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Identification and characterization of novel factors that act in the nonsense-mediated mRNA decay pathway in nematodes, flies and mammals

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Abstract

Nonsense-mediated mRNA decay (NMD) is a surveillance mechanism that degrades mRNAs harboring premature termination codons (PTCs). We have conducted a genome-wide RNAi screen in Caenorhabditis elegans that resulted in the identification of five novel NMD genes that are conserved throughout evolution. Two of their human homologs, GNL2 (ngp-1) and SEC13 (npp-20), are also required for NMD in human cells. We also show that the C. elegans gene noah-2, which is present in Drosophila melanogaster but absent in humans, is an NMD factor in fruit flies. Altogether, these data identify novel NMD factors that are conserved throughout evolution, highlighting the complexity of the NMD pathway and suggesting that yet uncovered novel factors may act to regulate this process.

Keywords C. elegans; nonsense-mediated decay; RNAi screen; smg genes

Subject Categories RNA Biology

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Introduction

The NMD pathway targets mRNAs harboring premature termination codons (PTCs) for degradation, but also regulates the stability of a wide array of endogenous transcripts [reviewed by 1–3]. Genetic screens in the nematode Caenorhabditis elegans resulted in the identification of seven genes required for NMD, termed smg-1-7 (for suppressor with morphological effect on genitalia). Likewise, a similar approach in Saccharomyces cerevisiae identified three NMD genes, UPF1-3 (for up-frameshift), that are orthologs of C. elegans smg-2, smg-3 and smg-4, respectively. Subsequently, orthologs for all the smg genes were identified in several species including insects, plants and mammals [reviewed by 4,5]. A genome-wide RNAi screen in C. elegans identified two additional NMD factors that are conserved throughout evolution and, unlike the core smg-1-7 genes, are essential for embryonic development [6]. Accordingly, they were termed smgl-1 and smgl-2 (for smg-lethal-1 and 2, respectively). Their human homologs, NBAS (for neuroblastoma amplified sequence) and DHX34, act in concert with core NMD factors to coordinate a large number of endogenous RNA targets [7].

The ATP-dependent RNA helicase, UPF1/SMG2, is a central NMD factor and undergoes cycles of phosphorylation and dephosphorylation that are essential for its activity. UPF1 is phosphorylated at multiple [S/T]Q motifs at its C- and N-terminus by the SMG1 complex, which contains the protein kinase SMG1 and the SMG8,9 subunits [3]. NMD is initiated by the assembly of the SURF complex, comprising SMG1, UPF1 and the translation release factors eRF1 and eRF3, in the vicinity of a PTC. Subsequently, an interaction of this complex with an exon junction complex (EJC), deposited downstream as a consequence of the splicing process, leads to the formation of the decay-inducing complex (DECID) that results in mRNA degradation [8]. The interaction of the SURF complex with the EJC allows the binding of UPF2 to the N-terminal domain of UPF1 resulting in a large conformational change that activates the UPF1 helicase activity [9, 10]. UPF1 dephosphorylation is carried out at a later stage and requires the activity of SMG5-7 together with protein phosphatase 2A (PP2A) [reviewed by 11].

In order to establish whether there are more factors that could regulate the NMD pathway within the context of a multicellular organism, we carried out a genome-wide RNAi screen in C. elegans that builds on the success of our previous effort [6], but using a different RNAi library that included many previously untested genes. We identified five novel NMD genes that are highly conserved throughout evolution and demonstrate that they participate in the NMD pathway in human cells and Drosophila embryos.

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**Results and Discussion**

**A genome-wide RNAi screen to identify new genes required for NMD**

Our previous RNAi screen in *C. elegans* led to the identification of smgl-1/NBAS and smgl-2/DHX34 that act in NMD in nematodes and vertebrates [6,12]. We revisited this approach with the use of a different RNAi library: the *C. elegans* ORF-RNAi library v.1.1 that contains 11,511 clones targeting 55% of the nematode genome [13]. This library includes dsRNAs against 1,736 genes that were not targeted previously [14]. As earlier, we used the *C. elegans* PTCxi strain that expresses a GFP-based reporter harboring a PTC that is integrated in the genome [6] (Fig 1). This PTCxi reporter has reduced GFP expression, since its transcript is subject to NMD-mediated degradation. Thus, novel NMD genes were identified by the criterion that their silencing by RNAi restores GFP expression. Accordingly, we searched for the appearance of green worms, dead or alive, following inactivation of individual genes by RNAi. As a negative control, we fed PTCxi animals empty RNAi vector, which had no effect on the level of GFP expression (Fig 2A, panel I), whereas inactivation of the core NMD factor, SMG-2/UPF1, which induced strong GFP expression, was used as a positive control (Fig 2A, panel II). Screening of the entire library resulted in the identification of five RNAi clones that scored positive by increased GFP expression (Fig 2A, panels III–VII). The clones identified in this screen are: T19A6.2, Y77E11A.13, Y87G2A.4, C47B2.4 and F52B11.3 (Table 1). Confirming that these newly identified *C. elegans* genes act as NMD factors, quantitative RT–PCR analysis showed that downregulation of each of these genes led to an increase in the GFP reporter mRNA level, as was seen with depletion of *smg-2* (Fig 2B).

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**Figure 1.** A genome-wide RNAi screen designed to identify novel NMD factors in *C. elegans*. The PTCx NMD reporter consists of a fusion between GFP (in green) and LacZ (in blue) genes; a PTC is present downstream of the GFP coding region, making the transcript an NMD substrate.
Novel NMD genes in *C. elegans*

All of the newly identified genes, with the exception of noah-2, are conserved throughout evolution and have clear orthologs in human, mouse, zebrafish and yeast (Supplementary Figs S1 and S2). The *C. elegans* gene *ngp-1* (T19A6.2) corresponds to the human *GNL2* gene and encodes a putative GTPase that comprises a GTP-binding domain formed by five G-motifs, which is typical of the HSR1_MMR1 GTP-binding protein subfamily. It also contains a conserved N-terminal domain (NGP1NT) (Supplementary Fig S1A). Its yeast homolog, Nog2p, is involved in ribosomal biogenesis playing a role in the processing of the pre-60S particles [15]. The *npp-20* gene

Figure 2. Newly identified NMD factors.

A RNAi was induced with an empty vector as a negative control (panel I), whereas a *smg-2* clone (panel II) was used as a positive control. Panels i and ii show brightfield images of the PTCx strain treated with the negative and positive controls, respectively. Depletion of five genes (panels III to VII) resulted in increased GFP expression. Panels iii to vii show brightfield images of the phenotypes of the affected worms. The scale bars correspond to 100 μm.

B Depletion of the novel NMD genes in *C. elegans* leads to upregulation of the PTCx NMD reporter mRNA, which was monitored by quantitative RT–PCR relative to the expression of *ama-1* reference gene. The values shown are the average fold-change (mean ± SEM) from at least three independent experiments relative to empty vector-depleted worms. Statistical analysis was performed using the Mann–Whitney U-test for non-parametric distributions. *P < 0.05.*
Table 1. List of novel putative NMD factors identified in this study.

| Clone ID | Gene name (C. elegans) | Predicted function in C. elegans | C. elegans phenotype | Gene name (H. sapiens) |
|----------|------------------------|----------------------------------|----------------------|-----------------------|
| T1946.2  | npp-1      | Nuclear/nucleolar GTP-binding protein family | Embryonic lethal Larval arrest Maternal sterile | GNL2 |
| Y77E11A.13 | npp-20    | Nuclear pore complex protein | Embryonic lethal Larval arrest | SEC13 |
| Y87C2A.4  | aex-6       | Rab protein involved in trafficking of vesicles | Aboc expulsion missing Constipated | RAB27A RAB27B |
| C47B24    | pbs-2       | Proteasome p subunit | Embryonic lethal Larval arrest | PSMB7 PSMB10 |
| F52B11.3  | noah-2      | PAN and ZP domain-containing protein | Embryonic lethal Larval arrest | Not conserved Conserved in Drosophila (nompA) |

(Y77E11A.13) corresponds to human SEC13, which encodes a protein that comprises six WD-40 domains (Supplementary Fig S1B) and is a constituent of the endoplasmic reticulum and the nuclear pore complex (NPC) [16]. The aex-6 gene (Y87G2A.4) is a member of the Rab small GTPase superfamily. It has two homologs in humans, RAB27A and RAB27B (Supplementary Fig S1C), with RAB27B functioning in the trafficking of dense-core vesicles [17]. The pbs-2 gene (C47B24) is a member of the proteasome B-type family and is a 20S core beta subunit of the proteasome (the affected worms were arrested at L1–L2 stages, compared to control worms that invariably reached adulthood within the time limit of the experiment (Fig 2A, panels i and iii, respectively). Those worms that escaped early arrest failed to reach adulthood and produced no embryos. Depletion of npp-20 resulted in worms arrested at L2–L3 larval stages. The majority of the worms were very fragile and died by bursting (Fig 2A, panel iv). By contrast, depletion of aex-6 resulted in a mild but highly consistent phenotype with worms able to progress through the developmental stages normally; however, adult worms were constipated, as previously reported [21], and also exhibited an egg-laying defect and reduced brood size (Fig 2A, panel v). Depletion of pbs-2 resulted in very sick and pale larvae that were arrested around L2 stage, displaying a swollen intestine in the majority of the affected worms (Fig 2A, panel vi). Finally, depletion of noah-2 led to an early larval arrest at L2–L3 stages and subsequent larval lethality (Fig 2A, panel vii).

**Drosophila nompA gene is required for NMD**

There is no ortholog of noah-2 in mammalian genomes; nonetheless, the gene is clearly present in Drosophila melanogaster (Supplementary Fig S2), suggesting that it most likely emerged at some point during the early evolution of the Ecdysozoa before the split between arthropods and nematodes. We assessed its potential role in NMD in Drosophila embryos by means of a previously described NMD fluorescent GFP reporter [22,23]. Unlike the previously characterized Drosophila NMD genes upf1, upf2 and upf3 that are ubiquitously expressed [22,24], expression of nompA in Drosophila embryos is confined to type I sense organs of the peripheral nervous system (PNS) [20]. Thus, we used the UAS/Gal4 system [25] to drive expression of a nompA RNAi construct within the PNS using a NompA-Gal4 line [26]. We found that nompA knockdown within its expression domain in the embryonic PNS led to a significant upregulation of the NMD reporter (Fig 3C, F and G), which was comparable to the effect observed following UPF1 depletion (Fig 3B, E and G), as compared to no RNAi treatment (Fig 3A, B and D), which showed no expression of the reporter. This demonstrates that the Drosophila ortholog of noah-2 behaves as a tissue-specific NMD factor in fruit fly embryos.

**GNL2 and SEC13 act in the NMD pathway in human cells**

Next, we investigated a potential role for the human homologs of the factors identified in this screen in NMD in human cells. HeLa cells stably expressing an integrated human -globin (HBB) gene, either in a wild-type version or carrying an NMD-inducing mutation (NS39) [27], were individually depleted of each of these genes. The level of depletion of these factors is shown in Supplementary Fig S3C. As expected, depletion of the human homologs of the novel NMD factors did not significantly affect the levels of the wild-type -globin mRNA (Fig 4A). By contrast, depletion of UPF2 (positive control) or of GNL2 and SEC13, but not of RAB27A-B (depleted individually or in combination), resulted in a significantly increased level of the -globin
NMD reporter (NS39) mRNA when compared to mock-depleted cells (Fig 4B). Whereas individual depletion of PSMB10 clearly showed no effect on the levels of the NMD reporter, knockdown of PSMB7 (either individually or in combination with PSMB10) led to an upregulation of both the wild-type and NMD reporters, making it difficult to conclude whether PSMB7 had a specific role in NMD (Supplementary Fig S3A and B). Altogether, these experiments show that GNL2 and SEC13 have a clear effect in the NMD response in human cells, whereas it still remains possible that the remaining tested genes may require that the NMD pathway.

To rule out indirect effects of GNL2 and SEC13, we first investigated whether these factors have a general role in mRNA translation, which would impact on NMD. This is unlikely, since the very nature of the RNAi screen in C. elegans requires that the NMD reporter is indeed translated. In agreement, knockdown of GNL2 or SEC13 did not result in a general inhibition of translation, as measured by metabolic labeling of HeLa cells (Supplementary Fig S3E and F). Next, we examined the half-life of translation, as measured by metabolic labeling of HeLa cells expressing a NMD reporter is indeed translated. In agreement, knockdown of GNL2 or SEC13 also resulted in an increased half-life of one of those NMD substrates (Fig 4C). By contrast, depletion of GNL2 or SEC13 led to a marked stabilization of a PTC-containing β-globin mRNA, confirming that both GNL2 and SEC13 act in the NMD pathway (Fig 4D). Furthermore, depletion of GNL2 or SEC13 in HeLa cells led to a marked upregulation of three endogenous transcripts that were previously reported to be sensitive to NMD regulation [7, 28] (Fig 4E). In agreement, knockdown of GNL2 or SEC13 also resulted in an increased half-life of one of those NMD substrates (ARHGEP18) mRNA (Supplementary Fig S3D). As further proof of the role of the novel factors identified in this screen in the NMD pathway, we probed for the interaction of GNL2 with the core NMD factor UPF1. We immunopurified Flag-tagged UPF1 expressed at physiological levels from transiently transfected HEK 293T cells that also co-expressed T7-tagged GNL2 in the presence of RNase A. We used transiently expressed Flag-empty vector (F-EV) co-expressed with T7-tagged GNL2, as a negative control. We observed that UPF1 specifically co-immunoprecipitated with T7-tagged GNL2 in an RNA-independent manner (Supplementary Fig S4). Future studies will aim to test the interaction of GNL2 and SEC13 with components of the NMD machinery.

**Conclusions**

Even though the NMD pathway is a highly conserved process, several mechanisms have evolved to define a PTC across different species [3]. Whereas in mammalian cells, NMD is linked to
Figure 4.
The NMD reporter is based on the mammalian cells and we chose HeLa cells as our experimental system. The only exception was the noah-2 gene that does not have a human counterpart. For this, we studied its functional homolog in Drosophila and found that it acts in the NMD pathway in insects. Importantly, we show that two human homologs, GNL2 (ngp-1) and SEC13 (np2-20), are also required for NMD in human cells. Only recently, we uncovered the mechanism by which the mRNA helicase DHX34, which was identified in our first RNAi screen, promotes mRNP remodeling and triggers the conversion from the SURF complex to the DECID complex resulting in NMD activation [35]. Further studies will help to delineate the mechanism by which SEC13 and GNL2 activate NMD in human cells, as well as their involvement in the described alternative NMD branches [36–38]. In summary, our work has led to the identification of novel NMD factors in nematodes, flies and mammals, revealing that the machinery underlying NMD is more complex than previously thought.

Materials and Methods

Genome-wide RNAi screen

The NMD reporter is based on the GFP::lacZ vector pDP96.04 and is driven by the ubiquitous sec-23 promoter [6]. The PTCxi transgenic strain carrying this GFP-based NMD reporter integrated in the genome was described previously [6]. PTCxi transgenic worms were grown on standard NGM plates seeded with OP50 E. coli bacteria at 20°C. The RNAi library used for the screen was created in the laboratory of Marc Vidal and is commercially available [13]. RNAi was performed in liquid format by feeding synchronized population of PTCxi L1 larvae with bacterial clones expressing dsRNA corresponding to individual genes in 96-well plates [6]. Worms were then scored for the appearance of GFP expression, indicating that the depleted protein is required for NMD in C. elegans.

Fly stocks

We used the following fly stocks all obtained from the Bloomington Stock Center (Indiana, USA): w1118; P{GMR29A10-GAL4}attP2, y¹ w¹; P{UAS-mCD8::GFP.L}LL5 [BM 5137], y¹ v¹; P{TRIP.JF02919}attP2 and y¹ v¹; P{TRIP.GLO1485}attP2. Animals were reared at 25°C on cornmeal, molasses and yeast medium.

For more detailed Materials and Methods see the Supplementary Information.

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Author contributions

AC, DL and JFC conceived, designed and interpreted the experiments in nematodes and human cells. AC, DL, NH and LD performed the experiments and analysis. RVB and CRA designed, performed and interpreted the experiments in Drosophila. The manuscript was co-written by all authors.

Conflict of interest

The authors declare that they have no conflict of interest.

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