The Nonsense-Mediated Decay RNA Surveillance Pathway

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Abstract

Nonsense-mediated mRNA decay (NMD) is a quality-control mechanism that selectively degrades mRNAs harboring premature termination (nonsense) codons. If translated, these mRNAs can produce truncated proteins with dominant-negative or deleterious gain-of-function activities. In this review, we describe the molecular mechanism of NMD. We first cover conserved factors known to be involved in NMD in all eukaryotes. We then describe a unique protein complex that is deposited on mammalian mRNAs during splicing, which defines a stop codon as premature. Interaction between this exon-junction complex (EJC) and NMD factors assembled at the upstream stop codon triggers a series of steps that ultimately lead to mRNA decay. We discuss whether these proofreading events preferentially occur during a "pioneer" round of translation in higher and lower eukaryotes, their cellular location, and whether they can use alternative EJC factors or act independent of the EJC.

Contents

INTRODUCTION

Forgive me my nonsense as I also forgive the nonsense of those who think they talk sense.

–Robert Frost (1874–1963)

Eukaryotic gene expression comprises a series of interconnected steps, including transcription, 5′ cap formation, mRNA splicing, polyadenylation, mRNA export, translation, and mRNA degradation. These steps are integrated with one another to augment their efficiency and fidelity (1, 2). To further ensure fidelity, many quality-control mechanisms have evolved (3, 4). One of these mechanisms is nonsense-mediated mRNA decay (NMD), a pathway that targets mRNAs harboring premature termination (nonsense) codons (PTCs) for degradation (5–12). This pathway is important because if PTC-containing messages were allowed to be translated they would produce truncated proteins with potentially deleterious gain-of-function or dominant-negative activity.

PTCs can arise in a variety of ways. The most obvious source is random nonsense and frameshift mutations in the DNA sequence that are subsequently transcribed into mRNA. Another source of PTCs is programmed DNA rearrangements. In mammals, the best-studied examples of this are the T-cell receptor (TCR) and immunoglobulin (Ig) genes, which undergo programmed DNA rearrangements to increase their repertoire of antigen receptors. Two out of three times, these rearrangements lead to the generation of frameshift mutations and consequent downstream PTCs, which activate the NMD response (13–15). Yet another common source of PTCs is errors in RNA splicing, including aberrant alternative splicing (7, 10, 16, 17).

NMD is a highly conserved pathway that exists in all eukaryotes examined to date (16). The core NMD machinery comprises three *trans*-acting factors, called up-frameshift (UPF) proteins, which were initially discovered in *Saccharomyces cerevisiae* and later identified in higher eukaryotes (18–20). One of these proteins, UPF1, is a group I helicase family member recruited to mRNAs upon recognition of stop codons by the translation apparatus (21, 22). Rapid decay of PTC-bearing mRNAs is triggered when UPF1 is allowed to interact with the two other UPF proteins, UPF2 and UPF3, by a mechanism that is still poorly understood. In mammalian cells, UPF2 and UPF3 are part of a large complex of proteins that is deposited on mRNAs at exon-exon junctions during RNA splicing in the nucleus. Other components of this exon-junction complex (EJC), including those in its tetramer core (eIF4AIII, MLN51, and the Y14/MAGOH heterodimer), also participate in NMD. In addition, NMD requires factors that regulate

PTC: premature termination (nonsense) codon

TCR: T-cell receptor

Ig: immunoglobulin

UPF1 phosphorylation. Initially discovered in *Caenorhabditis elegans*, the Suppressor with Morphogenetic effect on Genitalia-1 (SMG-1) protein phosphorylates UPF1, whereas SMG-5, SMG-6, and SMG-7 promote UPF1 dephosphorylation (23–26). The requirement for all four of these SMG factors implies that a cycle of UPF1 phosphorylation and dephosphorylation drives NMD (27).

In this review, we discuss the biochemistry and underlying molecular mechanism of NMD. We focus on NMD in mammals but also cover NMD in lower organisms to provide context. For a more complete description of NMD in *C. elegans*, *Drosophila melanogaster*, *S. cerevisiae*, and plants, readers can refer to many excellent reviews (5–8, 16, 17, 28–34). We first briefly describe the conserved factors that are essential for NMD in all eukaryotes. Next, we discuss the composition, structure, and function of the EJC and its unique involvement in mammalian NMD. We then describe how the assembly of NMD and EJC factors onto mammalian PTC-bearing mRNAs forms messenger ribonucleoproteins (mRNPs) that elicit rapid mRNA decay. Finally, we discuss putative alternative branches of the NMD pathway, some of which use alternative sets of EJC factors and others that appear to be completely independent of the EJC.

NMD FACTORS

Several proteins have been shown to be essential for NMD. The UPF1, UPF2, and UPF3 proteins comprise the core NMD machinery. The SMG-1, SMG-5, SMG-6, and SMG-7 proteins mediate the phosphorylation and dephosphorylation cycle of UPF1. In this section, we describe each of these proteins, their known biochemical functions, and their interactions.

UPF Proteins: The Core NMD Machinery

UPF1, a complex phosphoprotein harboring multiple domains, is recruited to mRNAs when they terminate translation (21, 35, 36). The N and C termini of UPF1 contain multiple serine residues that are of central importance in NMD because they are subject to regulation by a cycle of phosphorylation and dephosphorylation (discussed below) (25, 27, 37–39). UPF1 also contains domains with ATP-dependent 5′-to-3′ helicase activity and RNA-dependent ATPase activity in both human cell lines and *S. cerevisiae*, but the functional relevance of these biochemical activities is not well understood (40, 41). Consistent with its role in translation termination, human UPF1 is primarily a cytoplasmic protein. However, it has also been shown to shuttle in and out of the nucleus using novel nuclear localization and export sequences (42). The functional significance of the ability of UPF1 to shuttle between the nucleus and cytoplasm is not known.

Both loss- and gain-of-function approaches have been used to demonstrate that UPF1, as well as several other proteins, participate in mammalian NMD (**Table 1**). Loss of function was achieved by RNA interference (RNAi) or expression of dominant-negative proteins. Gain of function was achieved by fusing these proteins to the MS2 coat or λN protein, both of which are bacteriophage RNA-binding proteins. NMD activity was indicated when expression of such fusion proteins destabilized a reporter mRNA harboring high-affinity MS2- or λN-binding sites downstream of the stop codon.

The *Upf1* gene is essential for the survival of mice. Embryos from *Upf1*-null mice die by day 7.5 of gestation, and attempts at creating *Upf1*-null embryonic fibroblast lines failed because the cells underwent apoptosis after a brief growth period (43). This suggests that NMD is essential for mammalian cell survival, but this interpretation is clouded by the fact that UPF1 has roles in pathways other than NMD (44, 45). In contrast to mice, lower eukaryotes do not require UPF1 for survival, but its loss does cause defects in fermentation (in *S. cerevisiae*) and reproduction (in *C. elegans*) (16, 18, 40, 46–51).

Exon-junction complex (EJC): a dynamic protein complex deposited upstream of exon-exon junctions after RNA splicing; serves as a second signal for NMD

SMG: suppressor with morphogenetic effect on genitalia

Messenger ribonucleoprotein (mRNP): an mRNA and its associated proteins, many of which regulate mRNA transport, translation, and mRNA decay

RNAi: RNA interference

Table 1 Nonsense-mediated decay (NMD) and exon-junction (EJC) proteins

Protein	Cellular location	Characteristics and Functions	References[a,b]
UPF1	Mainly cytoplasmic, some nuclear (shuttles)	NMD; promotes translation; histone mRNA decay; Stau1-mediated mRNA decay; ATPase; helicase; phosphoprotein substrate for SMG-1; recruited by eRFs to stop codons	39[b], 41, 42[b], 44, 45[a,b], 53[a], 57[b], 87[a], 165
UPF2	Cytoplasmic (perinuclear) but has nuclear localization signals	NMD; promotes translation; EJC adapter protein that binds both UPF1 and UPF3; binds RNA in vitro	22[b], 45[a,b], 53[a], 54, 56, 57[b], 87[a]
UPF3a (UPF3)	Mainly nuclear, some cytoplasmic (shuttles)	NMD (weak); promotes translation (weak); EJC protein with short and long isoforms that differentially distribute into distinct cytoplasmic protein complexes with UPF1	45[a], 53[a], 54, 58[a], 150, 166
UPF3b (UPF3X)	Mainly nuclear, some cytoplasmic (shuttles)	NMD; promotes translation; EJC protein that directly interacts with UPF2; may also directly interact with Y14	39, 45[b], 53[a], 54, 56, 57[a], 58[a], 87[a], 132[a], 150, 166, 167[a]
SMG-1	Cytoplasmic	NMD; phosphoinositide 3-kinase-related kinase family member; phosphorylates UPF1	37[b], 63, 168[b]
SMG-5	Mainly cytoplasmic, some nuclear (shuttles)	NMD; interacts with PP2A and promotes UPF1 dephosphorylation	25, 37, 38, 39[b], 55[b]
SMG-6	Mainly cytoplasmic, some nuclear (shuttles)	NMD; interacts with PP2A and promotes UPF1 dephosphorylation	55[b], 67, 169[b]
SMG-7	Mainly cytoplasmic, some nuclear (shuttles)	NMD; interacts with PP2A and promotes UPF1 dephosphorylation; when overexpressed, it recruits UPF1 to P-bodies	55[b], 66[a]
Y14	Nuclear and cytoplasmic (shuttles)	NMD; promotes translation; EJC core protein that forms a stable heterodimer with MAGOH	57[a,b], 78, 87[a], 91[a], 132[a,b], 166, 167[a]
MAGOH	Nuclear and cytoplasmic (shuttles)	NMD; promotes translation; EJC core protein that forms a stable heterodimer with Y14; binds to TAP	57[a,b], 87[a], 95
eIF4AIII	Nuclear and cytoplasmic (shuttles)	NMD; RNA helicase; EJC core protein that probably anchors the other EJC proteins to the mRNA substrate	57[a,b], 91[b], 100[b], 102[b], 103
MLN51 (BTZ, CASC3)	Nuclear and cytoplasmic (shuttles)	NMD; EJC core protein that directly interacts with eIF4AIII; binds RNA in vitro	57[b], 91[a,b]
RNPS1	Nuclear and cytoplasmic (shuttles)	NMD; promotes translation; splicing coactivator; EJC factor that interacts with PININ, SAP18, and ACINUS	57[a,b], 78, 87[a], 167[a], 170
PYM	Cytoplasmic	NMD; EJC factor that directly interacts with Y14/MAGOH; RNA-binding protein	171
UAP56	Nuclear	RNA splicing; mRNA export; EJC factor that recruits REF/ALY	172
REF/ALY	Nuclear and cytoplasmic (shuttles)	mRNA export; EJC factor that recruits TAP/p15; interacts with UAP56 and Y14	85, 167, 173
TAP (NXF1)/p15	Nuclear	mRNA export; loosely associated with the EJC; interacts with REF/ALY and components of the nuclear pore complex	80, 173–175

Table 1 (*Continued*)

Protein	Cellular location	Characteristics and Functions	References[a,b]
SRm160	Nuclear and cytoplasmic (shuttles)	Splicing coactivator; EJC factor	62, 78
PININ	Nuclear	mRNA export; EJC factor that binds RNPS1	176
ACINUS	Nuclear	Apoptosis; EJC factor that forms a stable heterodimer with RNPS1	170, 176
SAP18	Nuclear and cytoplasmic (shuttles)	Function unknown; component of the transcriptional repression SIN3-HDAC complex; EJC factor that binds RNPS1	85, 170

[a]Used gain-of-function experiments (i.e., tethering) to show involvement in NMD.
[b]Used loss-of-function experiments (i.e., RNAi or expression of dominant-negative mutant) to show involvement in NMD.

Unlike UPF1, UPF3 has no known biochemical activity; instead, it probably participates in NMD in mammals (**Table 1**) by virtue of its being part of the second signal for NMD: the EJC (see below). Mammals possess two *UPF3* genes, whereas *C. elegans*, *D. melanogaster*, and *S. cerevisiae* have only one (52–55). Human *UPF3a* (also called *UPF3*) is on chromosome 13, and *UPF3b* (also called *UPF3X*) is on the X chromosome. The UPF3a and UPF3b proteins have some common characteristics. First, both directly interact with UPF2 (described below) through a domain in their N termini (**Figure 1**). Surprisingly, X-ray crystallographic analysis revealed that this binding is achieved using a canonical RNA-binding domain (RBD) in UPF3b (56). It is not known whether UPF3a employs the same strategy to interact with UPF2. Second, both UPF3a and UPF3b are predominantly nuclear proteins that shuttle to the cytoplasm (53, 54). Thus, like other

RBD: RNA-binding domain

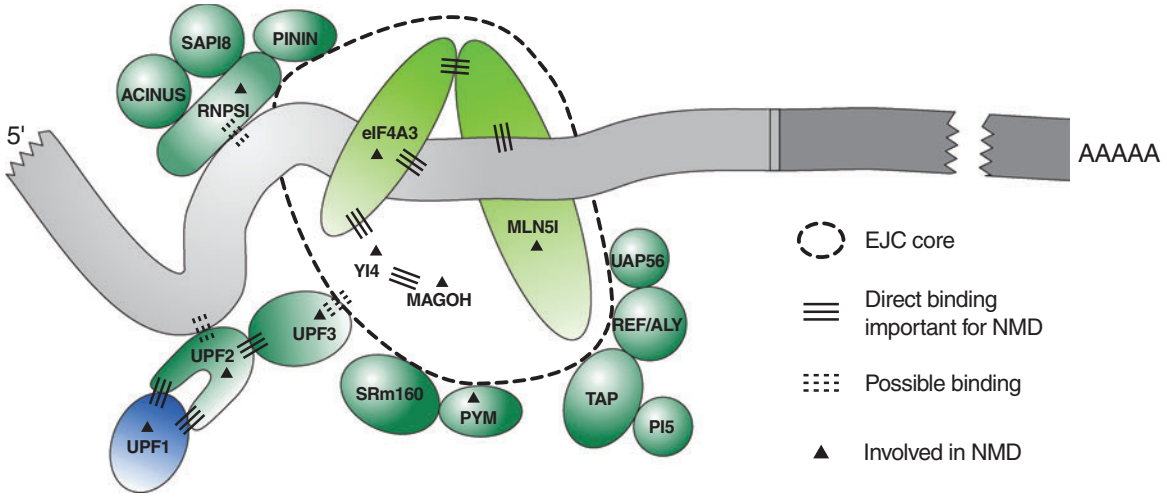


Figure 1

A model of the exon-junction complex (EJC). Four proteins form the RNA-binding EJC core (eIF4AIII, MAGOH, MLN51, and Y14); these interact with other EJC factors that associate more transiently with the mRNP during its journey from the nucleus to the cytoplasm. N- and C-terminal binding domains are shaded dark and light gray, respectively (53, 54, 60, 92, 94, 100). For additional details, refer to **Table 1**.

Phosphoinositide 3-kinase-related kinases: a protein kinase family whose members regulate various cellular events, such as DNA repair and the cell growth response

EJC proteins, both UPF3a and UPF3b are recruited to mRNAs during nuclear RNA splicing and then travel with the mature mRNAs to the cytoplasm.

Although UPF3a and UPF3b contain similar structural motifs, they differ in their ability to elicit NMD. UPF3b strongly elicits NMD when tethered downstream of a stop codon, whereas UPF3a has only modest activity (53, 57, 58). Further support for this difference is the finding that RNAi-mediated depletion of UPF3b, but not UPF3a, reverses the decay of a commonly used NMD substrate (45, 59). A crucial arginine residue in the C terminus of UPF3b, but not in UPF3a, is responsible for the dramatic difference in their NMD activities (58). It remains for future studies to determine why two *UPF3* genes evolved in higher eukaryotes and whether they have redundant or independent roles.

UPF2 is an adapter molecule that brings together UPF1 and UPF3 to elicit NMD. Consistent with this role, UPF2 has distinct domains that bind UPF1 and UPF3 (53, 54, 60). The UPF1-binding domains are in both the N and C termini of human UPF2 (**Figure 1**); their importance is underscored by the fact that both domains are necessary for NMD (56, 61). The UPF3-binding domain has sequence identity with the MIF4G protein-protein interaction motif that was originally identified in the translation initiation factor eIF4G (56). UPF2 accumulates in the cytoplasm, in the so-called perinuclear region (53, 60). However, like other EJC components, UPF2 is probably first recruited to mRNAs as a result of mRNA splicing in the nucleus, an idea supported by the fact that UPF2 contains several consensus nuclear localization sequences in its N terminus and can be immunoprecipitated from nuclear extracts (60, 62). Whether UPF2 is first recruited to mRNAs in the nucleus or the perinuclear region in the cytoplasm is an important issue to resolve because it influences models defining the steps in the NMD pathway. Construction of accurate models also depends on knowing whether UPF2 binds directly to RNA or "piggybacks" on another RNA-binding factor; the former possibility is consistent with the fact UPF2 can directly interact with RNA, at least in vitro (56).

SMG Proteins: Mediators of the UPF1 Phosphorylation Cycle

SMG-1, SMG-5, SMG-6, and SMG-7 control the phosphorylation status of UPF1 (27). All four are required for NMD, as mutation of any one of their corresponding genes completely eliminates NMD in *C. elegans* (49). Likewise, knockdown or inhibition of any one of the corresponding mammalian proteins partially reverses NMD in mammalian cells (**Table 1**).

SMG-1 is a kinase that phosphorylates serine and threonine residues (24, 37, 63). It is a member of the phosphoinositide 3-kinase-related kinase family, whose other members function in DNA damage and growth responses as well as other events (64, 65). SMG-1-mediated phosphorylation of UPF1 is likely to be crucial for NMD on the basis of many lines of evidence (27), including the fact that a kinase-deficient version of SMG-1 acts in a dominant-negative manner to inhibit NMD in mammalian cells (37).

SMG-5, SMG-6, and SMG-7 are three nonredundant proteins that promote the dephosphorylation of UPF1 (24, 25, 37–39). SMG-5 and SMG-7 form a stable heterodimer by interacting through their N-terminal domains, whereas SMG-6 appears to act more independently (25, 39, 66). None of these SMG factors are phosphatases themselves; instead they promote UPF1 dephosphorylation by other means, including recruitment of protein phosphatase 2A to UPF1 (25, 38, 39, 66). Consistent with this common goal, the SMG-5/7 heterodimer and SMG-6 have both been shown to form complexes containing protein phosphatase 2A (25, 38, 39).

X-ray crystallographic analysis of SMG-7 revealed that its N terminus has a 14-3-3-like domain, a motif known to bind to

phosphoserine residues (67). Thus, the 14-3-3-like domain in SMG-7 probably binds to phosphoserine residues in UPF1 (67). Consistent with this idea, disruption of this domain by mutation inhibits the ability of SMG-7 to bind to UPF1 in vitro and to recruit UPF1 to processing bodies (P-bodies), cytoplasmic compartments in which mRNA decay occurs (67). SMG-5 and SMG-6 also have a phosphoserine-binding 14-3-3-like domain on the basis of their amino-acid sequence (67), which suggests that they too use this domain to bind specifically to phosphorylated UPF1.

The discovery that NMD requires factors that mediate both UPF1 phosphorylation and dephosphorylation strongly suggests that a cycle of UPF1 phosphorylation and dephosphorylation drives NMD (27). As described below, the molecular events mediating UPF1 phosphorylation and dephosphorylation are becoming better understood, but the specific biochemical functions of this phosphorylation cycle in NMD has been difficult to determine.

DETECTING ABBERANT MESSAGES FOR DEGRADATION

A unique aspect of mammalian NMD is the involvement of the EJC, a complex of proteins deposited at exon-exon junctions during mRNA splicing. In this section, we describe the factors that make up the EJC and their known functions.

NMD and the Exon-Junction Complex

A major issue that is still only partially understood is how NMD distinguishes between premature and normal stop codons. The prevailing view is that a second signal downstream of the stop codon dictates whether a stop codon is premature or not. In *S. cerevisiae*, the second signal appears to be a loosely defined downstream sequence element or an abnormally long 3′ untranslated region (UTR) (68–70). Early studies revealed that the second signal in mammalian cells is quite different from that in yeast because it is somehow delivered by an intron downstream of the stop codon (71–74). This discovery clarified why normal stop codons do not elicit NMD, as most are in the last exon (75). It also explained why transcripts from mammalian genes lacking introns are typically immune to NMD (76, 77). However, the following paradox arose: How does an intron—an entity that is removed from precursor transcripts in the nucleus—participate in a mechanism that depends on the cytoplasmic translation apparatus? A clue to solving this paradox was the discovery that the downstream intron must be spliceable to elicit NMD (73). This suggested that the spliceosome leaves an imprint with the second signal on spliced mRNA that remains bound even when it enters the cytoplasm to be read by ribosomes (72, 73).

Indeed, subsequent research identified such an imprint, the EJC (78–81). This ~350-kDa protein complex contains at least 10 proteins that are deposited 20–24 nucleotides upstream of exon-exon junctions after RNA splicing. Consistent with its role as an imprint providing a second signal for NMD, the EJC remains bound to mRNPs after they enter the cytoplasm. Furthermore, the EJC contains several proteins involved in NMD, including the well-characterized NMD proteins UPF2 and UPF3b (80). Together with several other lines of evidence (7, 81–83), a strong case can be made for the idea that the EJC relays the previous location of introns relative to the stop codon to ultimately dictate whether or not rapid mRNA decay will be elicited.

In addition to acting as a signal to degrade aberrant transcripts, EJC factors have other functions (11, 81). The EJC factors RNPS1 and SRm160 enhance the export of normal spliced mRNAs from the nuclei of *Xenopus laevis* oocytes (80, 84, 85). Several lines of evidence suggest that translation in mammalian cells is stimulated by the EJC factors UPF2, UPF3a, UPF3b, RNPS1, Y14, and MAGOH (86, 87). This translational enhancement appears to require efficient splicing (88). The

Processing body (P-body): a cytoplasmic focus containing high concentrations of RNA decay enzymes and factors mediating translational repression

EJC factors Y14/Mago and Barentz collaborate to localize *oskar* mRNA to the posterior pole of *D. melanogaster* oocytes (89–91). It is not known whether EJC factors direct the cytoplasmic localization of mRNAs in mammalian cells.

Proteins that have been identified in the EJC, including all those known to be involved in NMD, are listed in **Table 1**. **Figure 1** provides a model of the EJC that indicates the known direct interactions between EJC/NMD proteins and distinguishes between core EJC proteins and those that are more transiently part of the EJC.

The Exon-Junction Complex Core Proteins

The EJC core consists of four proteins: Y14, MAGOH, eIF4AIII, and MLN51 (also known as BTZ or CASC3), all of which participate in NMD. Cross-linking, coimmunoprecipitation, mutation analysis, and RNase H footprinting studies showed that these four proteins form a highly stable complex on single-stranded RNA in vitro (78, 92). The EJC core probably serves as a platform to which the transient EJC components attach (**Figure 1**) (93, 94).

Y14 and MAGOH form a stable heterodimer that is deposited near exon-exon junctions on a mature mRNA during RNA splicing, where it remains bound after export of the mRNA into the cytoplasm (95–98). Y14 contains an RBD that was originally postulated to allow it to bind directly to RNA; however, X-ray crystallographic analysis showed that this domain instead interacts directly with MAGOH (97, 99). Because the Y14 RBD-MAGOH interaction is of very high affinity and completely masks the RNA-binding surface of Y14, it is unlikely that Y14 uses its RBD to bind RNA. Y14 also interacts directly with the C-terminal domain of eIF4AIII (92, 100).

eIF4AIII is a DEAD-box RNA helicase that serves as an anchor to attach the EJC to its RNA substrate (100). In addition to its role as a molecular anchor, eIF4AIII when bound by ATP stabilizes the EJC core, at least in vitro (92). Mutagenesis and X-ray crystallographic data indicate that a domain in eIF4AIII's C terminus is responsible for its interaction with Y14/MAGOH (92, 94). In turn, Y14/MAGOH inhibits the ATPase activity of eIF4AIII, stabilizing the interaction between eIF4AIII and other EJC components as well as the EJC's interaction with its mRNA substrate (92, 97, 99, 101). Like many EJC factors, eIF4AIII accumulates at high levels in the nucleus but also shuttles to the cytoplasm (100, 102). Its nuclear accumulation distinguishes it from eIF4AI and eIF4AII, two proteins highly related to eIF4AIII, which accumulate in the cytoplasm and function as translation initiation factors (102, 103).

MLN51 is the mammalian ortholog of *D. melanogaster* Barentz. Barentz was originally defined as a factor that localizes *oskar* mRNA to the posterior pole of the fly oocyte; later, its mammalian ortholog was discovered to have a role in NMD (55, 89, 91). Interestingly, MLN51 is overexpressed in some breast cancer cells (104), but whether it has a causal role in malignancy is not known. A study using ultraviolet light cross-linking analysis identified a conserved region in MLN51's N terminus that is sufficient to bind spliced mRNA in vitro, interact with MAGOH, and direct MLN51 to nuclear speckles in vivo (92, 105). MLN51 also binds to eIF4AIII's N terminus, which stimulates the ATPase activity of eIF4AIII and stabilizes its binding to RNA (92). MLN51 can directly bind single-stranded RNA in the absence of eIF4AIII, suggesting that it may function independently of the EJC in some situations (92).

Recruitment and Assembly of Exon-Junction Complexes

The EJC is a dynamic structure whose protein composition changes as the mRNA with which it associates is processed in the nucleus, exported to the cytoplasm, and translated (2, 7, 106, 107). Certain EJC proteins

are recruited to the pre-mRNA even before splicing, including REF (108). Others, such as the EJC core factors eIF4AIII and Y14/MAGOH, are recruited after the first step of splicing (107–109). Some EJC proteins dissociate shortly after nuclear export, as indicated by experiments in injected *X. laevis* oocytes (80, 110, 111). Other EJC proteins probably do not dissociate until the ribosome displaces them during the first round of translation. In support of this, Y14 is stripped from RNA after translation in HeLa cytoplasmic extracts (96). Whether the ribosome physically knocks off the EJC or also biochemically modifies the EJC to facilitate dissociation remains unclear. Y14 phosphorylation may have a role in the latter event because Y14 mutants unable to be phosphorylated cause other EJC factors to be retained on ribosome-bound mRNPs (98).

ASSEMBLY OF NMD FACTORS

Three aspects of the underlying mechanism of NMD have been under intense study: whether there is a unique pioneer round of translation that recognizes aberrant transcripts, how premature stop codons are distinguished from bona fide stop codons, and the molecular choreography that ultimately triggers the decay of aberrant PTC-bearing transcripts. In this section, we discuss the experimental evidence that sheds light on these three issues.

NMD May Occur During a Pioneer Round of Translation

If a PTC-containing transcript underwent multiple rounds of translation before being targeted by NMD, large amounts of truncated proteins with potentially deleterious activities would be produced. Thus, it is advantageous for the cell to degrade aberrant transcripts as early as possible. To achieve this, mRNAs must be scanned for PTCs and degraded during the first few rounds of translation. Early evidence for this notion was provided by the discovery that most mammalian mRNAs are downregulated by NMD in the nuclear fraction of cells (71, 73, 112, 113). This suggested that mRNAs are scanned either in the nucleus or soon after entering the cytoplasm when the mRNAs are still associated with the nucleus (4, 17, 114).

More recent studies have provided additional evidence that NMD occurs on newly synthesized mammalian mRNAs. These studies revolve around the discovery of the cap-binding complex (CBC), a marker unique to newly synthesized mRNA. The CBC is a heterodimer of CBP20 and CBP80 subunits that is added to the 5′ cap of pre-mRNAs during transcription (115–118), whereupon it promotes pre-mRNA splicing (118). CBCs remain bound to mRNAs after they are spliced and begin translation, indicating that CBC-bound mature mRNAs are potential targets for NMD (119–122). A key discovery was that the CBC is at some point replaced by another 5′ cap-binding factor, eIF4E, a translation initiation factor responsible for supporting bulk translation (119, 123). Thus, by comparing the properties of CBC and eIF4E-bound mRNAs, it has been possible to dissect events that occur on newly synthesized mRNAs compared to older mRNAs undergoing bulk translation.

This type of comparison has generated several lines of evidence that NMD occurs on newly synthesized CBC-bound mRNAs. First, PTCs decrease the level of mRNAs bound by the large CBC subunit CBP80; in contrast, there is no further decrease in the level of eIF4E-bound mRNA, implying that NMD acts only on CBP80-bound mRNAs (119). Second, several NMD and EJC factors, including SMG-1, UPF2, UPF3a, UPF3b, and eIF4AIII, are present in mRNPs containing either CBP80 or CBC20 but not eIF4E (22, 62, 119, 121). Third, knockdown of CBP80 by RNAi partially reverses NMD (124). Fourth, CBP80-bound, PTC-containing mRNA accumulates when translation is blocked (62, 119). Finally, 4E-BP1, a translation inhibitor that specifically

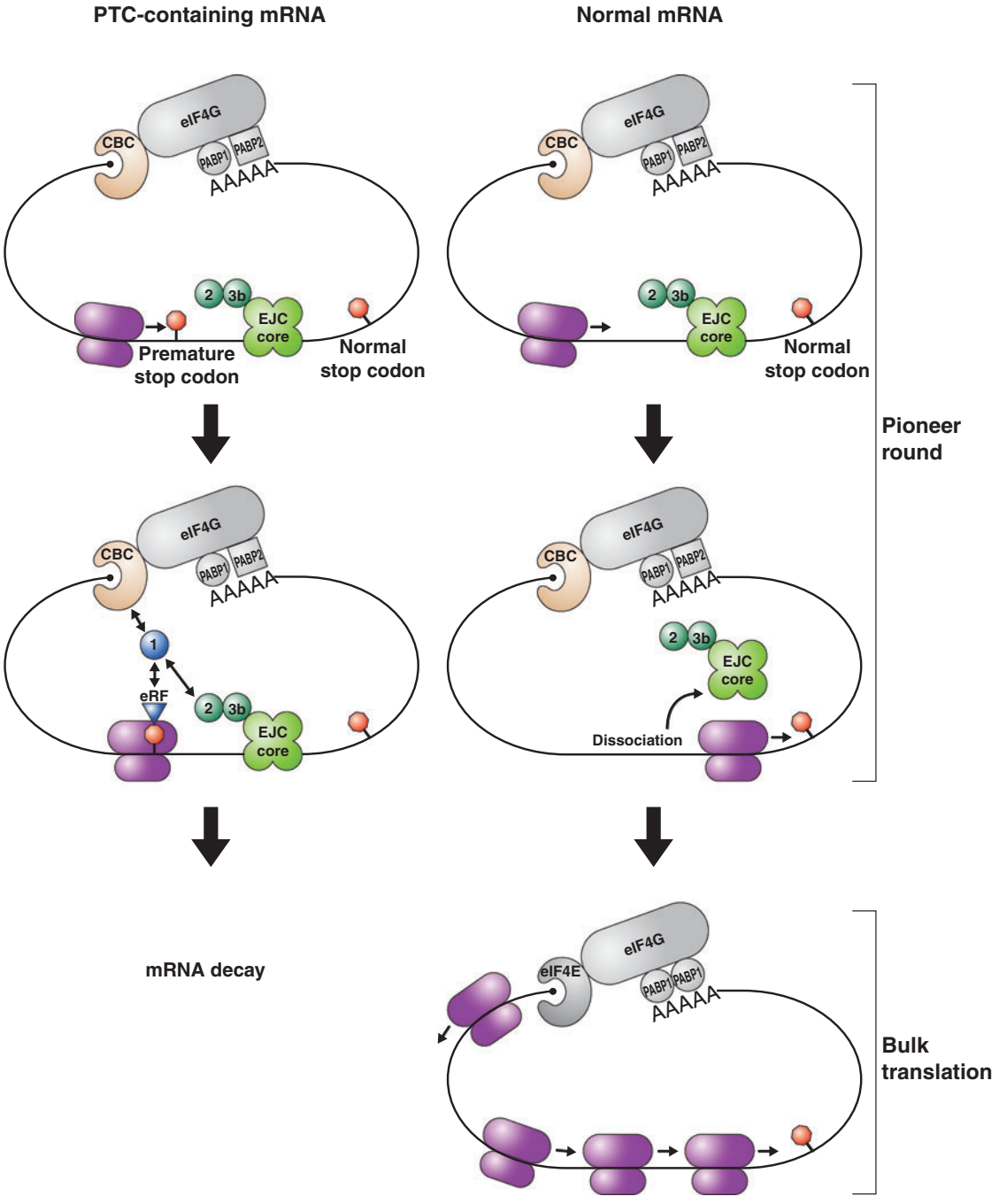
Pioneer round of translation: the first round of translation, which occurs on CBC-bound mRNPs and may function in proofreading mammalian mRNAs

CBC: cap-binding complex

Bulk translation: rounds of translation after the pioneer round that are devoted to making proteins; characterized by eIF4E-bound mRNPs

competes with eIF4E for binding to eIF4G but does not interact with the CBC (125), has no effect on NMD (121).

In addition to being a marker for NMD-targeted mRNAs, the CBC functions in NMD (**Figure 2**). Several lines of evidence suggest that its large subunit, CBP80, promotes NMD by recruiting UPF1 and enhancing the interaction between UPF1 and UPF2 (124). First, knockdown of CBP80 reduces the



magnitude of NMD elicited in response to UPF2 or UPF3a tethered downstream of a stop codon but has no effect on NMD elicited by tethered UPF1. Second, coimmunoprecipitation, far-Western, and pull-down assays demonstrated that CBP80 and UPF1 interact with each other, probably in a direct manner. Third, CBP80 enhances the binding of UPF1 to UPF2 in vitro. Conversely, knockdown of CBP80 reduces the interaction between UPF1 and UPF2 in vivo. Fourth, tethering CBP80 or CBP20 downstream of a stop codon reduces mRNA levels, consistent with the CBC recruiting UPF1 to elicit NMD.

In striking contrast to mammals, *S. cerevisiae* appears to activate NMD during any round of translation. One line of evidence for this is that *S. cerevisiae* NMD is very inefficient, implying that multiple rounds of translation (perhaps 200 or more) are required to degrade a significant fraction of the PTC-bearing transcripts (126). More direct evidence that NMD can degrade old aberrant yeast mRNAs comes from studies using a galactose-inducible promoter to rapidly express Upf1p, Upf2p, or Upf3p in yeast strains null for the corresponding *Upf* gene. These studies revealed that induction of either Upf1p, Upf2p, or Upf3p elicits decay of preexisting PTC-bearing mRNAs, implying that NMD does not degrade only newly synthesized PTC-bearing mRNAs (127). The idea that yeast does not have a unique pioneer round of translation devoted to NMD is further supported by the finding that *S. cerevisiae* strains lacking Cbc1p (the yeast ortholog of mammalian CBP80) are still capable of degrading PTC-bearing mRNAs; these mRNAs are bound to eIF4E (128).

It remains to be determined whether yeast and mammals truly differ with regard to what rounds of translation triggers NMD. It is possible, for example, that yeast NMD is more often triggered during early rather than later rounds of translation. Conversely, mammalian NMD may occur to some extent in later rounds. Consistent with this, the substrate for mammalian NMD does not absolutely require using a CBC-dependent mechanism because mRNAs containing internal ribosome entry sites can be degraded by NMD (129, 130).

Tethering: fusion of a protein of interest to a site-specific RNA-binding protein so that the protein binds a specific site in an mRNA

Molecular Interactions that Define Nonsense Codons

How are premature stop codons distinguished from bona fide stop codons? Several lines of investigation indicate that a second signal is required to define a stop codon as being premature and thereby trigger NMD (13, 16, 17, 69). While this second signal appears to vary in different species and possibly in different transcripts, a common feature is that it must be downstream of the stop codon to elicit NMD. The best-defined second signal on mammalian transcripts is the EJC (see the

Figure 2

Mammalian nonsense-mediated mRNA decay (NMD) likely occurs as a result of PTC recognition during a unique "pioneer" round of translation. NMD is specifically triggered when up-frameshift protein 1 (UPF1, labeled 1) is allowed to interact with UPF2 (2), which is bound to UPF3b (3b) (22). UPF2 and UPF3b are both part of the EJC (80), which is recruited to exon-exon junctions (not shown) during mRNA splicing (78). In an aberrant transcript (*left panels*), there is typically at least one EJC deposited downstream of the premature stop codon, allowing it to interact with UPF1 recruited by CBC and the eukaryotic release factors eRF1 and eRF3 (eRF) (22, 124). The interaction between UPF1 and UPF2 is enhanced by CBC (124), which is bound to the 5′ cap of mRNAs undergoing the pioneer round of translation (119). In contrast, a normal transcript (*right panels*) avoids NMD because all of the EJCs (only one is shown) are upstream of the stop codon and are thus displaced by the ribosome before UPF1 is recruited (96). After surviving this proofreading step, normal transcripts exchange proteins at both their 5′ and 3′ ends and proceed to bulk translation (119).

eRF: eukaryotic release factor

SURF: SMG-1-UPF1-eRF1-eRF3 complex

NMD and the Exon-Junction Complex section, above). Normal transcripts escape NMD because all of the EJCs are typically deposited upstream of the stop codon, permitting the ribosome to displace them. In contrast, an aberrant transcript harboring a stop codon in a premature position will typically have at least one EJC downstream of the PTC, thereby triggering NMD.

How does the second signal trigger NMD? The available evidence suggests that mRNA decay is elicited if the factors that constitute the second signal (such as the EJC) are allowed to interact with factors bound at stop codons. This does not occur on normal transcripts because the second signal factors are probably all stripped off by the ribosome. In this section, we explore what is known about the interaction of stop codon-bound factors with second-signal factors in mammalian cells. It should be borne in mind that the interaction between these two sets of factors does not necessarily cause destabilization of an inherently stable mRNA. Rather, it is possible that this interaction interferes with normal translation termination and/or normal remodeling of the mRNP that is required for its stabilization (69, 70, 131).

Translation termination is triggered by recognition of the stop codon by the eukaryotic release factors eRF1 and eRF3. UPF1 is probably recruited to the scene soon thereafter, as both eRF1 and eRF3 have been shown to interact with UPF1 (21, 35, 36). Using coimmunoprecipitation analysis, a recent study showed that UPF1, eRF1, and eRF3 are part of a single complex that also contains the UPF1 kinase SMG-1 (22). This SMG-1, UPF1, eRF (SURF) complex is proposed to assemble on ribosomes stalled at a stop codon, with eRF1 and eRF3 recruiting unphosphorylated UPF1, which in turn recruits SMG-1 (**Figure 3**).

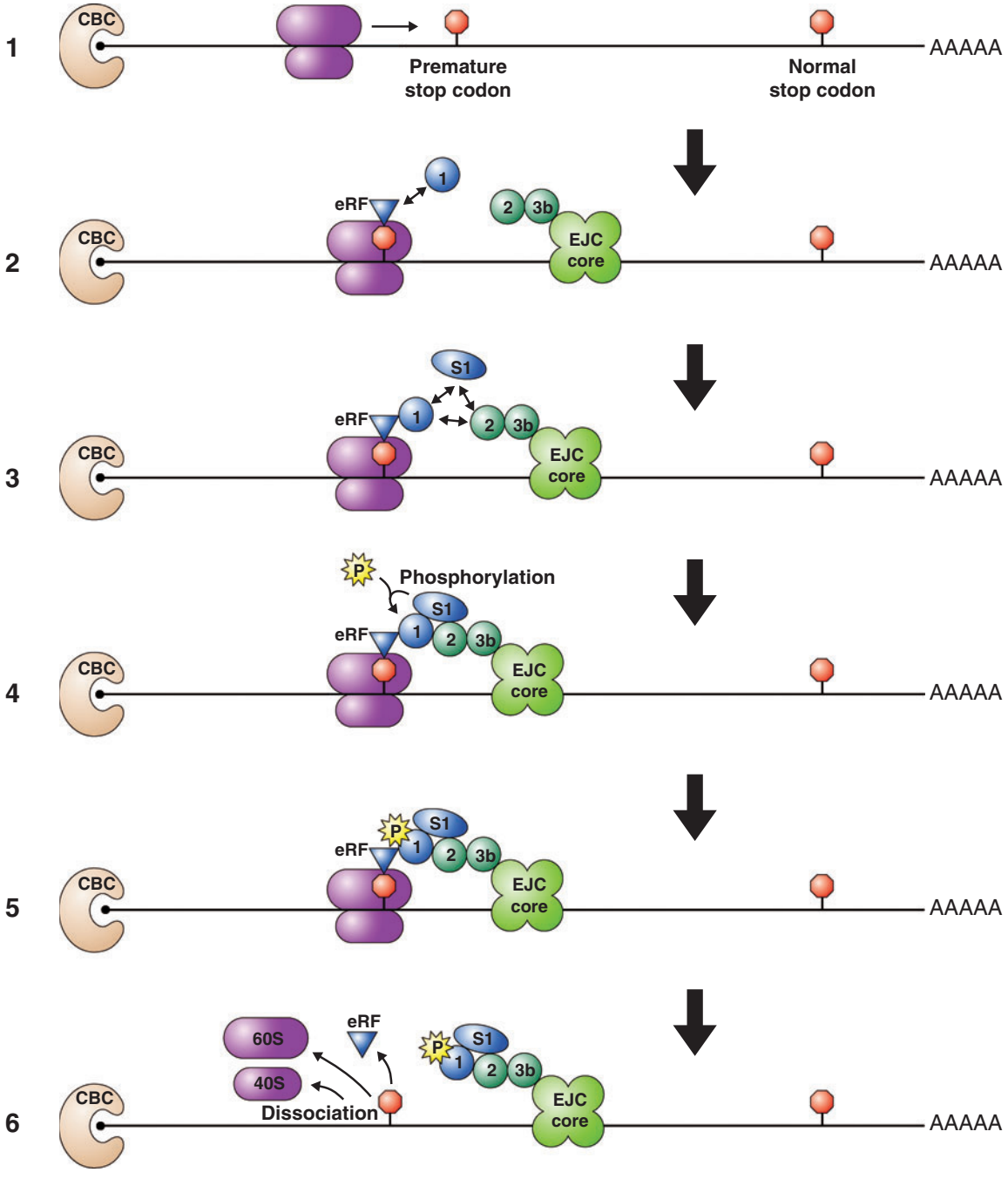
SURF is probably a transient complex formed after translation termination because evidence suggests it rapidly forms a larger complex that also contains the EJC components UPF3b, eIF4AIII, and Y14/MAGOH (22). The interaction between UPF1 in the SURF complex and UPF2 in the EJC acts as a molecular bridge that brings together these two complexes (**Figure 3**). As evidence for this, SURF and EJC interactions are disrupted by RNAi-mediated depletion of UPF2 or mutation of UPF2's UPF3b-binding site. This interaction is probably further enhanced by SMG-1, which uses two independent domains to bind UPF1 and UPF2 simultaneously. Evidence for a precursor-product relationship between the SURF complex and the putative SURF/EJC "super" complex is that the former accumulates when the EJC components Y14 or UPF2 are depleted by RNAi or when interactions between UPF1 in the SURF complex and UPF2 in the EJC are blocked by mutations (22).

The formation of the SURF/EJC complex probably activates SMG-1 to phosphorylate UPF1. As evidence, UPF1 phosphorylation is reduced by mutations that disrupt the

Figure 3

Early molecular events preparing an mRNA to be degraded by nonsense-mediated mRNA decay (NMD). Translation of an mRNA during the pioneer round of translation (step 1; also see **Figure 2**) leads to recognition of the stop codon by the eukaryotic release factors eRF1 and eRF3, which recruit the NMD factor UPF1 (labeled 1; step 2) (22, 124). UPF1, in turn, recruits the protein kinase SMG-1 (S1), which together with the eRFs forms a transient complex called SURF (step 3) (22). In an aberrant mRNA like the one shown, the SURF complex interacts with an EJC downstream (step 4). This interaction may be an obligate requirement for SMG-1 to phosphorylate UPF1 (step 5), which then probably triggers subsequent steps that ultimately degrade the mRNA (see **Figure 4**) and recycles release factors and the 40S and 60S ribosomal subunits (step 6). Abbreviations: CBC, cap-binding complex; EJC, exon-junction complex; S1, protein kinase SMG-1; SMG-1, suppressor with morphogenetic effect on genitalia-1; SURF complex, the SMG-1, UPF1, eRF complex; UPF1 (labeled 1), UPF2 (labeled 2), UPF3b (labeled 3b), up-frameshift proteins.

interaction between UPF1 and UPF2 or between UPF2 and UPF3b (22). Furthermore, RNAi-mediated depletion of EJC factors in mammalian cells also reduces UPF1 phosphorylation (22). Similarly, null mutations of the *C. elegans SMG-3* (*UPF2*) and *SMG-4* (*UPF3*) genes reduce SMG-2 (UPF1) phosphorylation (24, 49).



Polysomes: multiple ribosomes translating a single mRNA

Exoribonucleases: enzymes that degrade RNA from the exposed ends; some degrade 5′-to-3′ and others 3′-to-5′

Although these studies have begun to reveal the molecular mechanisms responsible for UPF1 phosphorylation, the function of UPF1 phosphorylation in NMD remains a mystery. One attractive possibility is that UPF1 must be phosphorylated to attract new molecules, such as SMG-7, that ultimately bring about the rapid destruction of the mRNA (see the next section). UPF1 phosphorylation may also drive the dissociation of eRF1 and eRF3 from the mRNP to allow for their recycling (**Figure 2**). In support of this, expression of a kinase-deficient SMG-1 mutant increases the level of eRF3 coimmunoprecipitated with UPF1 (22).

Several lines of evidence support the SURF/EJC model described above (22), but contradictory data also exist. One issue concerns the key role played by UPF2 in mediating an interaction between the SURF and the EJC. This is apparently at odds with the fact that UPF3b lacking its UPF2-interaction domain is still capable of eliciting NMD when tethered downstream of a stop codon (132). Although UPF2 independence could be attributed to the artificial nature of the λN/MS2 tethering system, knockdown of endogenous UPF2 by RNAi also has no effect on some NMD substrates (57). This suggests that NMD can be UPF2 independent, and in these cases, some alternative molecule links the SURF complex with the EJC. Similarly, RNAi-mediated knockdown experiments suggest that a UPF3b-independent branch of the NMD pathway exists (see the Alternative Branches of the NMD Pathway section, below) (59), which would require a substitute factor to link UPF2 and Y14 (**Figure 3**). Another unresolved issue concerns the finding that most phosphorylated UPF1 is found in the polysome fraction (133). The SURF/EJC model predicts that this would not be the case because the vast majority of polysomes would be expected to contain normal mRNAs that do not form SURF/EJC complexes and would thus have UPF1 in a nonphosphorylated state.

UPF1 Dephosphorylation, P-Body Recruitment, and mRNA Decay

Once UPF1 is phosphorylated, it recruits factors that mediate its dephosphorylation (**Figure 4**). Among these factors is SMG-7, which together with SMG-5 forms a heterodimer that directly binds phosphorylated UPF1 (39, 67). SMG-7 has been proposed to be the terminal effector in NMD, as tethering SMG-7 to any site in an mRNA (using the λN/BoxB system) rapidly destabilizes the mRNA (66). Tethering SMG-7's C terminus alone is sufficient to mediate mRNA decay. In contrast, all other NMD factors that have been tested destabilize mRNAs only when tethered downstream of the stop codon. Tethered SMG-5, although not able to elicit RNA decay when only endogenous SMG-7 is present, is able to trigger NMD when SMG-7 is overexpressed (66). In contrast, SMG-6 does not cooperate with SMG-7 but may have a role just upstream of SMG-5/SMG-7 (66).

The ribonucleases that carry out the actual destruction of most PTC-bearing mRNAs are the same as those that degrade most normal mRNAs. Both the decapping-dependent 5′-to-3′ exoribonuclease pathway and the exosome-mediated 3′-to-5′exoribonuclease pathway rapidly degrade mammalian mRNAs harboring PTCs (134–136). Similarly, *S. cerevisiae* uses both pathways, although the prominent one appears to be the 5′-to-3′ exoribonuclease pathway (137–139). In contrast, *D. melanogaster* uses a unique pathway involving endonucleolytic cleavage near the site of the PTC (140). Mammalian β-globin transcripts harboring PTCs are also degraded by an endonuclease-initiated mechanism in erythroleukemia cells (141, 142).

A controversial issue has been where the decay of PTC-bearing mRNAs occurs. Many mammalian mRNAs are downregulated by PTCs in the nuclear fraction (71, 73, 112, 113). This is surprising, given that translation has only been clearly demonstrated to occur in the cytoplasm. To date, it has not been resolved whether this

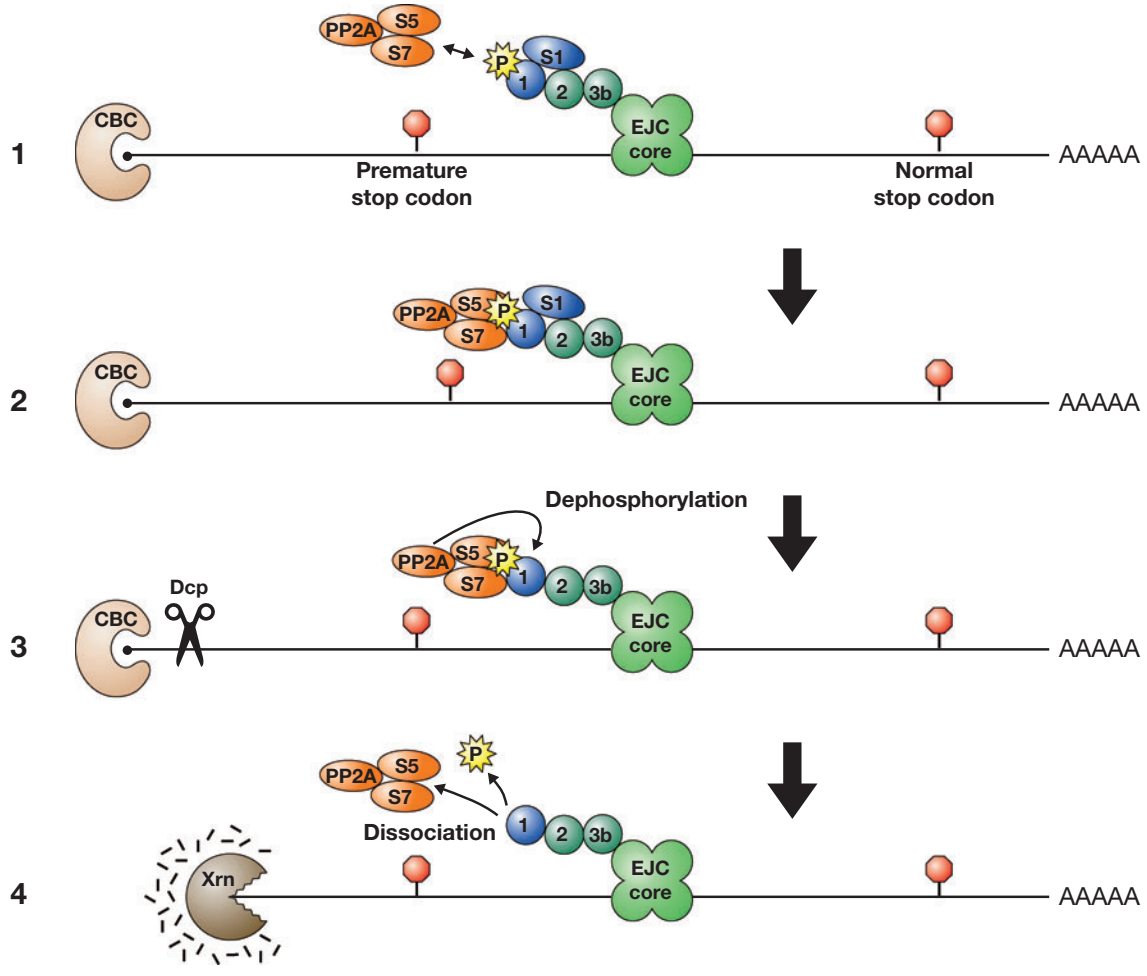


Figure 4

Late molecular events in nonsense-mediated mRNA decay (NMD) that ultimately degrade the mRNA. The phosphorylation of UPF1 recruits the SMG-5/SMG-7 heterodimers (labeled S5 and S7, respectively) and the phosphatase PP2A (steps 1 and 2) (39). It is uncertain whether PP2A is initially associated with SMG-5/SMG-7 (as shown) or recruited at a later stage. Unknown events (perhaps P-body recruitment) trigger PP2A to dephosphorylate UPF1 (step 3) (39), which is likely to lead to the dissociation of PP2A and SMG-5/SMG-7 from the mRNP (step 4). Also unknown is what specific event leads to loss of the mRNA 5′ cap by decapping enzymes (Dcp, step 3). Decapping may be triggered by the recruitment of SMG-7, which is sufficient to elicit mRNA decay when artificially tethered to an mRNA (see text and Reference 66). Alternatively, decapping may be triggered as a result of UPF1 dephosphorylation. Regardless, once decapping occurs, the mRNA body is susceptible to rapid decay by 5′-to-3′ exonucleases (Xrn, step 4) (135). Abbreviations: CBC, cap-binding complex; EJC, exon-junction complex; S1, protein kinase SMG-1; UPF1 (labeled 1), UPF2 (labeled 2), UPF3b (labeled 3b), up-frameshift proteins.

"nuclear-associated NMD" actually occurs in the nucleus or instead occurs in a portion of the cytoplasm that cofractionates with the nucleus. The former possibility is supported by the evidence that a fraction of translation occurs in the nucleus (114, 143), whereas the latter is indirectly supported by evidence against nuclear translation (144, 145). It is also

possible that certain mammalian transcripts are targeted for decay in the nucleus and that others are degraded in the cytoplasm, an idea consistent with the fact that mammalian NMD substrates can be sorted into two categories: those degraded in the cytoplasm and those degraded in the nuclear fraction (4, 17, 114).

One likely cytoplasmic site for mammalian NMD is the P-body, a dynamic cytoplasmic compartment that harbors high concentrations of RNA decay factors, including decapping enzymes and 5′-to-3′ exonucleases (146). Several lines of evidence indicate that the P-body is a major site of NMD in *S. cerevisiae* (147, 148). NMD might also occur in mammalian P-bodies because overexpressed SMG-7 concentrates in cytoplasmic foci with the characteristics of P-bodies (66). The C terminus of SMG-7 is likely to be responsible both for its P-body localization and for eliciting mRNA decay when tethered (66). Further support for the idea that SMG-7 participates in NMD in P-bodies is the finding that overexpressed SMG-7 recruits UPF1 into P-bodies. UPF1's P-body localization is only achieved with full-length SMG-7, not with its C-terminal region alone (66), consistent with the fact that SMG-7's N-terminal region houses its UPF1-binding domain (67). These results lead to a model in which SMG-7 is a key molecule that links upstream and downstream events in NMD by using its N terminus to interact with phosphorylated UPF1 and its C terminus to bring about the degradation of the mRNA. Selective destruction of PTC-bearing mRNAs is achieved because only such mRNAs form the SURF/EJC complexes essential to generate phosphorylated UPF1 (**Figure 3**), the binding surface that recruits SMG-7 (**Figure 4**).

A caveat with the interpretation of these experiments is that it is not known whether endogenous SMG-7 is localized in P-bodies because its expression is so low that its subcellular expression has not yet been determined (39, 149). Also, it is important to bear in mind that SMG-7 traffics to other locations, including the nucleus, so its activities are probably not restricted to the P-body (39, 66). In conclusion, although the penultimate (SMG-7 activation and UPF1 dephosphorylation) and terminal (mRNA decay) phases of NMD are becoming better understood, the cellular locations of these two NMD phases and how they are interconnected remain to be clearly defined.

ALTERNATIVE BRANCHES OF THE NMD PATHWAY

Early genetic and biochemical studies suggested the existence of only a single linear NMD pathway; however, more recent experiments suggest the existence of three distinct branches of the NMD pathway in mammals. The first branch is apparently independent of UPF2, as it retains normal functions when UPF2 is depleted by RNAi (57). This UPF2-independent branch requires the EJC factor UPF3b and the EJC core proteins. In contrast, the second branch is apparently independent of the EJC core proteins and instead depends on the RNA-binding EJC proteins RNPS1 and UPF2 (57). The third branch is not affected by the combined depletion of UPF3b and UPF3a and thus appears to be independent of these EJC factors (59).

Evidence for the existence of the UPF2-dependent and UPF2-independent branches came from a combination of loss- and gain-of-function approaches involving RNAi and the tethering assay described in the UPF Proteins: The Core NMD Machinery section, above. The primary evidence for the UPF2-independent pathway was the discovery that NMD elicited by tethered EJC core proteins or UPF3b is not affected by RNAi-mediated depletion of UPF2 (57). This was consistent with the finding that tethered UPF3b lacking its UPF2-interaction domain is still capable of eliciting NMD (132). It is also consistent with the finding that the related protein UPF3a is in a high-molecular-weight complex that lacks UPF2 (39, 150).

The UPF2-dependent pathway was supported by the finding that tethered RNPS1 requires its UPF2-interaction domain to elicit NMD (57). Furthermore, RNPS1-induced NMD is abrogated by depletion of UPF2. In contrast, RNPS1-induced NMD is not affected by knockdown of the EJC core components eIF4AIII and MLN51, suggesting that this branch is independent of the EJC core. RNPS1 and UPF2 may make do without the EJC core by binding to the mRNA substrate on their own. RNPS1 is an RNA-binding protein with a canonical RRM domain, and UPF2 binds RNA in vitro using a novel domain (56, 151).

The primary evidence for the UPF3-independent pathway was the discovery that RNAi-mediated depletion of UPF3a, UPF3b, or both has no effect on the downregulation of TCR-β transcripts in response to PTCs (59). In contrast, depletion of UPF3b reverses PTC-mediated downregulation of standard NMD substrates, including β-globin mRNA. Because TCR-β NMD is known to require both UPF2 (42, 129) and EJC core proteins (91, 100, 152), this suggests that TCR-β transcripts are not degraded by the UPF2-dependent or UPF2-independent pathways described above but instead by a unique third branch of the NMD pathway. TCR-β NMD is also unique in many other respects: It is an unusually robust response (153), it requires efficient splicing (88), it is elicited in a unique polar manner (5′ PTCs elicit much stronger downregulation than do 3′ PTCs), and it does not abide by the –55 boundary rule dictated by the classical EJC (73, 154).

All three pathways downregulate some naturally occurring transcripts. RNAi depletion studies identified unique subsets of human transcripts with NMD features regulated by the UPF2-dependent pathway (downregulated by UPF2 and RNPS1 but not MLN51), the UPF2-independent pathway (downregulated by MLN51 but not UPF2), and the UPF3-independent pathway (not downregulated by UPF3b). Transcripts in all three pathways are downregulated by UPF1, suggesting that although several different combinations of EJC proteins are employed as "input branches" for NMD, these are all funneled together into a common UPF1-dependent tributary that elicits mRNA decay.

RSV: Rous sarcoma virus

NOVEL NMD SECOND SIGNALS

The EJC is not universally required for NMD. Yeast lack most EJC components (155) and are thus incapable of depositing a classical EJC on spliced mRNAs. Flies have all the major EJC components, but RNAi-mediated depletion of these EJC components has no effect on NMD in *D. melanogaster* cell lines, and NMD occurs efficiently even when no exon-exon junction is downstream of the PTC (55, 156). Thus, the EJC appears to have evolved for other purposes before it was co-opted for use in NMD in mammals (11, 81).

The fact that lower eukaryotes execute NMD without the EJC raises the possibility that higher eukaryotes may also, under some circumstances, engage in NMD independent of the EJC. In support of this, some transcripts have been identified that are degraded by NMD in mammalian cells even when the PTC is not followed by an exon-exon junction. Human β-hexosaminidase, mouse Ig-μ, Rous sarcoma virus (RSV), and hybrid mouse/human β-globin are all examples of transcripts that do not require an intron downstream of the PTC to be degraded by NMD (157–160).

Mapping experiments have begun to assign the transcript regions responsible for these apparent examples of EJC-independent NMD. Deletion analysis in β-globin revealed a region in the second exon that is likely to be responsible for promoting NMD (157). The location of this NMD-promoting region relative to the PTC makes it reminiscent of *S. cerevisiae* downstream sequence elements that elicit NMD (155), but there is no obvious sequence identity between the β-globin region and the yeast downstream sequence element consensus sequence (157). A curious aspect of the regulation is that only the

hybrid mouse/human version of β-globin, and not normal human β-globin, displays intron-independent NMD (161). In contrast to β-globin, RSV and Ig-μ transcripts have NMD-regulatory regions that map to the 3′ UTR. The RSV 3′ UTR houses a putative NMD-inhibitory *cis* element that stabilizes RSV transcripts when introduced downstream of the PTC (162). Nonmutant RSV transcripts also depend on this 3′ UTR element for stability (162). It remains for future studies to identify a minimal *cis* element in the RSV 3′ UTR and the *trans*-acting factors recruited to it. Ig-μ transcripts appear to require a minimal 3′ UTR length, rather than a particular 3′ UTR sequence, to elicit EJC-independent NMD. A deletion that reduced the distance between the PTC and the poly(A) tail abolished Ig-μ NMD, whereas insertion of a stuffer downstream of the normal Ig-μ stop codon triggered NMD (160). The notion that the execution of NMD is decided by the distance between the stop codon and the poly(A) tail is an attractive one, especially given that evidence suggests this may be the case in yeast (163, 164). However, a simple inverse relationship between 3′ UTR length and NMD induction is clearly not the case because mammalian transcripts with long 3′ UTRs are not necessarily targeted by NMD (161).

Although the above examples suggest the existence of an alternative non-EJC second signal for NMD, it is important to note that there is no direct evidence for EJC independence other than a study in which Ig-μ NMD was shown to be unaffected by knockdown of the EJC component eIF4AIII (160). It is possible, for example, that the deletion of the β-globin intron downstream of the PTC generated a cryptic intron or splice site that is capable of recruiting the EJC. It is also possible that EJCs can be deposited on exons in a splicing-independent manner. In summary, even though progress has been made in the identification of regulatory regions required for intron-independent NMD, the experiments to date have not definitively determined whether it is truly EJC independent, and they have not yet yielded a simple or a universal mechanism.

SUMMARY POINTS

1. NMD is an evolutionarily conserved RNA surveillance pathway that targets mRNAs harboring PTCs for decay.
2. The UPF1, UPF2, and UPF3 proteins are core factors participating in NMD. UPF1 is an RNA helicase that is recruited to mRNAs upon translation termination and undergoes a cycle of phosphorylation and dephosphorylation.
3. In mammalian cells, UPF2 and UPF3 are part of the EJC, a large dynamic protein complex deposited just upstream of exon-exon junctions during RNA splicing. Many EJC components, including UPF2 and UPF3, remain bound to the mRNA after its export to the cytoplasm, where they function as a second signal to elicit NMD when the mRNA is proofread during translation.
4. The EJC contains four core proteins that form a stable tetramer that binds to mRNA. All EJC core proteins are involved in NMD.
5. Mammalian NMD appears to occur as a result of PTC recognition during an early (pioneer) round of translation. The substrate for this proofreading round of translation has a different mRNP composition than subsequent (bulk) rounds of translation that generate large amounts of protein.

6. Upon translation termination, release factors recruit UPF1 and the UPF1 kinase SMG-1 to form the SURF complex. SURF is a transient complex that rapidly interacts with the EJC through the EJC component UPF2. This SURF-EJC interaction defines the stop codon as premature and probably promotes the phosphorylation of UPF1, a likely key step in NMD.
7. Once phosphorylated, UPF1 attracts the phosphoserine-binding domain proteins SMG-5, SMG-6, and SMG-7. These three proteins are each essential for NMD; together, they promote the dephosphorylation of UPF1. SMG-7 is thought to be the terminal effector of NMD because it accumulates in P-bodies (cytoplasmic sites of mRNA decay) and is unique among NMD proteins in that it elicits rapid decay when tethered to any position within an mRNA.
8. Recent studies suggest that rather than being a single linear pathway, mammalian NMD has several branches, some of which may use alternative EJCs and others that may operate independent of EJCs.

FUTURE ISSUES

1. How does UPF1 function in NMD? Although UPF1 has been shown to have ATP-dependent helicase and RNA-dependent ATPase activities, their role in NMD is not clear. UPF1 undergoes a cycle of phosphorylation and dephosphorylation, but the functional role of this cycle is still poorly understood.
2. What is the molecular switch responsible for activating the kinase activity of SMG-1? Its substrate, UPF1, is phosphorylated when the SMG-1-containing SURF complex interacts with the EJC, but the molecular choreography responsible for SMG-1 activation is not known.
3. Is the P-body a major site of NMD in mammalian cells?
4. How does SMG-7 trigger mRNA decay? Is its ability to promote UPF1 dephosphorylation integral to its function in NMD? Does it directly or indirectly recruit RNA decay-associated factors, including decapping enzymes? Does it direct mRNAs into P-bodies?
5. What is the role of SMG-6 in NMD? It interacts with the PP2A phosphatase and phosphorylated UPF1 and is essential for NMD in *C. elegans*, but its precise functional role in NMD remains obscure.
6. What role do EJCs have in NMD? Are they obligate signals for some branches of the NMD pathway? Or are they merely NMD amplifiers? This is a difficult question to address for technical reasons because RNAi-mediated knockdown of EJC components is never complete. In addition, it may not be feasible to completely knock out EJC components, as this is probably lethal to mammalian cells.
7. Conversely, do EJC-independent branches of NMD exist in mammals? If so, do they use molecules and molecular steps orthologous to those used by EJC-independent NMD mechanisms in lower eukaryotes? This is an intriguing possibility because it

suggests that the EJC-independent signal drives the primordial NMD mechanism and that the EJC was co-opted later in evolution for use in NMD.

8. How do the putative alternative branches of the NMD pathway function? For example, how do the UPF2- and UPF3-independent branches operate, given that both UPF2 and UPF3 are integral components of the SURF/EJC-containing "super complex" thought to be essential to elicit NMD? How does the UPF2/RNPS1-dependent pathway operate in the absence of some or all of the core EJC components?

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