Caenorhabditis elegans SMG-2 Selectively Marks mRNAs Containing Premature Translation Termination Codons^{▽†}

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Eukaryotic mRNAs containing premature translation termination codons (PTCs) are rapidly degraded by a process termed “nonsense-mediated mRNA decay” (NMD). We examined protein-protein and protein-RNA interactions among *Caenorhabditis elegans* proteins required for NMD. SMG-2, SMG-3, and SMG-4 are orthologs of yeast (*Saccharomyces cerevisiae*) and mammalian Upf1, Upf2, and Upf3, respectively. A combination of immunoprecipitation and yeast two-hybrid experiments indicated that SMG-2 interacts with SMG-3, SMG-3 interacts with SMG-4, and SMG-2 interacts indirectly with SMG-4 via shared interactions with SMG-3. Such interactions are similar to those observed in yeast and mammalian cells. SMG-2–SMG-3–SMG-4 interactions require neither SMG-2 phosphorylation, which is abolished in *smg-1* mutants, nor SMG-2 dephosphorylation, which is reduced or eliminated in *smg-5* mutants. SMG-2 preferentially associates with PTC-containing mRNAs. We monitored the association of SMG-2, SMG-3, and SMG-4 with mRNAs of five endogenous genes whose mRNAs are alternatively spliced to either contain or not contain PTCs. SMG-2 associates with both PTC-free and PTC-containing mRNPs, but it strongly and preferentially associates with (“marks”) those containing PTCs. SMG-2 marking of PTC-mRNPs is enhanced by SMG-3 and SMG-4, but SMG-3 and SMG-4 are not detectably associated with the same mRNPs. Neither SMG-2 phosphorylation nor dephosphorylation is required for selective association of SMG-2 with PTC-containing mRNPs, indicating that SMG-2 is phosphorylated only after premature terminations have been discriminated from normal terminations. We discuss these observations with regard to the functions of SMG-2 and its phosphorylation during NMD.

Eukaryotic mRNAs containing premature translation termination codons (PTCs) are rapidly and selectively degraded by a conserved system termed “nonsense-mediated mRNA decay” (NMD). NMD is thought to improve the fidelity of gene expression by eliminating mRNAs that encode truncated proteins, which are potentially deleterious (14, 27). NMD influences expression of a wide variety of mRNAs in wild-type cells, including, for example, unspliced or unproductively spliced mRNAs, mRNAs that contain upstream open reading frames or engage in “leaky scanning,” mRNAs having introns within their 3′ untranslated regions, mRNAs of unproductively rearranged genes, and many others (reviewed in references 1, 21, 39, 40, 56, and 61). Indeed, NMD affects expression of many genes and plays a role in numerous biological processes. NMD is not essential for viability in yeasts and nematodes, but NMD components are essential for viability in mice (44), human cells (6), *Drosophila* (48, 56), and *Arabidopsis* (4, 65).

Genes required for NMD have been identified in many eukaryotes. Three genes (*UPF1*, *UPF2*, and *UPF3*) discovered in yeast (*Saccharomyces cerevisiae*) are evident in the genomes of sequenced eukaryotes (reviewed in references 17 and 18) and constitute a core machinery of NMD. Upf1 is the central regulator of NMD. Premature translation termination activates Upf1, which in turn activates decapping of yeast and mammalian nonsense mutant mRNAs. Upf1 interacts directly or indirectly with other important regulators of NMD (reviewed in reference 9). Yeast Upf1p interacts with Upf2p, which in turn interacts with Upf3p, and these interactions are conserved in *Drosophila* and humans (31, 42). Mammalian pathways of mRNA turnover that require Upf1 but not Upf2 or Upf3 have been defined (24, 33, 35), but it appears that most mammalian NMD requires all three components of the core machinery. Upf3 shuttles through the nucleus and is exported to the cytoplasm as part of mRNP particles (5, 16, 42, 46, 57, 58). Additional components of the surveillance complex then join the mRNPs in the cytoplasm.

The context of translation termination determines whether NMD will occur. Abnormal 3′ untranslated regions (2, 51) and/or “downstream elements” of yeast (60, 66) elicit NMD by accelerating decapping of nonsense mRNAs (8). The distance between the termination event and the poly(A) tail can be an important determinant during NMD, with termination events occurring close to the poly(A) tail being less sensitive to NMD (2, 10, 13). The exon junction complex (EJC) of mammals greatly enhances NMD, although it may not be absolutely required (13). Human Upf3 (hUpf3) associates with mRNAs

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as part of the EJC (25, 34, 37) and likely recruits hUpf2 to the mRNP. hUpf1 associates with nuclear cap binding complex (30), with translation release factors (19, 32), and with the EJC-associated complex to activate NMD (32). Elements that signal the context of *Caenorhabditis elegans* termination have not been described in detail, but like *Drosophila* (23), downstream introns are not required for *C. elegans* NMD (41, 55).

Upf1 of metazoa undergoes cycles of phosphorylation and dephosphorylation that are required for NMD (53; reviewed in reference 63). Phosphorylation of *C. elegans* SMG-2, the ortholog of Upf1, requires SMG-1, SMG-3, and SMG-4 (53). SMG-1 is the SMG-2 kinase (20, 26, 64), while SMG-3 and SMG-4 are the *C. elegans* orthologs of Upf2 and Upf3, respectively (5 and see below). Three additional proteins (SMG-5, SMG-6, and SMG-7) are required for efficient SMG-2 dephosphorylation (16, 32, 52, 53). SMG-5 may direct protein phosphatase 2A to its SMG-2 substrate via shared interactions with SMG-2 and protein phosphatase 2A (3), but the functions of SMG-6 and SMG-7 are less well understood.

Both phosphorylation and dephosphorylation of Upf1 are required for NMD, but the precise functions of these modifications are uncertain. Phosphorylation of hUpf1 is enriched in polysomal fractions (54), requires hUpf2 (62), and occurs following association of hUpf1 with the EJC (32). Phosphorylated hUpf1 forms distinct complexes with differing isoforms of hUpf3 (52), suggesting that hUpf1 phosphorylation modulates its interactions in the surveillance complex, possibly by destabilizing interactions between Upf1 and translation release factors (32). Phosphorylated hUpf1 binds hSMG-7 (22) and may target bound mRNAs for decay (59).

We investigated protein-protein and protein-RNA interactions between endogenous *C. elegans* SMG-2, SMG-3, and SMG-4 and mRNA targets of NMD. We describe those interactions, examine their dependencies on SMG-2 phosphorylation and dephosphorylation, and discuss potential functions of SMG-2 phosphorylation during NMD.

MATERIALS AND METHODS

Strains and genetic methods. Genetic methods for *C. elegans* were performed as described by Brenner (11). The alleles used in this study were as follows: wild-type strain N2, *smg-1(r904)*, *smg-2(r908)*, *smg-3(r930)*, *smg-3(r929)*, *smg-4(r1169)*, *smg-5(r860)*, *smg-6(r1217)*, *smg-7(r1197)*, and *unc-54(r293)*. All *smg*(-) alleles used in this paper are known from this or previous work to express none of the encoded protein (3, 5, 15, 26, 53; H. Shang and P. Anderson, unpublished observations).

Transformation rescue of *smg-3*. Motile *smg-3(r930) unc-54(r293)* hermaphrodites were coinjected with DNA to be tested and marker plasmid pRF4[*rol-6(su1006)*] by established methods (45). Independent heritable transformants were established and subsequently scored for motility. Animals rescued for their *smg-3* phenotype exhibited the flaccid paralysis characteristic of *unc-54(r293) smg-3(+)* strains. To confirm that the transforming DNA was supplying *smg-3(+)* activity and not directly causing a dominant Unc phenotype, rescued animals were crossed with wild-type males. Array-containing cross-progeny exhibited normal motility. Genomic rescue was achieved both by injection of YAC Y73B6 and by injection of two overlapping fragments spanning the *smg-3* genomic region, according to established methods (43). cDNA expression construct TR#393 was constructed by inserting the *smg-3* cDNA into the expression vector pPD96.52 (45).

Antibodies. An SMG-3 antiserum was generated by immunizing rabbits with recombinant glutathione *S*-transferase (GST)-tagged SMG-3 (GST-SMG-3 [amino acids 208 to 415]), which was purified using glutathione-Sepharose 4B columns (pGEX4T system; Amersham Biosciences). Anti-SMG-3 antibodies were affinity purified by coupling GST-SMG-3 to Actigel ALD (Sterogene) and passing serum over the GST-SMG-3-conjugated resin. Anti-SMG-3 antibodies

were eluted with ActiSep (Sterogene), desalted, and concentrated. Anti-SMG-4 antibodies were generated essentially as described above, except that a full-length SMG-4-GST fusion protein was used to immunize rabbits. Anti-SMG-2 antibodies were previously described (53). The anti-DYN-1 antibody is an affinity-purified peptide rabbit antibody that was a gift from Ahna Skop. The anti-phospho(Ser/Thr)Gln antibody was purchased from Cell Signaling Technology. The antirabbit secondary antibody was purchased from Amersham Biosciences, the mouse anti-actin antibody was purchased from MP Biomedicals, and the antimouse secondary antibody was purchased from BD Biosciences.

Western blotting. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to Immobilon-P membranes (Millipore) using a Trans-Blot semidry transfer cell (Bio-Rad). Western blotting was performed using standard procedures (28) and the ECL enhanced chemiluminescence detection system (Amersham Biosciences).

IP. Mixed-stage animals were suspended in immunoprecipitation (IP) buffer (20 mM MOPS [morpholinepropanesulfonic acid, pH 7.2], 100 mM NaCl, 0.01% NP-40, a cocktail of protease inhibitors [Sigma], pepstatin A [Sigma], and phenylmethylsulfonyl fluoride [Fluka]), sonicated with five 1-s pulses, and centrifuged at $14,000 \times g$ for 25 min, retaining the supernatant fraction. The Bio-Rad protein assay was used to measure the concentration of the extracts. Extracts were diluted to 1 mg/ml total protein with the IP buffer and protease inhibitors. Extracts were incubated with affinity-purified antibody followed by addition of protein A-Sepharose (Amersham Biosciences). Precipitated proteins were collected by centrifugation and washed three to five times with the IP buffer. RNase-treated extracts were incubated with 1 mg/ml RNase A for 1 h on ice prior to IP. This treatment and an otherwise identical treatment with 100 μ g/ml RNase A were sufficient to degrade rRNA to completion, as judged by the disappearance of rRNA bands on an ethidium bromide-stained agarose gel.

RT-PCR. RNA was extracted from IP pellets and inputs using TRIzol (Invitrogen). First-strand synthesis was performed using random hexamers and SuperScript III reverse transcriptase (RT) (Invitrogen). *rpl-12*, *rpl-10a*, *rpl-7a*, *rsp-2*, and *rsp-4* were amplified using primers on either side of the alternatively spliced intron (49, 50; B. Schmidt and P. Anderson, unpublished observations). Products were separated on 1.5% ethidium bromide-stained agarose gel and quantified using ImageQuant software (GE Healthcare, Inc.).

RESULTS

SMG-3 and Upf2 are orthologs. We identified transcript Y73B6BL.18 as *smg-3* based on the following four lines of evidence. (i) Y73B6BL.18 is similar to Upf2p of yeast and its orthologs in other species (reviewed in reference 17). (ii) We established that a transgene containing a genomic fragment of Y73B6BL.18 fully rescues the NMD defect of *smg-3(r930)*. (iii) We assembled a full-length cDNA clone of Y73B6BL.18 (GenBank accession no. EF472000). The cDNA is trans-spliced to SL2 at its 5' end, and its expression fully rescues the NMD defect of *smg-3(r930)* (see Materials and Methods). (iv) We sequenced two *smg-3* alleles, and they both contain mutations of Y73B6BL.18. In particular, *smg-3(r930)* is a deletion of 13,791 nucleotides (coordinates 6370181 to 6383971 of LGIV) that removes 2.9 kb of the 5' end of Y73B6BL.18 and expresses no detectable SMG-3 protein (Fig. 1) or mRNA (data not shown).

In vivo interactions between SMG-2, SMG-3, and SMG-4. We investigated interactions among SMG-2, SMG-3, and SMG-4 in crude extracts by co-IP assays. Our general strategy was to immunoprecipitate SMG-2 (or SMG-3 or SMG-4) from wild-type and *smg*(-) mutant extracts and then to probe the IP pellets for the presence of other SMG proteins by Western blotting. All *smg*(-) alleles used in this paper are known from this or previous work to express none of the encoded protein (3, 5, 15, 26, 53; H. Shang and P. Anderson, unpublished observations for *smg-6*). We prepared affinity-purified polyclonal antibodies against SMG-3 and SMG-4 (see Materials and Methods), and we used an anti-SMG-2 antibody described

FIG. 1. (A) Demonstration of antibody specificity. A Western blot of total protein from 40 adult worms was probed with anti-SMG-2 (α -SMG-2) (panel 1), anti-phospho(Ser/Thr)Gln dipeptides [α -Phospho(S/T)Q] (panel 2), anti-SMG-3 (α -SMG-3) (panel 3), and anti-ACT-1 (α -ACT-1) (panel 4) antibodies (Ab). WT (lane 1), wild-type N2 strain. All *smg* alleles used are known from this or previous work to express none of the encoded protein. The strain containing *smg-7(r1197)*, which is temperature sensitive despite being a null allele, was grown at 25°C. (B) Demonstration of IP specificity. The IP pellets and the input following IP with the anti-SMG-2 (left), anti-SMG-3 (center), and anti-SMG-4 (right) antibodies from crude WT and *smg-2(-)*, *smg-3(-)*, and *smg-4(-)* protein extracts were probed by Western blotting for the presence of SMG-2, SMG-3, and DYN-1 (as a negative control).

previously (53). The specificity of our anti-SMG-2 and anti-SMG-3 antibodies is demonstrated in Fig. 1. The more slowly migrating SMG-2 band observed in *smg-5*, *smg-6*, and *smg-7* mutants is a phosphorylated isoform (53), as demonstrated in panel 2 using an antibody specific for phospho-(Ser/Thr)Gln dipeptides known from previous studies (12) to react with phosphorylated hUpf1/SMG-2. On Western blots, SMG-3 migrates as a single band with a relative mobility of 130 kDa. It is absent in the null allele *smg-3(r930)* and is expressed in approximately normal abundance in all other *smg* mutants except *smg-4*. We observe a slight but reproducible (approximately twofold) decrease in the steady-state abundance of SMG-3 in the absence of its binding partner, SMG-4 (Fig. 1, panel 3, and Fig. 2 [for quantification, see

FIG. 2. Interactions of SMG-2, SMG-3, and SMG-4. (A) Proteins contained in crude extracts and in the pellets following IP of SMG-2 were electrophoresed and probed on Western blots with an anti-SMG-3 antibody. Blots were then stripped and reprobed with an anti-SMG-2 antibody. The input was also probed with an antiactin antibody as a loading control. (B and C) As in panel A, except IPs were performed with an anti-SMG-3 antibody (B) or an anti-SMG-4 antibody (C). Western blots were probed first for SMG-2 and subsequently for SMG-3.

Fig. S1 in the supplemental material]). Our anti-SMG-4 antibody does not react with SMG-4 on a Western blot and is not shown in Fig. 1. However, this antibody appears to effectively and specifically immunoprecipitate native SMG-4 from crude extracts. Both SMG-2 and SMG-3, two proteins that interact with SMG-4 in other species, are observed in the IP pellets following IP of SMG-4 (Fig. 1B and Fig. 2C). SMG-2 and SMG-3 copurify with immunoprecipitated SMG-4 from wild-type crude extracts but not from *smg-4(-)* mutant extracts, thus demonstrating the specificity of our anti-SMG-4 antibody. We, therefore, believe our antibody reacts with native SMG-4 but not with SMG-4 that has been through the denaturation and renaturation of a Western blot. Antibodies that recognize such “conformational epitopes” present only on native proteins are well documented (7).

SMG-2 and SMG-3 copurify from wild-type extracts following IP of either individual protein (Fig. 2A and B, lanes 1). Our antibodies and IP protocols are specific for SMG-2 and SMG-3, as little or none of the other protein is observed in the IP pellets of null mutant controls. SMG-2–SMG-3 interactions are not altered in *smg-1*, *smg-4*, and *smg-5* mutants (Fig. 2A and B, lanes 2, 5, and 6). The slight reduction in the quantity of SMG-3 that copurifies with SMG-2 in Fig. 2A (lane 5) results from an approximately twofold reduction in the steady-state abundance of SMG-3 in *smg-4* mutants (Fig. 2, lower panels; see also Fig. S1 in the supplemental material).

Both SMG-2 and SMG-3 copurify with immunoprecipitated SMG-4 from wild-type extracts (Fig. 2C, lane 1) but not from *smg-4* null mutant control extracts (Fig. 2C, lanes 5 and 6). SMG-4–SMG-3 interactions are unaltered in *smg-2* mutants (Fig. 2C, lane 3), but SMG-4–SMG-2 interactions are reduced in *smg-3* mutants (Fig. 2C, lane 4). Treatment of crude extracts with sufficient RNase A to completely eliminate ethidium bromide-stained rRNA bands does not alter observed SMG-2–SMG-3, SMG-2–SMG-4, or SMG-3–SMG-4 interactions (data not shown). We interpret these results to indicate that, as in yeast, SMG-3 bridges an interaction between SMG-2 and SMG-4 (see Discussion). Two-hybrid experiments indicate that SMG-2–SMG-3 and SMG-3–SMG-4 interactions are likely to be direct in yeast cells (data not shown).

Three lines of evidence demonstrate that the state of SMG-2 phosphorylation does not strongly influence assembly or stability of SMG-2–SMG-3–SMG-4 complexes. First, SMG-2–SMG-3, SMG-3–SMG-4, and SMG-2–SMG-4 interactions are approximately normal in *smg-1* mutants, which lack all detectable SMG-2 phosphorylation (Fig. 2A, B, and C, lanes 2) (26, 53). Second, SMG-2–SMG-3–SMG-4 interactions are unaffected in *smg-5* mutants, which are defective for SMG-2 dephosphorylation (Fig. 2A, B, and C, lanes 6). Third, both phosphorylated SMG-2 and unphosphorylated SMG-2 copurify with immunoprecipitated SMG-3 and SMG-4 in *smg-5* mutants (Fig. 2B and C, lanes 6). We conclude that SMG-2, SMG-3, and SMG-4 interact as a complex in crude extracts and that SMG-2–SMG-3–SMG-4 interactions do not require, nor are they inhibited by, SMG-2 phosphorylation.

SMG-2 preferentially marks PTC-containing mRNA. We investigated the association of SMG-2 with normal and PTC-containing mRNAs in crude extracts. We purified SMG-2-containing mRNPs from wild-type and *smg(-)* mutant extracts by IP, and then examined the IP pellets for presence of copu-

rifying *rpl-12*, *rpl-10a*, *rpl-7a*, *rsp-2*, and *rsp-4* mRNAs. We used an *smg-2* deletion allele that expresses no SMG-2 protein (53) to control for nonspecific mRNAs that might contaminate the IP pellets. Pre-mRNAs of *rpl-12*, *rpl-10a*, *rpl-7a*, *rsp-2*, and *rsp-4* are alternatively spliced to yield both PTC-containing and PTC-free mature mRNAs. For example, productively spliced *rpl-12* mRNA, which we term “*rpl-12(+)*,” encodes RPL-12, does not contain a PTC, and is not a target of NMD. Unproductively spliced *rpl-12* mRNA, which we term “*rpl-12(PTC)*,” contains a PTC and is efficiently degraded by NMD. *rpl-12(PTC)* mRNA is barely detected in the wild type, but it constitutes approximately half of total *rpl-12* mRNA in *smg(-)* mutants (49) (Fig. 3B). *rpl-7a*, *rpl-10a*, *rsp-2*, and *rsp-4* pre-mRNAs are alternatively spliced in a similar manner, and the PTC-containing spliced products are substrates of NMD (Fig. 3C and D) (49, 50; B. Schmidt and P. Anderson, unpublished observations).

We measured the relative abundance of PTC-containing and PTC-free mRNAs in crude extracts before SMG-2 IP and in the IP pellet afterwards. By measuring relative, as opposed to absolute, abundance of each mRNA, we control for slight variation in the quantities of mRNA from sample to sample and in the efficiencies of IP or RT-PCR. A significant increase or decrease in the ratio of PTC-containing to PTC-free RT-PCR products (termed the “PTC/+ ratio” below) in the IP pellets compared to the crude extracts indicates a preferential association of SMG-2 with one or the other of the mRNAs. Reconstruction experiments demonstrate that (i) both PTC-containing and PTC-free cDNAs amplify with equal efficiencies, (ii) varying the amount of added RNA does not alter the measured PTC/+ ratio, and (iii) the measured final PTC/+ ratio is the same as that of the starting mRNA as judged by Northern blotting (49) (see Fig. S2 in the supplemental material).

SMG-2 copurifies with both PTC-free and PTC-containing mRNAs of all five genes, but it preferentially copurifies with those containing PTCs (Fig. 3). For example, *rpl-12(PTC)* mRNA is barely detected in wild-type extracts (Fig. 2B). This mRNA is a substrate of NMD, and we previously showed that it constitutes 5 to 10% of total *rpl-12* mRNA in the wild type (49). *rpl-12(PTC)* mRNA is strongly enriched among the mRNAs that coimmunoprecipitate with SMG-2, constituting approximately 40% of total *rpl-12* mRNA. In *smg(-)* mutants, *rpl-12(PTC)* mRNAs are similarly enriched in the SMG-2 IP pellet. *rpl-12* PTC/+ ratios of *smg-1* through *smg-5* mutants ranged from 1.0 to 2.3 in extracts and 2.9 to 8.9 among mRNAs coimmunoprecipitated with SMG-2 (Fig. 3B). Enrichment of *rpl-12(PTC)* mRNAs in the IP pellet is particularly striking for *smg-1* and *smg-5* mutants (Fig. 3B, lanes 2 and 6). The measured PTC/+ ratios in the IP pellets of *smg-1* and *smg-5* mutants are 5.6-fold and 6.8-fold greater, respectively, than those in the input samples. SMG-2 preferentially copurifies with *rpl-12(PTC)* mRNA in *smg-4* mutants, but the enrichment is modest, with a PTC/+ ratio of 3.4 in the IP pellet compared to 1.0 in the input. We did not observe significant enrichment of *rpl-12(PTC)* mRNA in the IP pellet of *smg-3* mutants, but, as described below, we observed modest enrichment of other PTC-containing mRNAs.

We investigated the selective association of SMG-2 with PTC-containing, alternatively spliced mRNAs of four addi-

FIG. 3. SMG-2 preferentially associates with PTC-containing *rpl-12*, *rpl-10a*, and *rsp-4* mRNA. (A) Schematic diagram of *rpl-12* alternative splicing. *rpl-10a* and *rsp-4* are alternatively spliced in a similar manner (49, 50). (B) SMG-2-containing mRNPs were immunoprecipitated from wild type (WT) and *smg(-)* mutant extracts as described in Materials and Methods, and RNA was extracted from the inputs and IP pellets. The entire IP pellet sample and about 5% of the input sample were then analyzed by RT-PCR. The intensity of each band was quantified from a digital image of the stained gel. Numbers below each lane represent the measured ratio of *rpl-12(PTC)* to *rpl-12(+)* band intensities from five (lanes 1 to 3 and 6 and 7) or eight (lanes 4 and 5) independent IP experiments followed by RT-PCR (average $\pm$ standard deviation). Measured quantities of mRNAs are shown in the graph below; error bars indicate standard deviations. (C and D) As in panel A, except RT-PCR was performed for *rpl-10a* or *rsp-4*. Numbers below each lane represent the measured PTC/+ ratios and standard deviations from 3 (lanes 1 to 3 and 6) and 4 (lanes 4 and 5) independent IP or RT-PCR experiments. ND, not determined.

FIG. 4. Summary of results for selective association of PTC-containing mRNAs with SMG-2. The PTC/+ ratios of five tested mRNAs were measured in crude extracts and in IP pellets after IP of SMG-2 (Fig. 3). The PTC/+ ratios of the IP pellets divided by the PTC/+ ratios of the input are shown for four different *smg* mutants and five tested genes. Bar heights represent average ratios of three to eight independent IPs (see Fig. 3). Such ratios indicate the increase (fold) in PTC/+ ratios after IP.

tional genes and obtained results similar to those described above. Results with *rpl-10a* and *rsp-4* mRNAs are shown in Fig. 3C and D, and those of all five genes are summarized in Fig. 4. As exemplified above by *rpl-12*, mRNAs that copurify with SMG-2 from wild-type, *smg-1*, and *smg-5* mutant extracts are strongly enriched for those containing PTCs. The average increases in the measured PTC/+ ratio when comparing the IP pellets to the crude extracts of all five genes are 12-fold for *smg-1* mutants and 16-fold for *smg-5* mutants. We observed a modest enrichment of PTC-containing mRNAs in the IP pellets of both *smg-3* mutants (1.6-fold increase) and *smg-4* mutants (3.4-fold increase) (Fig. 3C and D and Fig. 4).

As a further control for specificity, we investigated association of SMG-2 with *thp-1* mRNA, which is not alternatively spliced, does not contain PTCs, and is not elevated in abundance in *smg* mutants. The quantities of *thp-1* mRNA copurifying with SMG-2 were readily detected and approximately equal for the wild type and all *smg* mutants except the *smg-2* negative control (data not shown). Thus, association of SMG-2

with normal (PTC free) mRNAs is general and not restricted to mRNAs that are alternatively spliced.

We conclude from these experiments that (i) SMG-2 associates with both PTC-containing and PTC-free mRNAs in both the wild type and in *smg* mutants, but it selectively associates with ("marks") mRNAs containing PTCs; (ii) SMG-3 and SMG-4 enhance the selective marking of PTC-containing mRNAs by SMG-2; and (iii) neither SMG-2 phosphorylation (which is eliminated in *smg-1* mutants) nor SMG-2 dephosphorylation (which is eliminated in *smg-5* mutants) is required for such marking.

SMG-3 and SMG-4 do not selectively associate with PTC-containing mRNA. We investigated the association of SMG-3 and SMG-4 with PTC-containing and PTC-free mRNAs in experiments similar to those described above. We immunoprecipitated SMG-3 or SMG-4 from crude extracts and examined the pellets for copurifying mRNAs of the five genes discussed above. IP of SMG-3 from *smg-3* null mutants and SMG-4 from *smg-4* null mutants served as negative controls for mRNAs that might nonspecifically contaminate the IP pellets.

We were unable to consistently immunoprecipitate above background any *rpl-7a*, *rpl-10a*, *rpl-12*, *rsp-2*, or *rsp-4* mRNAs using anti-SMG-3 or anti-SMG-4 antibodies (Fig. 5) (data not shown). The IP pellets contained barely detectable quantities of mRNA, but those quantities were not elevated above those of *smg-3* or *smg-4* null mutant controls. Analysis of *rpl-12* is shown in Fig. 5, and results for the remaining four genes were qualitatively similar. The small quantities of mRNA detected in the pellets were not enriched for either PTC-containing or PTC-free mRNA (Fig. 5). Thus, although SMG-3 and SMG-4 are needed for the selective association of SMG-2 with PTC-containing mRNA, SMG-3 and SMG-4 do not appear to be a part of those mRNPs. Perhaps SMG-3 and SMG-4 are removed from PTC-containing mRNPs after their marking by SMG-2. Or perhaps SMG-3 and SMG-4 are associated with SMG-2-marked mRNPs, but their interactions are simply weak and not maintained during our IP and washing protocols. The IP and washing conditions we used to detect SMG-2-mRNA

FIG. 5. SMG-3 and SMG-4 do not associate with *rpl-12* mRNA. SMG-3-containing mRNPs or SMG-4-containing mRNPs were immunoprecipitated in three independent experiments and analyzed as described in the legend to Fig. 3. RT-PCR products from the IP pellets were consistently too faint to accurately quantify. In the upper panels, the exposure times of the anti-SMG-3 (IP αSMG-3) and anti-SMG-4 (IP αSMG-4) images are the same as that of the input. In the lower panels, the band intensities in the IP pellets have been digitally enhanced to show that the small quantity of mRNAs present is not enriched for either *rpl-12*(+) or *rpl-12*(PTC).

interactions (Fig. 3 to 5) are essentially identical to those used to detect SMG-2–SMG-3–SMG-4 protein interactions (Fig. 2). SMG-2, SMG-3, and SMG-4 coimmunoprecipitate under these conditions, but only SMG-2 is associated with PTC-containing mRNA. Thus, we are detecting different complexes. The SMG-2–SMG-3–SMG-4 protein complex we detect is not associated with mRNA, and the SMG-2–mRNA complex is not associated with SMG-3 or SMG-4 (see Discussion).

DISCUSSION

smg-3 is the *C. elegans* ortholog of yeast and human *UPF2*, a gene required for NMD in all eukaryotes tested (23, 42, 47, 57). Previous work demonstrated that *smg-2* and *smg-4* are the *C. elegans* orthologs of yeast *UPF1* and *UPF3*, respectively (5, 53). These three genes (*UPF1*, *UPF2*, and *UPF3*) constitute the core components of NMD and are evident in the genomes of all sequenced eukaryotes (17, 18). Four additional *C. elegans* genes (*smg-1*, -5, -6, and -7) are required for cycles of SMG-2 phosphorylation and dephosphorylation, but unambiguous yeast orthologs are not apparent. Yeast Upf1p and Upf2p, however, are phosphoproteins, and phosphorylated residues of Upf2p are necessary for NMD (60). It is presently uncertain whether phosphorylation of yeast NMD proteins has functions similar to those of higher eukaryotes.

We investigated interactions among the core components of *C. elegans* NMD and between those proteins and the mRNA substrates of NMD. Our general strategy was to immunoprecipitate an SMG protein from crude extracts and to then examine the IP pellets for the presence of interacting components. We detected a *C. elegans* SMG-2–SMG-3–SMG-4 complex analogous to that described for both yeast and human Upf1, Upf2, and Upf3 (29, 42, 47, 52, 57). SMG-2 interacts with SMG-3, SMG-3 interacts with SMG-4, and SMG-2 interacts with SMG-4 only if SMG-3 is present (Fig. 2). Such interactions represent conserved interactions of the core “surveillance complex.” When and where this SMG-2–SMG-3–SMG-4 complex assembles in *C. elegans* is uncertain. Mammalian Upf3 is loaded on mRNAs in the nucleus as part of the EJC, after which Upf2 is thought to be recruited to mRNPs in the perinuclear region of the cytoplasm (25, 31, 34, 37, 38, 42, 57). Upf1 interacts with Upf2 as part of a larger complex involving the EJC, Upf proteins, and translation release factors. However, NMD in *C. elegans*, like that of *Drosophila*, does not require downstream introns (23, 41, 55). We assume that *C. elegans* SMG-4 is recruited to mRNPs, but interactions responsible for this are presently unknown.

Upf1 likely interacts with mRNPs initially as a complex containing translation release factors and the Upf1 kinase SMG-1 (19, 32, 36, 64). SMG-2 of *C. elegans* copurifies with SMG-1 (26), but interactions of SMG-2 with *C. elegans* release factors have not been examined. Both SMG-2–SMG-3–SMG-4 and SMG-2–SMG-1 interactions (26) do not require and are not inhibited by SMG-2 phosphorylation. As discussed above, it is unlikely that the SMG-2–SMG-3–SMG-4 complex we detected by co-IP is associated with target mRNAs. Rather, the SMG-2–SMG-3–SMG-4 complex could represent an intermediate of assembly and/or disassembly of the core surveillance complex, a complex that is not associated with mRNA, or perhaps a storage form of the proteins.

SMG-2 selectively associates with mRNAs that contain premature termination codons, thereby distinguishing them from mRNAs that do not contain PTCs (Fig. 3 and 4). Selective co-IP of SMG-2 with PTC-containing mRNAs is especially striking in the wild type and in *smg-1* and *smg-5* mutants. Averaging the results of five tested genes, the ratio of PTC-containing to PTC-free mRNA (the “PTC/+ ratio”) in the IP pellets compared to those in the extracts increases 12-fold in *smg-1* mutants and 16-fold in *smg-5* mutants (Fig. 4). Enrichment was similarly striking in the wild type (Fig. 3B to D, lanes 1), but we cannot accurately quantify the increase due to the low abundance of PTC-containing mRNA in NMD-competent strains. Thus, marking of PTC-containing mRNAs by SMG-2 is not an artifact of studying *smg*(–) strains. Rather, we view the marked mRNAs as intermediates of mRNA decay whose abundance increases or decreases in specific *smg*(–) mutants (see below).

SMG-2 also copurifies with PTC-containing mRNAs in *smg-3* and *smg-4* mutants, but enrichment of those mRNAs in the IP pellets is modest. Averaging the results of all genes tested, the measured PTC/+ ratios increase 2- to 4-fold in *smg-3* and *smg-4* mutants (Fig. 4), compared to 12- to 16-fold in *smg-1* and *smg-5* mutants. We interpret this to indicate that SMG-3 and SMG-4 function to promote assembly or remodeling of mRNP complexes, such that SMG-2 binds preferentially and selectively to mRNPs that contain PTCs. SMG-2, therefore, “marks” such mRNPs as containing PTCs and distinguishes them from PTC-free mRNPs. Remodeling presumably occurs after translation termination, and it does not require either SMG-2 phosphorylation or dephosphorylation (see below). SMG-2 associates not only with PTC-containing mRNAs but also with PTC-free mRNAs (Fig. 3B to D; compare lanes 1 and 3). Thus, SMG-2 likely assembles on all mRNPs, and those discriminated as containing PTCs are then remodeled such that SMG-2 is more tightly or more persistently bound. Such remodeling requires or is enhanced by SMG-3 and SMG-4.

Discrimination of stop codons as being premature causes SMG-2 to be tightly associated with PTC-mRNPs, either directly by binding mRNA or indirectly by binding as-yet-undefined components of the mRNP. Tethering of mammalian Upf1 to an mRNA, either artificially such as to mRNAs of expressed transgenes (42) or naturally such as during Staufen-mediated decay (35), is sufficient to elicit NMD. The “SURF” complex of mammalian NMD, which contains Upf1 and SMG-1, interacts via Upf2 with components of the EJC following termination (32; reviewed in reference 9). Two lines of evidence suggest that mRNP remodeling in *C. elegans* may be somewhat different: (i) NMD in *C. elegans* does not require downstream introns (41, 55), and (ii) SMG-3 and SMG-4 are not stably associated with SMG-2-bound, PTC-containing mRNAs (Fig. 5). It is possible that *C. elegans* SMG-3 and SMG-4 actually are associated with SMG-2-marked mRNPs, but their interactions with those mRNPs are simply weak and do not survive our IP and washing protocols. In that case, the SMG-2–mRNP complex we describe could be equivalent to the “DECID” complex of Kashima et al. (32). Alternatively, perhaps the complex described by Kashima et al. (32) represents an intermediate of remodeling that occurs in both organisms but is followed by exit of Upf2 and Upf3 from the mRNP.

A later complex, containing SMG-2/Upf1 but not SMG-3/Upf2 or SMG-4/Upf3, may be the complex we detect in *C. elegans*. Despite some apparent differences, NMD in mammals and NMD in *C. elegans* exhibit many striking similarities. In both organisms, Upf1 interacts with SMG-1 and SMG-5 (16, 26, 32), Upf2 and Upf3 are required for Upf1 phosphorylation (32, 53), SMG-5 is required for Upf1 dephosphorylation (32, 52, 53), and Upf1 phosphorylation occurs only on mRNPs and only after PTCs have been discriminated from normal termination codons (32; also described above).

Our results indicate that SMG-2 phosphorylation occurs only after PTCs have been discriminated and after PTC-mRNPs have been marked with SMG-2. SMG-1, which is required for SMG-2 phosphorylation, is not required for marking of PTC-mRNPs by SMG-2 (Fig. 3). Mutants that are blocked in an ordered pathway of dependent gene function are expected to complete steps of the pathway before the block and to accumulate intermediates prior to the block. Assuming that only a single step is blocked in *smg-1* mutants, SMG-1 must function only after PTC discrimination and after SMG-2 marking of mRNAs. The point during NMD at which SMG-2 dephosphorylation occurs is less certain. Almost all SMG-2 present in *smg-5* mutants is phosphorylated (Fig. 1C), and we assume that phosphorylated SMG-2 is the isoform being copurified with PTC-defined mRNPs in *smg-5* mutants. Thus, although SMG-2 phosphorylation occurs after SMG-2 is specifically associated with PTC-containing mRNPs, its dephosphorylation could occur at any later step.

What then does SMG-2 phosphorylation and dephosphorylation regulate? Ohnishi et al. (52) describe remodeling of mRNPs that occurs after Upf1 phosphorylation and involves both SMG-5 and SMG-7 (52). Tethered SMG-7, which is required for Upf1 dephosphorylation, is sufficient to elicit mammalian NMD and bypasses the need for Upf1, SMG-5, and SMG-6 (59). Perhaps such interactions are regulated by SMG-2 phosphorylation or dephosphorylation. At this point, however, the list of candidate processes regulated by phosphorylation must include all events occurring after PTC discrimination, including deadenylation, decapping, trafficking of mRNAs to cytoplasmic processing bodies, or subsequent mRNP processing. One attractive possibility is that phosphorylation and dephosphorylation of SMG-2 are required for its disassembly and recycling in order to make it available for future rounds of NMD on other mRNAs. Phosphorylation might be needed to release SMG-2 from marked mRNPs, and dephosphorylation might be needed to make released SMG-2 available for subsequent NMD.

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