yamashita et al and Anderson).[18-20] Thus, the N- and C-termini of hUpf1, which are missing from yeast Upf1p, are involved in regulating the protein by a phosphorylation and dephosphorylation cycle.

hUpf1 primarily localizes to the cytoplasm.[11,12] However, evidence shows that hUpf1 shuttles in and out of the nucleus, and its export out of the nucleus is mediated by Crm1.[14] A conventional nuclear export signal (NES) or nuclear localization signal (NLS) has not been identified in hUpf1. However, specific regions of hUpf1 have been shown to possess NLS (amino acids 596-697) and NES (amino acids 55-416) function (Fig. 1).[14]

hUpf2 and hUpf3 Function in NMD

hUpf2 interacts with both hUpf1 and hUpf3.[10-12] hUpf2 contains three conserved middle of eIF4G-like (MIF4G) domains with similarity to a domain in the middle of eukaryotic translation initiation factor 4G(eIF4G) (Fig. 1). The region overlapping with the last of the three MIF4G domains is responsible for interaction with hUpf3.[10,11] The importance of the MIF4G domains in NMD has been studied in *Schizosaccharomyces pombe* where mutations in the phylogenetically conserved "FIGEL" motif of the last two MIF4G domains of Upf2 inhibit NMD.[10] In addition to the MIF4G domains, hUpf2 contains more than one putative NLS in its N-terminus. Even though the N-terminal region of hUpf2 (amino acids 1-120) can target a heterologous protein to the nucleus,[10] it is currently unknown whether hUpf2, like hUpf1, is a nucleocytoplasmic shuttling protein (Fig. 1).[10-12] However, at steady state, hUpf2 is cytoplasmic and concentrated in the perinuclear region.[11,12]

hUpf3 is the least conserved component of the hUpf proteins.[4] The human genome contains two different hUPF3 genes, encoding hUpf3a (also known as hUpf3) and hUpf3b (also known as hUpf3X since the corresponding gene maps to the X-chromosome), respectively.[11,12] hUpf3a and hUpf3b transcripts are alternatively spliced to encode two isoforms for each protein (Fig. 1). hUpf3 proteins are nucleocytoplasmic shuttling proteins that contain putative NLS and NES motifs and primarily localize in the nucleus.[11,12] The hUpf3 proteins also contain an RNA recognition motif (RRM) in the N-terminal region (Fig. 1). The hUpf3 RRM, which was originally hypothesized to constitute an RNA-binding surface,[12] lacks critical aromatic amino acid residues for RNA-binding.[21] Recently, the RRM domain of hUpf3b was shown to constitute the surface that interacts with hUpf2 (see below).[21]

Interactions between hUpf Proteins

The three yeast Upf proteins are known to form a complex in which Upf2p serves as a bridge between Upf1p and Upf3p.[2] A similar complex exists between hUpf proteins.[10-12] The regions of hUpf proteins that mediate these interactions were predicted based on mapped interaction domains of yeast Upf proteins, and tested by deletion analyses. The hUpf2-interaction domain of hUpf1 is found in the N-terminus spanning the zinc-finger domain (amino acids 1-415). This conserved N-terminal region interacts with the C-terminal region of hUpf2 (amino acids 1084-1272) (Fig. 1). However, a hUpf2 deletion protein lacking these residues shows residual weak interaction with hUpf1,[10,11] possibly mediated by an N-terminal region of hUpf2 (amino acids 94-133).[11] Thus, there are two potential hUpf1-interacting regions in hUpf2.

Deletion analysis mapped the region of hUpf2 necessary for interaction with hUpf3 to its third MIF4G domain, while the hUpf2-interacting surface of hUpf3 overlaps with its RRM motif (Fig. 1).[11,12] A recent crystal structure of this MIF4G domain of hUpf2 in complex with the RRM domain of hUpf3b provides detailed insight into how hUpf2 interacts with hUpf3b.[21] The α-helices formed by the MIF4G domain interact with the β-sheet surface of the hUpf3b RRM domain. The interaction between hUpf2 and hUpf3b is mediated by positively charged residues of the ribonucleoprotein motif 2 (RNP2) of the hUpf3b RRM domain and negatively charged residues in hUpf2. Specific mutations in these residues abolish complex formation.[21]

Even though the interaction between Upf3 and Upf2 is conserved between species, hUpf3b lacking its hUpf2-interacting domain is capable of destabilizing an mRNA when tethered downstream of a stop codon.[22] This observation suggests that hUpf3b does not require an interaction with hUpf2 to trigger tethered decay of an mRNA. However, it is unknown if this is also true in the natural NMD pathway.

Interactions between hUpf Proteins and the Translation Machinery

Translation is essential for NMD.[23-25] NMD substrates need to undergo at least one round of translation during which the PTC is recognized. Evidence suggests that this occurs during a first, so-called pioneer, round of translation (see chapter by Maquat).[26-29] However, experiments in yeast show that while NMD may normally take place during a pioneer round of translation,[30] this is not an absolute requirement, because an mRNA that has undergone several rounds of translation can be a target of NMD (see chapters by Baker and Parker, and Amrani and Jacobson).[31]

The key to understanding how the hUpf complex functions in NMD will be to understand how it communicates with the translational machinery. Translation initiation is the rate-limiting and the most tightly regulated step of translation. The translation factor eIF4G acts as a scaffold for assembly of the translation initiation complex and plays a central role in translation initiation. eIF4G interacts with the mRNA cap binding proteins, CBC (cap-binding complex)[28,32] and eIF4E[33], which predominantly localize to the nucleus and cytoplasm, respectively (Fig. 2).[29,33] Evidence in mammalian cells suggests that NMD is associated with CBC-initiated translation (see chapter by Maquat).[26,28] eIF4G helps recruit the 40S ribosomal subunit via the eIF3 complex and other associated factors.[33,34] The release factors eRF1 and eRF3 are essential for translation termination. eRF1 recognizes the stop codon in the A-site of the ribosome and catalyzes the release of the nascent peptide.[31] eRF3 may promote release of eRF1 from the ribosome and ribosome recycling via its interaction with poly(A) binding protein (PABP) C1.[33,35] PABPC1 also interacts with eIF4G at the cap to circularize the mRNA, a key feature of efficient translation in eukaryotes.[33]

Several interactions between hUpf proteins and the translation factors described above have been reported. Both yeast and human Upf1 proteins have been shown to interact with the yeast translation termination factors eRF1 and eRF3.[36] However, the role of this critical interaction in discriminating a premature from a normal termination codon is poorly understood. In yeast, Upf proteins have been shown to play a role in translation termination. Yeast strains depleted of Upf proteins are reported to show increased readthrough at termination codons,[37-39] although conflicting evidence about the role of yeast Upf proteins in translation termination exists.[39,40] A similar role for human Upf proteins has not been tested. The interplay between hUpf1 and eRFs 1 and 3 likely holds a key to understanding the mechanism of PTC-containing mRNA recognition and should be an important topic for future studies.

hUpf1 has also been found to interact with PABPC1,[41] but the significance of this interaction in NMD is unknown. Another NMD factor that may link the hUpf complex to the translation process is hUpf2. Two-hybrid assays indicate that the third middle portion of the eIF4G (MIF4G) domain of hUpf2 can interact with the translation initiation factors eIF4AI and hSui1, the latter of which is an eIF3 subunit.[10] Since the MIF4G domain of eIF4G interacts with the same factors, it has been speculated that hUpf2 competes with eIF4G for MIF4G-mediated

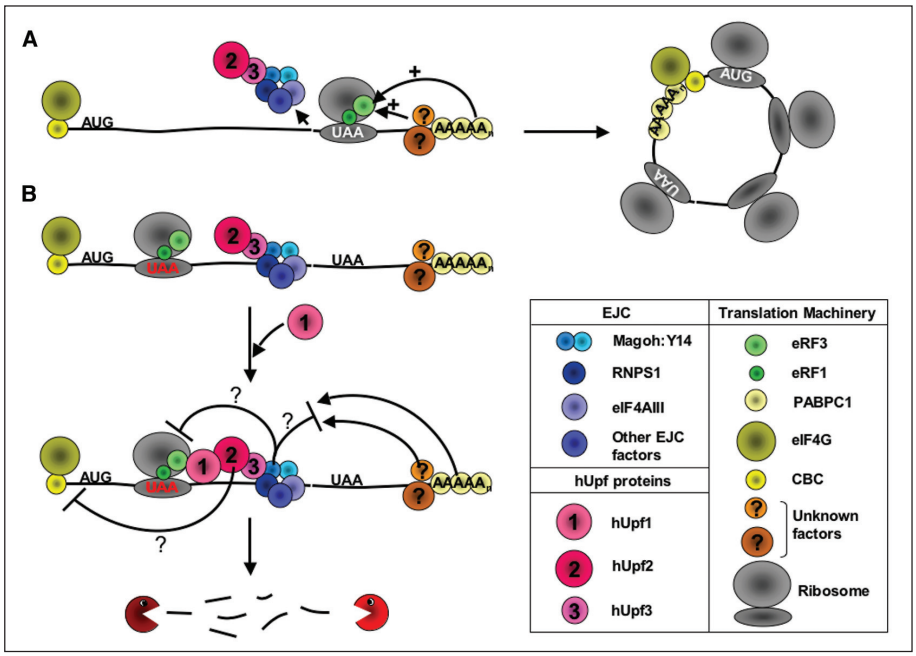

Figure 2. Models of events after translation termination on normal and PTC-containing mRNAs in mammalian cells. A) On normal mRNAs, the absence of an exon junction complex (EJC) downstream of the termination codon at the time of translation termination promotes efficient translation termination. This may require interactions (indicated by arrows) between PABPC1 and eRF3 and potentially other unknown factors as indicated. An efficient translation termination event channels normal mRNAs into the translational pool (shown on the right). B) A translation termination event at a PTC (5'-most UAA, shown in red) that is more than ~50-55 nucleotides upstream of the last exon-exon junction leads to NMD. An EJC that remains bound to the mRNA 3'-untranslated region (UTR) at the time of translation termination may promote inefficient termination, perhaps due to the inhibition (indicated by ⊥) of interactions between the termination complex and PABPC1 or other unknown factors the at 3'UTR. The recruitment of the hUpf complex triggers degradation by the mRNA decay machinery. The code for specific proteins is given on the right. The identities of mRNA decay factors (specified by pac-men), are at present only poorly defined and not shown.

interactions, so as to inhibit translation initiation on NMD-target mRNAs (Fig. 2).[10] However, there is no direct evidence to support this otherwise attractive model of hUpf2 function in NMD. It is possible that the interactions of hUpf proteins with translation initiation factors and PABPC1 could destabilize the closed-loop structure of the mRNP and thereby inhibit translation and expose the mRNA cap and poly(A) tail to decay enzymes.[10]

hUpf Proteins are Recruited to mRNAs

A major unanswered question is how hUpf proteins are recruited to mRNAs. Intriguingly, hUpf proteins concentrate in different sub-cellular compartments: hUpf1 and hUpf2 proteins in the cytoplasm, and hUpf3 proteins primarily in the nucleus.[11,12] Nevertheless, each hUpf protein should be recruited to a target mRNA before it can undergo NMD. This observation suggests that the assembly of the hUpf complex on mRNA targets is a dynamic process.

Several lines of evidence indicate that hUpf3 proteins are recruited to mRNAs in a splicing-dependent manner. The hUpf3 proteins associate specifically with mRNAs that have

undergone splicing in human cells.[12,42-44] Moreover, *Xenopus* Upf3 specifically interacts with spliced RNA in *Xenopus* oocyte extracts.[45] A hallmark of splicing in mammalian cells is the deposition of an exon-junction complex (EJC) ~20-24 nucleotides upstream of exon-exon junctions after splicing (see chapter by Maquat).[46] The interaction of hUpf3 with spliced mRNA was shown to be mediated by the EJC.[43-45] The hUpf3 proteins coimmunoprecipitate with core EJC subunits, RNPS1,[44] Y14,[43] and eIF4AIII (G. Singh and J. Lykke-Andersen, unpublished data), although it is not clear which interactions are direct.[43,47] The interaction of hUpf3b with Y14 has been studied in most detail and depends on a 14-amino acid conserved region in the C-terminal region of hUpf3b.[22] Deletion of this conserved stretch or an arginine-to-alanine mutation at residue 423 abolishes the ability of hUpf3b to form a complex with Y14 in cell extracts.[22] It is currently unknown whether hUpf3b interacts with other EJC components through the same C-terminal region.

hUpf2, like hUpf3, associates with spliced mRNA,[45] and is detected in mRNPs purified with antibodies against the nuclear cap binding protein, which also contain EJC components and hUpf3.[26,27] This suggests that hUpf2 may be recruited to the mRNA via the EJC and hUpf3. hUpf2 may also interact with mRNA directly. The crystal structure of the third MIF4G domain of hUpf2 revealed a basic patch that possesses RNA binding activity. This RNA-binding region of the MIF4G domain is not part of the hUpf3b-binding surface, and directly binds RNA in vitro with or without hUpf3b.[21] However, the significance of this RNA binding property of hUpf2 to the NMD pathway is unknown.

Unlike hUpf3 and hUpf2, hUpf1 has not been reported to specifically associate with spliced mRNAs. The cellular pool of hUpf1 evenly distributes between polysomes, a sub-polysomal fraction and a free cytoplasmic fraction.[48] How hUpf1 is recruited to mRNAs is not clear, and will be an important topic for future studies. eRF1 and eRF3,[36] hUpf2 (as a part of the EJC:hUpf3 complex) or PABPC1[41] or CBC (N. Hosoda and L.E. Maquat, personal communication) are all candidates for proteins responsible for recruiting hUpf1 to target mRNAs. In addition, hUpf1 can itself bind RNA via its RNA helicase motif. The RNA binding activity of hUpf1 is modulated by ATP, a cofactor of hUpf1.[15] The ability of hUpf1 to bind RNA is reduced in the presence of ATP, suggesting that binding of ATP to hUpf1 would cause the protein to dissociate from RNA while hydrolysis of bound ATP would allow it to bind RNA.[15,49] However, the specific role in NMD of the RNA helicase function of hUpf1 is currently unclear.

These observations taken together have led to the hypothesis that hUpf3 and hUpf2 are recruited to mRNAs through splicing via the EJC, whereas hUpf1 may be recruited by the hUpf2:hUpf3:EJC complex or alternatively via eRFs after translation termination (Fig. 2). Another possibility, which is more in line with the role of Upf proteins in translation termination in yeast, is that the hUpf proteins all enter the mRNA with the termination complex. However, these models need more experimental testing, and the question still remains how the hUpf proteins, once recruited to mRNA, help distinguish NMD targets from normal stable mRNAs.

hUpf Proteins Help Discriminate PTC-Containing from Normal mRNAs

The key step in the NMD pathway is the discrimination between a normal termination codon and a PTC that triggers. The high degree of conservation in the translation process between eukaryotes would suggest that the basic mechanism of PTC recognition is similar in all eukaryotes. Yet, only mammalian cells appear to rely on pre-mRNA splicing for recognition of PTCs. Evidence suggests that in the majority of cases, PTCs in mammalian cells are recognized when they are situated more than 50-55 nucleotides upstream of last exon-exon junction[24,50-53] in a way that depends on the EJC that is deposited upstream of the exon-exon junctions (see chapter by Maquat). By contrast, no EJC has been observed in yeast, and in flies the EJC plays no apparent role in NMD (see chapter by Behm-Ansmant and Izaurralde).[54] Evidence in yeast suggests that translation termination at a PTC is kinetically different from translation termination at a normal stop codon and depends on the nature of the downstream

3' untranslated region (UTR) (see chapter by Amrani and Jacobson).[55] Moreover, a PTC is recognized as a normal termination codon when poly(A) binding protein (Pab1p) is tethered 40-75 nucleotides downstream.[55] These data are consistent with a so-called faux 3'UTR model, in which a normal termination event takes place only in the presence of a normal 3'UTR whereas an aberrant 3'UTR triggers abnormal termination and NMD (see chapter by Amrani and Jacobson).[55-57] Although similar experiments have yet to be performed in mammalian cells, the conservation of the translational machinery and the Upf complex, and the observation that hUpf1 associates with PABPC1 and eRFs 1 and 3, suggests that mammalian NMD may function in a similar manner.

How could these observations be reconciled with the involvement of the EJC in mammalian NMD? Perhaps in mammalian cells, the presence of an EJC that remains bound to an mRNA 3'UTR after translation termination due to the presence of an exon-exon junction more than 50-55 nucleotides downstream of the PTC[50-52,58] signals an aberrant 3'UTR, which negatively influences the termination process (Fig. 2). The eRFs have been shown in both yeast and humans to associate with poly(A) binding protein.[35,59] Possibly, an EJC that remains bound to an mRNA 3'UTR after a premature translation termination event disrupts the communication between these translation factors, which in turn influences translation termination. hUpf3 may have evolved to acquire interactions with EJC components so as to link splicing with NMD in mammalian cells. Consistent with this hypothesis the Y14-binding region of hUpf3 is not conserved in yeast.[22] Clearly, more experiments are needed to understand how PTCs are recognized in mammalian cells and how this may differ from, or resemble the mechanism in other organisms.

hUpf Proteins Trigger mRNA Decay

The last step in the elimination of an mRNA that harbors a PTC is its destruction by the cellular mRNA decay machinery. In yeast, where the events in degradation of NMD substrates are best understood, decay mainly proceeds from the 5' end (see chapter by Baker and Parker). By contrast, in *Drosophila*, the decay of NMD substrates is initiated by endonucleolytic cleavage followed by exonucleolytic decay of the 5' and 3' fragments (see chapter by Behm-Ansmant and Izaurralde).[60]

The degradation of NMD substrates in human cells is poorly understood but has been found to depend on enzymes involved in decapping, deadenylation, and 5' to 3' and 3' to 5' exonucleolytic decay.[61] In addition, rapid deadenylation of NMD substrates has been detected.[62] hUpf1 has been found in complex with the human decapping proteins, hDcp1 and hDcp2,[61,63] as well as with components of the deadenylation machinery, and the 5' to 3' and 3' to 5' exonucleolytic decay machineries.[61] Preliminary evidence suggests that decay of the body of the transcript may proceed from both ends with similar kinetics.[61] However, the relative contribution of specific mRNA decay enzymes in human NMD is poorly understood. Moreover, it is unknown whether an endonucleolytic cleavage takes place, although endonucleolytic intermediates of PTC-containing β-globin mRNA have been detected in mouse erythroid cells (see chapter by Maquat).[64-68]

How does the hUpf complex, once assembled onto PTC-containing mRNAs, communicate with the mRNA decay machinery? Although little is known about this process, new evidence suggests a link between a phosphorylation-dephosphorylation cycle of hUpf1 and the delivery of PTC-containing mRNAs to the decay machinery.[5,69,70] The proteins hSmg5 and hSmg7 have been found in complexes containing phosphorylated hUpf1 and may mediate hUpf1 dephosphorylation via protein phosphatase 2A (see chapters by Yamashita et al and Anderson).[18,19] When exogenously expressed, these proteins localize to sub-cytoplasmic structures enriched in mRNA decay factors, called processing bodies.[69] Evidence in yeast and human cells suggests that processing bodies constitute cytoplasmic sites of mRNA decay.[71,72] Exogenous expression of hSmg7 also leads to detection of coexpressed hUpf1 protein in processing bodies.[69] Moreover, this hSmg7-mediated recruitment of hUpf1 to processing bodies

was disrupted by mutation of residues in hSmg7 critical for binding to hUpf1 in vitro.[70] This suggests that the complex that associates with phosphorylated hUpf1 and triggers dephosphorylation may also deliver PTC-containing mRNAs to the mRNA decay machinery. However, this model needs to be further tested in future experiments.

Conclusions and Future Perspectives

The hUpf complex is essential to human NMD. However, the mechanism by which this complex helps recognize PTC-containing mRNAs and divert them from the translational pool to the mRNA decay machinery is poorly understood. Key issues that need to be addressed in the future are how the hUpf proteins, which concentrate in different sub-cellular compartments, are recruited to mRNA targets, and what events lead to recognition of a termination codon as a PTC. Experiments aimed at studying the communication between the hUpf complex and translation termination factors should be critical to understanding these processes. Another important goal is to understand how mRNA decay factors are recruited to PTC-containing mRNAs. Future research should provide insight into these issues and help to elucidate the unsolved mysteries of NMD.

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