Nonsense-mediated mRNA decay factors act in concert to regulate common mRNA targets

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ABSTRACT

Nonsense-mediated mRNA decay (NMD) is a surveillance pathway that degrades mRNAs containing nonsense codons, and regulates the expression of naturally occurring transcripts. While NMD is not essential in yeast or nematodes, UPF1, a key NMD effector, is essential in mice. Here we show that NMD components are required for cell proliferation in *Drosophila*. This raises the question of whether NMD effectors diverged functionally during evolution. To address this question, we examined expression profiles in *Drosophila* cells depleted of all known metazoan NMD components. We show that UPF1, UPF2, UPF3, SMG1, SMG5, and SMG6 regulate in concert the expression of a cohort of genes with functions in a wide range of cellular activities, including cell cycle progression. Only a few transcripts were regulated exclusively by individual factors, suggesting that these proteins act mainly in the NMD pathway and their role in mRNA decay has not diverged substantially. Finally, the vast majority of NMD targets in *Drosophila* are not orthologs of targets previously identified in yeast or human cells. Thus phenotypic differences observed across species following inhibition of NMD can be largely attributed to changes in the repertoire of regulated genes.

Keywords: Est1; mRNA decay; mRNA surveillance; NMD; SMG; telomeres; UPF

INTRODUCTION

The gene expression pathway involves a number of interlinked post-transcriptional steps that are subject to several quality control mechanisms, to ensure that only fully processed and error-free mRNAs are translated. Among these mechanisms, the nonsense-mediated mRNA decay (NMD) pathway recognizes and targets for degradation mRNAs containing premature translation termination codons (PTCs), which could give rise to truncated and potentially harmful proteins (Holbrook et al. 2004; Conti and Izaurralde 2005; Lejeune and Maquat 2005).

A key molecular component of the NMD pathway is UPF1. Deletion or silencing of the *upf1* gene results in the stabilization of PTC-containing mRNAs in all organisms in which NMD has been investigated (Conti and Izaurralde 2005; Lejeune and Maquat 2005). Two additional proteins, UPF2 and UPF3, interact with UPF1 to form a complex whose function in NMD is conserved in eukaryotes (Conti and Izaurralde 2005; Lejeune and Maquat 2005). UPF1 activity is regulated by phosphorylation in multicellular organisms, and this requires UPF2, UPF3, and four additional proteins that have no clear orthologs in yeast: SMG1, SMG5, SMG6, and SMG7 (Pulak and Anderson 1993; Cali et al. 1999; Page et al. 1999). SMG1 is a phosphoinositide-3-kinase-related protein kinase required for UPF1 phosphorylation (Denning et al. 2001; Pal et al. 2001; Yamashita et al. 2001; Grimson et al. 2004). SMG5, SMG6, and SMG7 recognize phosphorylated UPF1 and are thought to trigger its dephosphorylation by recruiting protein phosphatase 2A (Anders et al. 2003; Chiu et al. 2003; Ohnishi et al. 2003; Fukuhara et al. 2005).

Despite conservation of the *trans*-acting factors required for NMD, different species have evolved different mechanisms to discriminate natural from premature translation termination codons and to degrade transcripts that have been identified as NMD substrates (Conti and Izaurralde 2005). In mammals, recognition of premature stop codons results from a conjunction of terminating ribosomes and an exon-exon boundary located at least 50 nucleotides downstream of the PTC (Lejeune and Maquat 2005). In contrast, in both *Drosophila* and *Saccharomyces cerevisiae* PTC recognition occurs independently of exon–exon boundaries (Conti and Izaurralde 2005).

Once a PTC is recognized by the NMD machinery, enzymes involved in general mRNA decay are recruited

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and the aberrant transcript is rapidly degraded. In yeast and human cells decay of PTC-containing mRNAs occurs by exonucleolytic degradation at either end of the message (Baker and Parker 2004). In *Drosophila*, degradation of nonsense transcripts is initiated by endonucleolytic cleavage near the PTC. The resulting 5′ decay intermediate is degraded by the exosome, while the 3′ fragment is degraded by XRN1 (Gatfield and Izaurralde 2004).

The NMD pathway not only degrades aberrant mRNAs containing PTCs as a result of mutations or errors during transcription or RNA processing, but is also implicated in the post-transcriptional regulation of wild-type transcripts (Holbrook et al. 2004; Lejeune and Maquat 2005). Nevertheless, NMD components are not essential in yeast (Leeds et al. 1991). Similarly, inhibition of the NMD pathway in *Caenorhabditis elegans* leads to viable worms with defects in the male bursa and the hermaphrodite vulva (Hodgkin et al. 1989; Pulak and Anderson 1993). In contrast, *UPF1* null mice die early in embryonic development and attempts to establish homozygous $UPF1^{-/-}$ ES cells have failed, indicating that UPF1 is required for cell viability in mice (Medghalchi et al. 2001). The simplest explanation for these phenotypic differences is that NMD factors regulate the expression of essential transcripts in mice, but not in *S. cerevisiae* or *C. elegans*. Alternative explanations for these differences include the possibility that NMD factors (e.g., UPF1) have acquired additional functions in higher eukaryotes.

Gene expression profiling of yeast strains lacking Upf1p, Upf2p, or Upf3p indicates that these proteins act as obligate partners to regulate the expression of a common set of transcripts, representing ~10% of the transcriptome (Lelivelt and Culbertson, 1999; He et al. 2003). Similarly, in human cells depleted of UPF1, about 10% of the transcriptome shows differential expression (Mendell et al. 2004). A subset of human UPF1 targets is also regulated by UPF2; however, the additional human NMD effectors have not been investigated. The lack of genome-wide information on genes regulated by the additional components of the NMD machinery in metazoa leaves open the question of functional diversification.

To investigate whether NMD factors have additional roles in mRNA turnover in multicellular organisms, and to shed light on the physiological role of NMD across species, we examined for the first time changes in gene expression associated with the depletion of all known metazoan NMD effectors in *Drosophila* cells (i.e., UPF1, UPF2, UPF3, SMG1, SMG5, and SMG6; no SMG7 ortholog has been identified in *Drosophila*) (Gatfield et al. 2003). Our analysis identified a core set of transcripts regulated in concert by all NMD factors. Among these we found smg5 and smg6 mRNAs, revealing the existence of a feedback mechanism. We show further that, with a few exceptions, orthologs of yeast or human NMD targets are not regulated by NMD in *Drosophila*. Finally, very few transcripts are regulated exclusively in the individual knockdowns, indicating that NMD factors act in the same pathway and their role in post-transcriptional mRNA regulation has not diverged substantially.

RESULTS

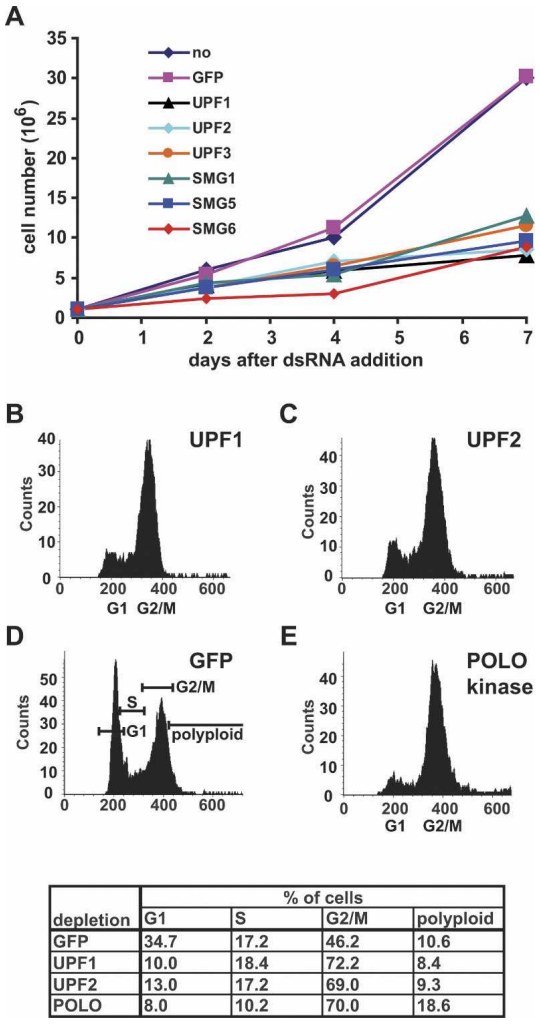
NMD is required for cell proliferation in *Drosophila*

To shed light on the physiological role of NMD in *Drosophila*, we depleted all known NMD factors (i.e., UPF1, UPF2, UPF3, SMG1, SMG5, and SMG6) from Schneider cells (SL2 cells). The depletions were carried out by RNA interference under conditions that lead to the stabilization of NMD reporters (Gatfield et al. 2003). The efficiency of the depletions was confirmed by RT-PCR (see Supplementary Fig. S1 at http://www-db.embl.de/jss/EmblGroupsHD/g_127?sP=4.) and by the stabilization of the ornithine decarboxylase antizyme mRNA (oda), an endogenous transcript known to be regulated by NMD (see below, and Gatfield and Izaurralde 2004). Depletion of NMD proteins impaired cell proliferation (Fig. 1A).

To understand better the basis for this inhibition, we analyzed DNA profiles using flow cytometry. Cells depleted of UPF1 and UPF2 were arrested at the G2/M phase of the cell cycle, when compared to cells treated with a dsRNA targeting green fluorescence protein (GFP dsRNA) (Fig. 1B–D). An increase in the proportion of cells in the G2/M phase was also observed following depletions of UPF3, SMG5, and SMG6 (Supplementary Fig. S2). Depletion of POLO kinase, a key regulator of mitotic onset as well as progression through mitosis (Ohi and Gould 1999), resulted in a cell cycle profile very similar to that of UPF1- or UPF2-deficient cells (Fig. 1E). These results reveal an important role for NMD in cell cycle progression in *Drosophila*.

Genome-wide identification of transcripts regulated by the NMD pathway

To identify a comprehensive set of targets of the NMD pathway, we analyzed RNA expression profiles of SL2 cells depleted of UPF1, UPF2, UPF3, SMG1, SMG5, or SMG6, using oligonucleotide microarrays. For each NMD component we obtained RNA expression profiles from two independent knockdowns. As a reference, RNA samples were isolated from mock-treated cells. To exclude mRNAs nonspecifically regulated in response to the dsRNA treatment, transcripts exhibiting altered expression in cells treated with GFP dsRNA were not considered in further analysis (see Materials and Methods). Detectable transcripts in all experiments (5379 RNAs) were assigned to three classes according to their relative expression levels in the two independent profiles of each factor (Fig. 2). These include mRNAs that were at least 1.5-fold underrepresented relative to the control sample (blue), not substantially changed (less than 1.5-fold different, yellow) or more than 1.5-fold over-



depletion	% of cells			
	G1	S	G2/M	polyploid
GFP	34.7	17.2	46.2	10.6
UPF1	10.0	18.4	72.2	8.4
UPF2	13.0	17.2	69.0	9.3
POLO	8.0	10.2	70.0	18.6

FIGURE 1. Depletion of NMD factors impairs cell proliferation and leads to G2/M-cell cycle arrest. (*A*) *Drosophila* SL2 cells were treated with the indicated dsRNAs. Cell numbers were determined up to 7 d after addition of dsRNAs. (*B–E*) FACS analysis of asynchronously growing SL2 cells. Cells were treated with the indicated dsRNAs, stained with propidium iodide and analyzed using a flow cytometer. The table shows the proportion of cells in different phases of the cell cycle as shown in (*D*).

represented (red; see Materials and Methods for an explanation of the 1.5-fold cutoff).

In cells depleted of UPF1, 75.3% of detected mRNAs were less than 1.5-fold different from the control samples, 14.3% of mRNAs were at least 1.5-fold upregulated, and 10.4% were underrepresented (Fig. 2). Although knockdowns of UPF3, SMG1, and SMG5 all resulted in the stabilization of the oda mRNA (Fig. 3) and impaired cell proliferation (Fig. 1A), overrepresented and underrepresented transcripts constituted only a minor fraction of detectable mRNAs (Fig. 2). An intermediate phenotype was observed in cells depleted of UPF2 or SMG6 (Fig. 2). The differences in the number of transcripts showing altered expression in the individual knockdowns might be explained by differences in depletion efficiencies and/or different levels of dependence on one or more NMD factors by specific transcripts.

We confirmed the microarray results using Northern blot to assay the expression levels of selected mRNAs (Fig. 3). Transcripts that were over- or underrepresented relative to the control samples in the microarray experiment showed the same positive or negative trend when detected by Northern blot. Differences in the extent of the regulation of specific transcripts in the different depletions (e.g., *tra* mRNA), were also confirmed when the transcript levels were measured by Northern blot.

Depletion of NMD factors leads to similar expression profiles

To ensure that the global changes of expression profiles observed in cells depleted of NMD factors are caused by the inhibition of NMD, rather than being a nonspecific response to the depletion of essential proteins, we com-

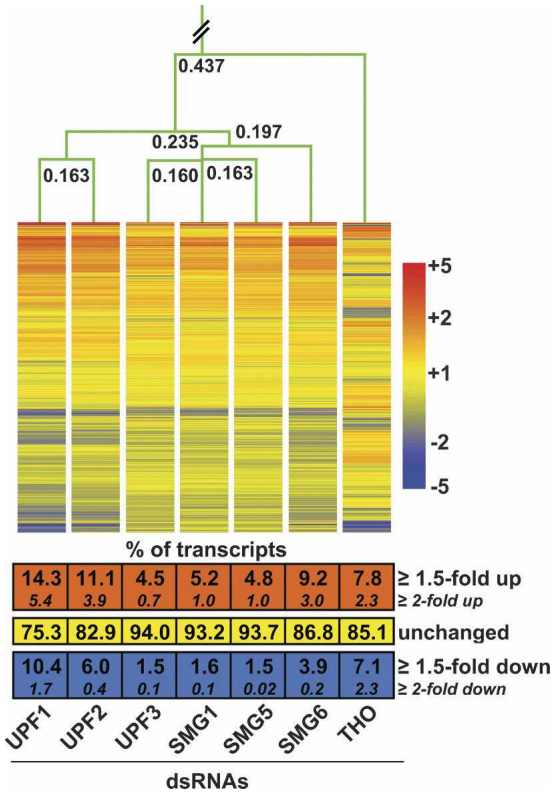


FIGURE 2. Expression profiles of *Drosophila* cells depleted of NMD factors. RNAs are represented as lines and colored relative to their expression levels, as indicated. Average expression levels of two independent profiles are shown. The experiment tree was calculated using the distance option of the genespring software (Euclidian distance). Although 5379 mRNAs were detected in the 12 profiles obtained for the NMD factors, the *top* panel of this figure displays 4940 mRNAs, which were detectable both in the 12 profiles obtained in NMD-deficient cells and in the two profiles of THO-depleted cells. The fractions of regulated transcripts in two independent profiles for each factor are indicated *underneath*.

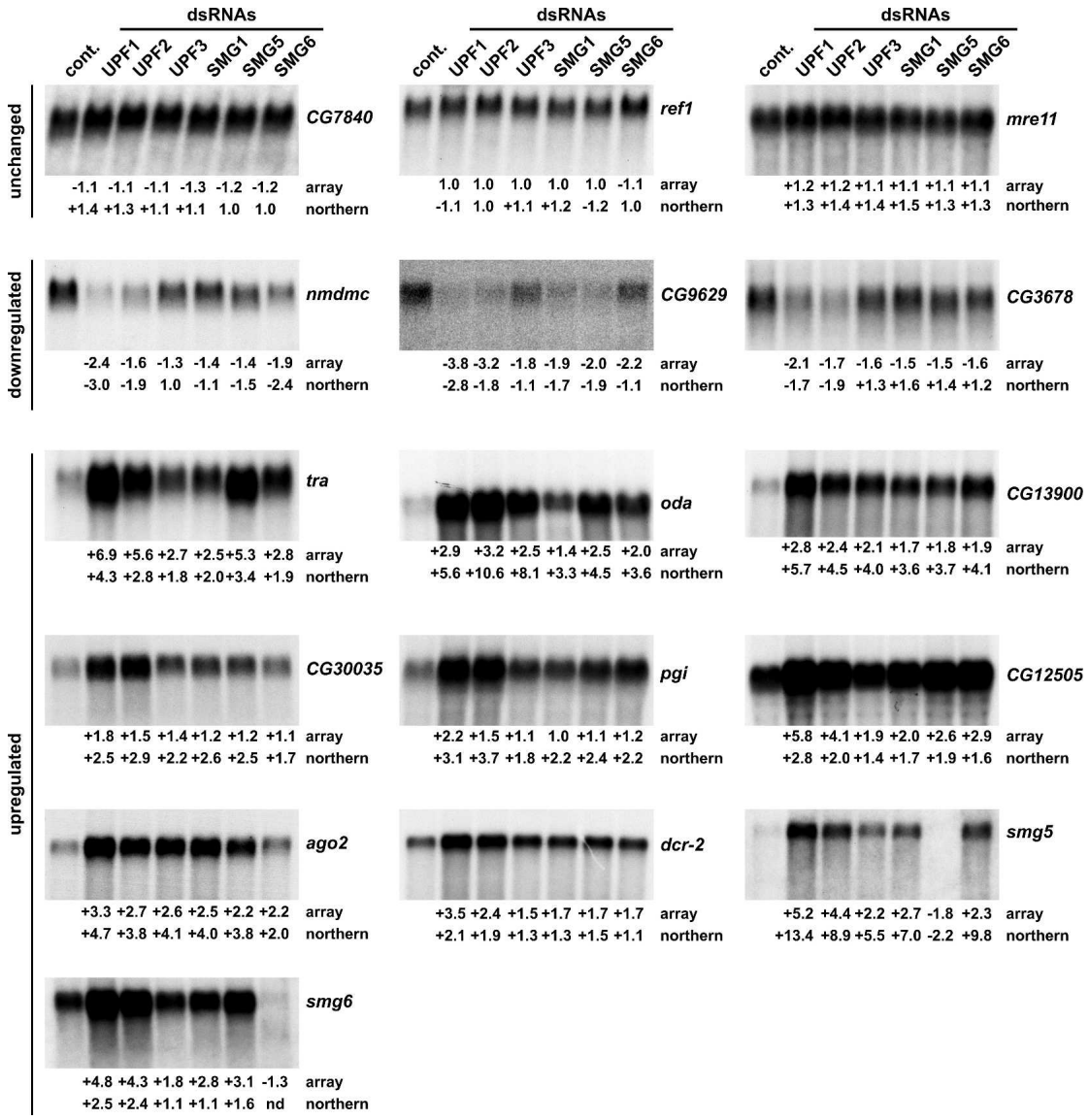


FIGURE 3. Validation of microarray results by Northern blot analysis. The identity of the selected transcripts is indicated on the *right*. The signals from the Northern blot were normalized to 18S rRNA (not shown). These values were compared with the values measured by microarray (average of two independent profiles). Values are given as fold changes relative to the values obtained in mock treated (cont.) cells (positive values, overrepresented; negative values, underrepresented).

pared these profiles to the profiles observed when proteins involved in a different step of gene expression were depleted, in particular when components of the THO complex were depleted. The THO complex is involved in mRNP assembly and export; its depletion impairs cell proliferation and leads to an increase in the proportion of cells in the G2/M phase similar to that observed in cells depleted of SMG5 or SMG6 (data not shown) (Rehwinkel et al. 2004). In addition, THO depletion alters the expression of a similar fraction of the transcriptome (Fig. 2; Rehwinkel et al. 2004). When compared to the expression profiles observed in the THO knockdown, the profiles displayed by cells depleted of NMD factors were similar to each other and clustered to one branch of the experimental tree (Fig. 2), suggesting that these profiles represent a specific signature of the NMD pathway.

To investigate further the similarity of the cellular response to the depletion of NMD factors, we selected mRNAs belonging to specific classes in the UPF1 and UPF3 knockdowns (at least 1.5-fold over- or 1.5-fold underrepresented, respectively) and analyzed their levels in the other knockdowns (Fig. 4A–D). A high degree of overlap was observed between upregulated transcripts in the individual knockdowns. This is consistent with the prediction that direct targets of the pathway would be upregulated in NMD-deficient cells. Indeed, of the 525 mRNAs that were

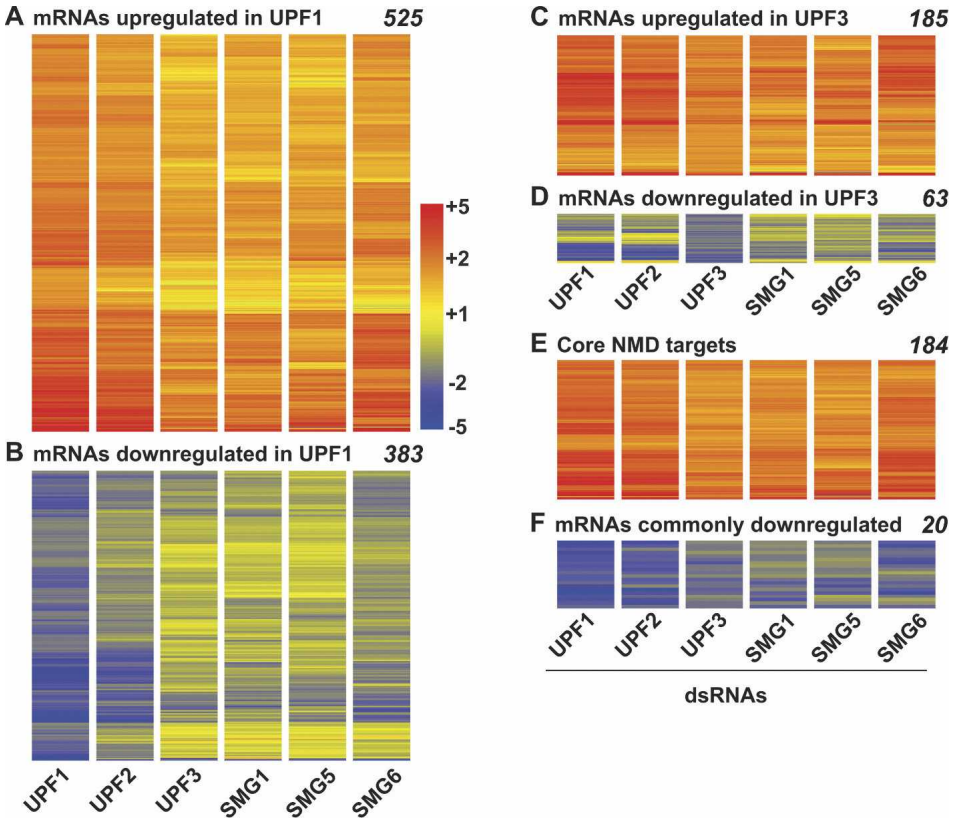


FIGURE 4. NMD factors regulate a common set of transcripts. (*A–D*) Expression profiles of RNAs at least 1.5-fold over- and underrepresented, respectively, in UPF1-depleted cells (*A*,*B*), or in UPF3-depleted cells (*C*,*D*) and detectable in all depletions. Average expression levels of two independent profiles per protein are shown. (*E*) Core NMD targets corresponding to transcripts at least 1.5-fold upregulated in 10 out of 12 profiles of cells depleted of NMD factors. (*F*) Transcripts at least 1.5-fold downregulated in 10 out of 12 profiles of cells depleted of NMD factors. RNAs are represented as lines and colored relative to their expression levels, as indicated. Note that the lines are wider in (*F*). The number of mRNAs displayed per panel is indicated in italics.

at least 1.5-fold overrepresented in the UPF1 knockdown, 88.4% and 74.9% changed levels coordinately in UPF2 and SMG6 depleted cells. Moreover, ca. 50% of these were 1.5-fold upregulated in UPF3, SMG1, and SMG5 knockdowns (Fig. 4A). Conversely, of the 185 mRNAs at least 1.5-fold overrepresented in the UPF3 knockdown, >80% were upregulated in the other knockdowns (Fig. 4C).

Downregulated transcripts overlapped to a lesser extent. Of the 383 mRNAs at least 1.5-fold underrepresented in the UPF1 knockdown, 66.6% and 45.5% were underrepresented in UPF2 and SMG6-depleted cells, respectively, and ca. 18% in the other knockdowns (Fig. 4B). Finally, between 27% and 68% of the 63 transcripts downregulated in the UPF3 knockdown were also underrepresented in the other knockdowns (Fig. 4D). Taken together, these results demonstrate that depletion of NMD proteins affects the expression of a common set of RNAs.

Identification of a core set of transcripts regulated by the NMD pathway

To identify a core set of transcripts regulated by the NMD pathway, we generated a list of 184 mRNAs that were at least 1.5-fold upregulated in 10 out of 12 profiles (six factors were depleted and two independent profiles were obtained for each factor) (Fig. 4E; Supplementary Table S1). We used this filtering criterion to avoid the exclusion of mRNAs that were only weakly affected in a specific depletion. As expected from the profiles shown in Figure 4B and D, when the same filtering criterion was applied to downregulated transcripts, only 20 mRNAs were found to be at least 1.5-fold underrepresented in 10 out of 12 profiles (Fig. 4F; Supplementary Table S2). These results are in agreement with those reported in yeast (He et al. 2003) and show that the majority of mRNAs commonly affected by the depletion of NMD factors are upregulated.

Core transcripts represent 3.4% of detectable transcripts and include oda mRNA, which is the only endogenous NMD target that has been experimentally validated (Gatfield and Izaurralde, 2004). Core transcripts also include smg5 and smg6 mRNAs (Supplementary Table S1). Both mRNAs were at least 1.5-fold overrepresented in depletions of all other NMD factors (Fig. 3), indicating that smg5 and smg6 mRNAs are targets of a feedback mechanism. In contrast, upf1, upf2, and smg1 transcript levels remained unchanged in cells depleted of other NMD factors, and upf3

mRNA levels were reduced as judged by RT-PCR (Supplementary Fig. S1).

To assess the fraction of core transcripts that represent authentic NMD targets, we selected eight mRNAs involved in diverse cellular processes and analyzed whether 3′-decay intermediates could be observed in cells depleted of XRN1. The rationale behind this experiment is that decay via the NMD pathway is initiated by endonucleolytic cleavage in *Drosophila,* and the resulting 3′-fragment is degraded by XRN1. Thus, for direct targets, 3′-decay intermediates are expected to accumulate in XRN1-depleted cells (Gatfield and Izaurralde 2004).

We observed decay intermediates for all eight selected mRNAs, including smg5 and smg6, and also for oda, which served as a positive control (Fig. 5A–I). In contrast, no decay intermediates were observed for two mRNAs (pgi, CG30035) (Fig. 5J,K) that were not included in the list of core targets, as the expression levels of these mRNAs remained unchanged in cells depleted of SMG1, SMG5, and SMG6, although these mRNAs were upregulated in the UPF1 or UPF2 knockdowns (Fig. 3). These results validate our filtering criteria and suggest that the majority of core transcripts represent primary NMD targets.

Features of NMD targets

We next examined the chromosomal positions of genes encoding core transcripts. In addition to the X and Y chromosomes, there are two large autosomes (chromosomes 2 and 3), and a small fourth chromosome in *Drosophila*. NMD targets exhibited an unbiased distribution toward the arms of chromosomes 2 and 3 (designated 2L, 2R, 3L, and 3R), with core transcripts representing 1.39%–1.64% of all transcripts derived from these chromosomes arms (Fig. 6A). Remarkably, on the X chromosome, only 0.54% of transcripts were upregulated, although the fraction of detectable transcripts derived from this chromosome was very similar to that of the autosomes (Fig. 6A). The probability to obtain by chance an equally low or lower number of regulated genes on the X chromosome is P value = 2×10^{-5}. A similar underrepresentation of potential endogenous NMD targets has been reported for the

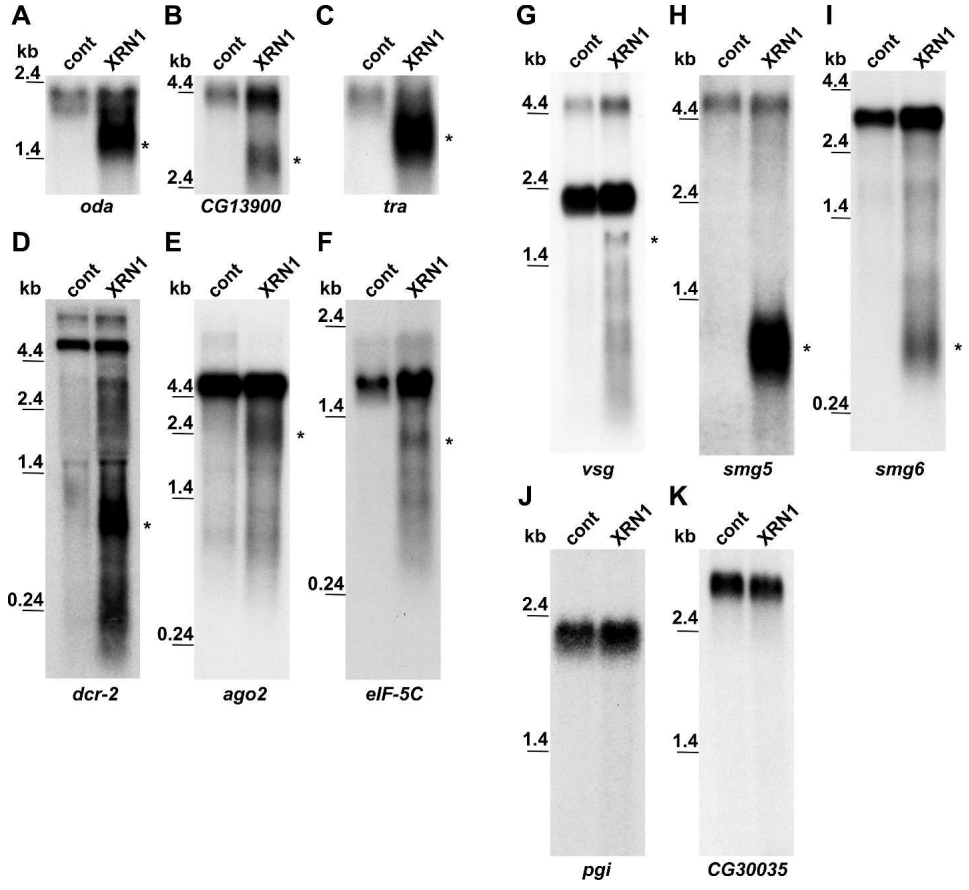


FIGURE 5. Core transcripts represent authentic NMD targets. (*A–K*) RNA samples isolated from control cells (cont.) or cells depleted of XRN1 were analyzed by Northern blot using probes complementary to the mRNAs indicated *below* the panels. Asterisks indicate the positions of the 3′-decay intermediates. No decay intermediates were detected for CG30035 or pgi. To detect the ago2, dcr-2, smg6 decay intermediates, poly(A)$^+$ RNAs was isolated and analyzed on a 1.6% agarose gel. The position of RNA size markers is indicated on the *left*.

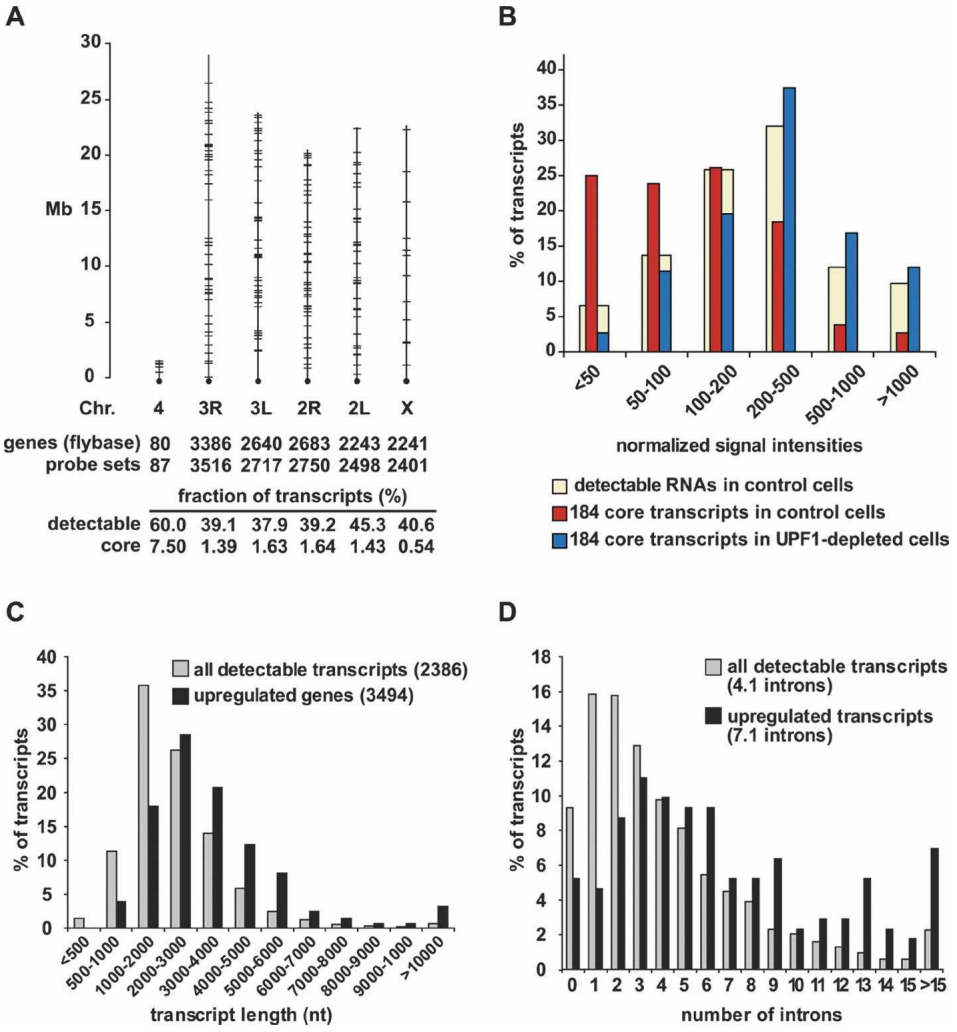


FIGURE 6. Features of NMD targets. (*A*) *Drosophila* chromosomes are represented as vertical lines. Centromeres are located at the *bottom* of the panel and are shown as dots. The scale bar on the *left* corresponds to the euchromatic sequence. Horizontal lines indicate the positions of core transcripts shown in Figure 4E. The number of genes and of probe sets present in the array per chromosome is indicated *below* the panel. The fractions of detectable and upregulated transcripts are given in percentages. (*B*) Signal intensities (averages of two independent experiments) after normalization are shown in a histogram plot for all detectable transcripts in control cells (yellow bars) as well as for core transcripts in control cells (red bars) or in UPF1-depleted cells (blue bars). (*C*) The distribution of transcript lengths is shown for detectable and core transcripts. Numbers in brackets indicate the average length in nucleotides. (*D*) The distribution of intron counts per gene is shown for detectable and core transcripts. Numbers in brackets indicate the average number of introns.

human X chromosome (Xing and Lee 2004). On the short chromosome 4, 7.5% of detectable transcripts were upregulated (Fig. 6A). The enrichment for NMD targets on this chromosome is significant (P value = 5.5×10^{-3}), despite the small number of genes found on this chromosome (Fig. 6A).

Previous studies in yeast have shown that mRNAs regulated by the NMD pathway are enriched in low abundance transcripts (He et al. 2003). Compared to the distribution of abundance of detectable transcripts (Fig. 6B, yellow bars), upregulated transcripts show a significant bias toward low abundance in control cells (red bars), but the usual distribution in UPF1-depleted cells (blue bars) indicates that these transcripts are not intrinsically of low abundance, but that their levels are downregulated by the NMD pathway in wild-type cells.

Next, we analyzed core mRNAs with respect to the GC content, the length of the transcript and of the coding sequence, the number of introns, and the presence of upstream open reading frames (uORFs). No apparent difference in GC content was observed (data not shown). The lengths of 5′ and 3′ UTRs were very similar for core and all detectable transcripts. However, core transcripts were on average longer than detectable transcripts (average length 3494 nt for core RNAs versus 2386 nt for all detectable transcripts, Fig. 6C). This increased length could be attrib-

uted to the coding sequences, which were skewed toward long ORFs (2701 nt versus 1714 nt, respectively).

Intronless genes or genes having one or two introns were underrepresented among core transcripts, while genes with six or more introns were enriched (Fig. 6D). Nevertheless, the density of introns per 500 nucleotides (nt) was 0.6 for both core and detectable transcripts (data not shown). These results suggest that long genes with a high number of introns are more likely to generate aberrant mRNA isoforms as a result of alternative splicing events and/or splicing errors. Consistent with this, the average number of annotated splice isoforms per gene was 1.9 for core transcripts and 1.5 for all detectable mRNAs. Transcripts with uORFs are often regulated by NMD, as in these cases the stop codon of the uORFs is interpreted as a PTC. We detected uORFs (of at least 10 codons) in 46% of the core transcripts, but only in 33% of detected mRNAs.

NMD targets are associated with a broad range of cellular functions

Inspection of gene ontology (GO) terms associated with core transcripts (Ashburner et al. 2000) revealed that NMD plays a widespread role in diverse cellular processes by regulating the expression of several transcripts associated with these processes (Fig. 7A; Supplementary Table S1). These include 35 transcripts involved in developmental processes, 28 transcripts involved in signal transduction, 22 mRNAs involved in cytoskeleton organization and biogenesis, 16 transcripts associated with transcription regulatory activity, eight transcripts

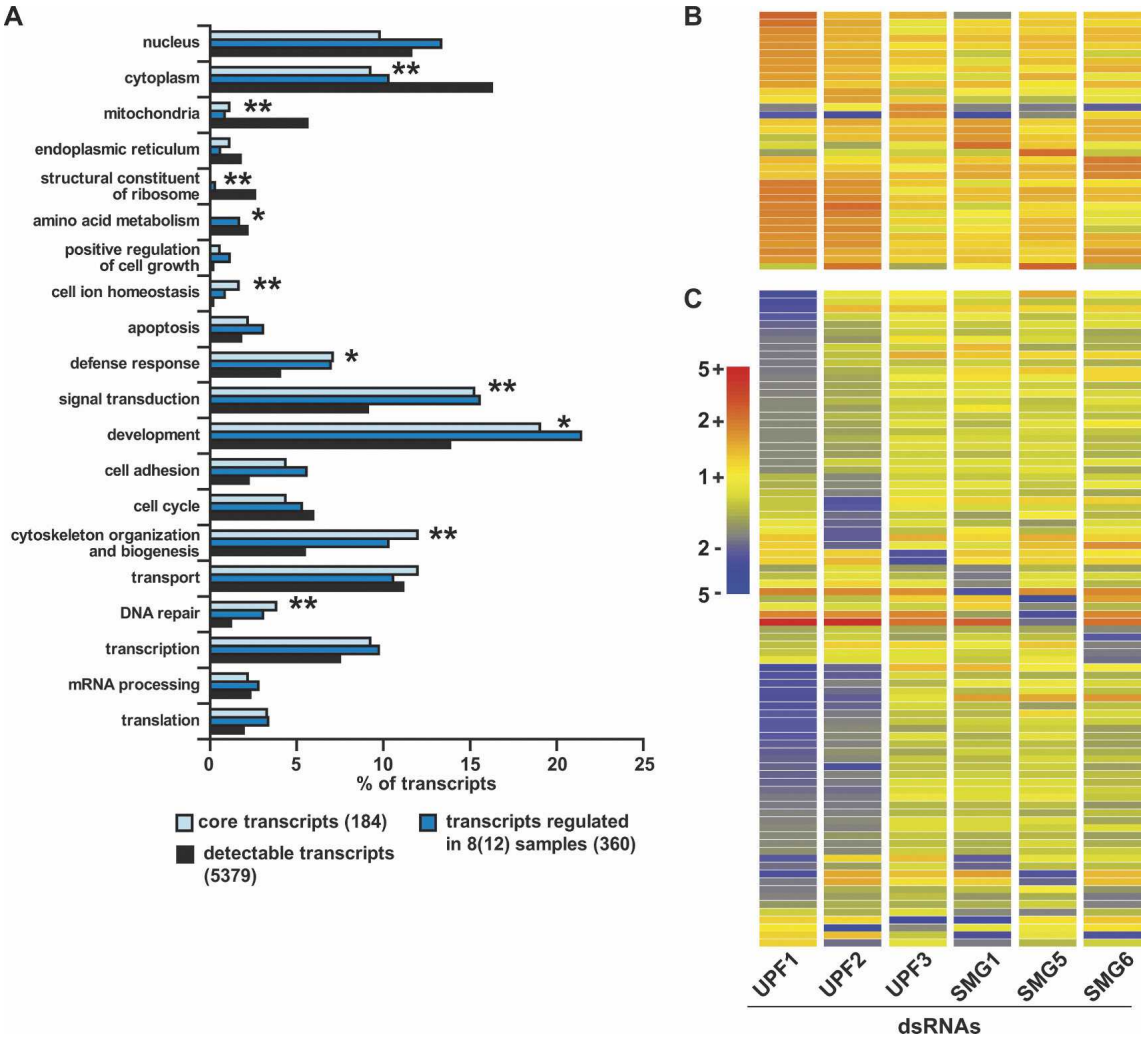


FIGURE 7. NMD regulates the expression of transcripts associated with diverse cellular processes. (*A*) Percentage of genes associated with the functional categories shown on the *left*. Black bars: 5379 detectable RNAs; cyan bars: core transcripts (184 mRNAs); blue bars: upregulated transcripts in eight out of 12 profiles obtained for NMD factors (360 mRNAs). Asterisks indicate functional categories for which the enrichment or the underrepresentation among core transcripts is significant (two asterisks, P value $< 1 \times 10^{-2}$, one asterisk, P value $< 5 \times 10^{-2}$). (*B*,*C*) Transcripts exclusively regulated in the individual knockdowns. Transcripts up- or downregulated in cells depleted of one or two NMD factors but unaffected in the other depletions.

involved in cell cycle, eight transcripts associated with cell adhesion, and seven transcripts involved in DNA repair. The role of NMD in regulating the expression of genes associated with these cellular activities is also reflected by the overrepresentation of the following descriptions of the molecular function ontology: damaged DNA binding (P value = 1.2×10^{-3}), protein–tyrosine kinase activity (P value = 3.7×10^{-2}), and protein–tyrosine phosphatase activity (P value = 1.3×10^{-2}), which were significantly enriched relative to their representation among detectable transcripts.

In human cells, UPF1 targets include genes involved in amino acid metabolism and in the response to amino acid starvation (Mendell et al. 2004). Transcripts associated with amino acid homeostasis where present, although not enriched amongst yeast NMD targets (He et al. 2003; Mendell et al. 2004). Based on these observations Mendell et al. (2004) hypothesized that NMD has a conserved role in regulating the expression of transcripts involved in the response to amino acid deprivation.

The list of core transcripts includes only one transcript associated with amino acid metabolism. Since the choice of the filtering criterium for selecting NMD targets is rather stringent (upregulation in 10 out of 12 profiles), it is possible that some authentic targets are overlooked. We therefore analyzed transcripts upregulated at least 1.5-fold in eight out of 12 profiles (360 RNAs representing 6.7% of the transcriptome, Supplementary Table S3). Overall, the representation of functional categories associated with this set of transcripts remained unchanged (Fig. 7A, blue bars). This list includes only three additional transcripts involved in amino acid metabolism, and in particular CG2107, whose homolog YAT2/YER024w was also among yeast NMD targets (He et al. 2003). Thus, transcripts involved in amino acid metabolism are not overrepresented among *Drosophila* NMD targets.

The response to starvation is complex, and involves genes associated with a broad range of cellular activities (Zinke et al. 2002; Harbison et al. 2005). We therefore analyzed whether core NMD targets were enriched in transcripts shown to be upregulated in response to starvation stress in two independent studies (Zinke et al. 2002; Harbison et al. 2005). We found 29 and 50 transcripts identified by Zinke et al. (2002) and Harbison et al. (2005), respectively, among core NMD targets. Of these, 13 transcripts were identified in the two studies and include smg5 and smg6, suggesting a potential link between NMD and the starvation response. Validation of this link requires further studies and, in particular, the investigation of the response to amino acid deprivation in NMD-deficient cells.

NMD regulates the expression of heterologous genes in different species

Targets of the NMD pathway have been identified in both yeast and human cells (He et al. 2003; Mendell et al. 2004). We therefore investigated whether *Drosophila* orthologs of these targets were regulated in NMD-deficient SL2 cells. Using the STRING database (von Mering et al. 2005), which employs clusters of orthologous groups of proteins (KOGs) (Tatusov et al. 1997), *Drosophila*, human, and yeast NMD targets could be assigned to 159, 170, and 316 KOGs, respectively (not shown). Remarkably, only two KOGs were represented in all three organisms: "monocarboxylate transporter" (KOG2504) and "nonsense-mediated mRNA decay protein" (KOG2162; Table 1). The monocarboxylate transporter group includes *Drosophila* CG8399, yeast ESBP6, and human SLC16A3 and SLC16A6. The NMD group includes *Drosophila* SMG5 and SMG6, human SMG5, and yeast Ebs1p. Ebs1p is related to Est1p (which was also among yeast NMD targets); both proteins are partially redundant and are involved in telomere maintenance (Zhou et al. 2000).

Eight additional KOGs were represented in the list of *Drosophila* and yeast NMD targets, but not in the list of transcripts regulated by human UPF1 (Table 1). Of these, KOG0892 includes yeast Tel1 and *Drosophila* ATM (CG6535). These proteins are involved in DNA repair and maintenance of telomere ends (Pandita 2002). Moreover, homologs of nine yeast NMD targets were present in the list of transcripts upregulated in eight out of 12 profiles (Supplementary Table S3). Two of the yeast transcripts (YAT2/YER024W and HFM1/YGL251C) have uORFs. In contrast, we could not detect uORFs in the corresponding *Drosophila* homologs (CG2107 and CG5205, respectively). These results indicate that even in cases in which related genes are regulated by NMD in diverse species, the features recognized by the NMD machinery may not be conserved.

The comparison of human and *Drosophila* NMD targets revealed 10 additional KOGs represented in both organisms, but not in yeast. These include "RhoGTPase-activating proteins" (KOG4406), "protein tyrosine phosphatases" (KOG4228) and "tyrosine kinases" (KOG0197) (Table 1). KOG4406 is represented by the RhoGTPase activating protein 1 (Rho GAP1) in human and by RhoGAP68F in *Drosophila*. For the human *RhoGAP1* gene an alternative splicing event generates a PTC-containing isoform (Mendell et al. 2004). In *Drosophila*, there are no alternative splice isoforms annotated, although the gene contains five introns, and alternative splicing events cannot be ruled out. Other KOGs represented among yeast and *Drosophila* or human and *Drosophila* NMD targets were loosely defined by protein domains (ankyrin repeat, Zinc-finger, RNA recognition motif, PDZ domain) and may not reflect true orthologous relationships (Table 1).

In summary, the KOG analysis reveals that the majority of NMD targets in *Drosophila* are not orthologs of genes regulated by NMD in yeast or human cells, indicating that the repertoire of genes targeted by NMD has not been conserved during evolution.

TABLE 1. NMD regulates heterologous gene across species

KOG	*Drosophila*	Yeast	Human
KOG2162 (nonsense-mediated mRNA decay protein)	*Smg5* *Smg6*	*EBS1*	*SMG5*
KOG2504 (monocarboxylate transporter)	*CG8389*	*ESBP6*	*SLC16A3* *SLC16A6*
KOG0504 (FOG: ankyrin repeat)	*cact*	*AVO2*	
NOG08584 (nonsupervised orthologous group)	*CG3532*	*ADY3*	
KOG0892 (protein kinase ATM/Tel1, involved in telomere length regulation and DNA repair)	*CG6535*	*TEL1*	
KOG0255 (synaptic vesicle transporter SVOP and related transporters [major facilitator superfamily])	*CG4630*	*FLR1* *QDR3* *QDR1*	
KOG2283 (clathrin coat dissociation kinase GAK/PTEN/Auxilin and related tyrosine phosphatases)	*auxillin*	*TEP1*	
KOG0867 (Glutathione S-transferase)	*GstE3*	*GTT2*	
KOG3022 (Predicted ATPase, nucleotide binding)	*CG3262*	*CFD1*	
KOG1721 (FOG: Zn-finger)	*CG10462* *MTF-1* *CG2199* *CG1233* *CG8092* *Aef1*	*FZF1* *RGM1* *MIG3*	
KOG0118 (FOG: RRM domain)	*CG33070*		*HNRPA1*
KOG3528 (FOG: PDZ domain)	*CG30023*		*PDZK3*
KOG4228 (protein tyrosine phosphatase)	*Ptp69D*		*PTPRF*
KOG1812 (predicted E3 ubiquitin ligase)	*ari-2*		*TRIAD3*
KOG4406 (CDC42 Rho GTPase-activating protein)	*RhoGAP68F*		*ARHGAP1*
KOG1218 (proteins containing Ca^{2+}-binding EGF-like domains)	*CG8942*		*DKK1*
KOG3520 (predicted guanine nucleotide exchange factor)	*RhoGEF2*		*P114-RHO-GEF* *ARHGEF2*
KOG0197 (tyrosine kinases)	*hop*		*FYN*
KOG2836 (protein tyrosine phosphatase IVA1)	*PRL-1*		*PTP4A2*
KOG1716 (dual specificity phosphatase)	*CG14211*		*DUSP3* *DUSP1* *DUSP10*

Yeast, *Drosophila*, and human NMD targets were sorted in clusters of orthologous groups of proteins (KOGs). Conserved KOGs are shown.

A few transcripts are regulated exclusively in the individual knockdowns

To investigate whether some NMD factors may have acquired additional roles in mRNA turnover we asked whether the expression levels of specific RNAs were regulated only in depletions of one or two NMD factors (at least 1.5-fold), while remaining unaffected in depletions of the other factors (i.e., less than 1.5-fold; Supplementary Table S4). As shown in Figure 7B, we found 10 and two transcripts exclusively upregulated in cells depleted of UPF1 and UPF2, respectively, and nine upregulated in the two knockdowns. The possibility that these transcripts represent authentic NMD targets that are only weakly affected in the other knockdowns cannot be ruled out.

We were able to identify a small number of transcripts exclusively upregulated in the UPF3, SMG1, SMG5, and SMG6 knockdowns or commonly regulated by UPF1 and SMG6 or by UPF2 and SMG5 (Fig. 7B; Supplementary Table S4). The expression levels of these transcripts remained unchanged in cells depleted of UPF1 and/or UPF2, and in some cases showed inverse correlation in the other depletions (i.e., transcripts regulated by UPF3), so it is unlikely that these transcripts represent authentic NMD targets.

Similar filters were applied to identify transcripts uniquely downregulated in one or more depletions and unchanged in the other knockdowns (Supplementary Table S4). Again, only a small number of transcripts were found to be exclusively downregulated in specific depletions (Fig. 7C). We therefore conclude that NMD factors function together, and only a small fraction of transcripts depends on individual NMD components for optimal expression.

DISCUSSION

Eukaryotic cells have evolved elaborate mRNA quality control mechanisms to ensure the fidelity of gene expression by detecting and degrading aberrant transcripts. The NMD pathway is among the best characterized mRNA

surveillance mechanism. It eliminates mRNAs containing nonsense mutations and regulates the expression of wild-type transcripts having features interpreted as aberrant by the NMD machinery. In this study we show that NMD plays an important role in post-transcriptional regulation of a broad range of biological activities, including cell proliferation in *Drosophila*. By comparing NMD targets identified in this study with those previously identified in yeast and human cells (He et al. 2003; Mendell et al. 2004), we demonstrated that NMD regulates the expression of heterologous genes across species. Finally, we provide evidence that the major role of UPF1, UPF2, UPF3, SMG1, SMG5, and SMG6 in mRNA turnover is to act as partners in the NMD pathway.

It has been reported that mutations in the *smg1* gene do not affect NMD in *Drosophila* embryos (Chen et al. 2005). These observations led Chen et al. (2005) to conclude that SMG1 is not required for NMD in *Drosophila*. Although it is possible that UPF1 is phosphorylated by another redundant kinase, in previous studies we showed that depletion of SMG1 stabilizes different PTC-containing reporters in SL2 to a similar extent as UPF3 depletion (Gatfield et al. 2003). The results shown in this study extend our previous observations and clearly demonstrate that depletion of SMG1 leads to similar changes in mRNA expression levels as the depletion of UPF3 or SMG5. We therefore conclude that SMG1 is a bona fide component of the NMD machinery in *Drosophila*.

Features of endogenous transcripts regulated by NMD

There are only few common features associated with the NMD targets identified in this study. First, these transcripts are expressed at low levels in wild-type cells, but their expression increases in NMD-deficient cells. This observation is consistent with the conclusion that these transcripts are post-transcriptionally regulated by NMD. Second, these transcripts are on average longer than the ensemble of detectable transcripts. This is explained by an increase in the average length of the ORFs rather than of the 5′ or 3′ UTRs. Finally, *Drosophila* NMD targets are underrepresented on the X chromosome and overrepresented on Chromosome IV.

Bioinformatic approaches have revealed that in human cells a substantial fraction of alternatively spliced transcripts contain PTCs, and represent potential NMD targets (Green et al. 2003; Lewis et al. 2003). The overall PTC rate is about threefold lower on the human X chromosome relative to the autosomes (Xing and Lee 2004). It has been proposed that negative selection pressure against aberrant PTC-containing transcripts is stronger for genes expressed as a single copy (Xing and Lee 2004). Although X chromosome inactivation does not occur in *Drosophila*, we observed an underrepresentation of NMD targets on this chromosome, suggesting that haploidy alone in males is sufficient to maintain negative selection pressure against the accumulation of PTC-containing isoforms. It would be of interest to determine whether the overrepresentation of NMD targets on chromosome 4 correlates with the absence of recombination on this chromosome (Nachman 2002).

In yeast and human cells, several classes of wild-type transcripts are regulated by NMD. These include: (1) mRNAs with upstream open reading frames (uORFs) in the 5′ unstranslated region (UTRs); (2) mRNAs with nonsense codons or frameshifts introduced by an alternative splicing event; (3) mRNAs undergoing "leaky-scanning" for translation initiation; (4) mRNAs regulated by programmed frameshifting; (5) mRNAs with selenocysteine codons; and (6) mRNAs regulated by stop codon readthrough (Lelivelt and Culbertson 1999; He et al. 2003; Mendell et al. 2004; for review, see Lejeune and Maquat 2005).

The specific features recognized by the *Drosophila* NMD machinery are known for a few of the targets. For instance, oda mRNA is regulated by programmed frameshifting and the transformer (*tra*) gene generates two mRNA isoforms of which one has a PTC (Boggs et al. 1987; Ivanov et al. 1998). Moreover, four transcripts found in the extended list of targets have been predicted to be regulated by stop codon readthrough (zfh1, Ipp, Fs[2]Ket and capu) (Sato et al. 2003). *Drosophila* mRNAs undergoing NMD are cleaved in the vicinity of the PTC (Gatfield and Izaurralde, 2004), so the size of the 3′-decay intermediate provides information on the position of the aberrant translation termination event. For smg6 mRNA for instance, the cleavage occurs in the vicinity of the natural stop (Fig. 5; data not shown), suggesting that this mRNA could be regulated by stop codon read-through. These findings illustrate that NMD targets are not unified by a single common feature, but represent a heterogeneous group of mRNAs. Hence, detailed studies are required to elucidate the mechanism by which the individual targets are regulated.

NMD target-genes function in diverse cellular processes

Analyses of the gene ontology terms associated with transcripts regulated by NMD revealed that some fall into functional categories that are overrepresented relative to the genome (e.g., damaged DNA binding, protein–tyrosine kinase activity, and protein–tyrosine phosphatase activity). However, it is the diversity of functional categories that is most striking, of which regulation of developmental processes, signal transduction, and cytoskeletal biogenesis are associated with the largest number of genes.

These functional categories are also represented among NMD targets identified in yeast and human cells, but, with a few exceptions, targets in different species do not represent orthologous genes. Indeed, analysis of orthologous groups (KOGs) represented amongst yeast, *Drosophila* and human NMD targets revealed that only two KOGs are

conserved: "monocarboxylate transporter" and "nonsense-mediated mRNA decay protein." Monocarboxylate transporters (MCTs) are involved in the transport of monocarboxylates such as lactate and pyruvate, and thus in the regulation of food intake and glucose homeostasis (Halestrap and Price 1999). This finding should, nevertheless, be interpreted with caution, as *Drosophila* CG8389 has not been characterized and might transport different monocarboxylates or unrelated substrates (Halestrap and Price 1999).

The NMD KOG is represented by *Drosophila* SMG5 and SMG6, human SMG5, and yeast Esb1p. Esb1p is related and partially redundant with the telomerase-associated protein Est1p (Zhou et al. 2000), and both are closely related to SMG5–7 (Reichenbach et al. 2003). Est1p is also regulated by NMD in yeast (He et al. 2003). In addition to *EST1* and *ESB1*, five genes involved in telomere maintenance are regulated by NMD in yeast, including *EST2*, *EST3*, *STN1*, *YKu70*, and *TEL1* (Dahlseid et al. 2003; He et al. 2003). This regulation has functional implications, as inhibition of NMD in yeast leads to telomere shortening and derepression of silenced telomeric loci (Lew et al. 1998; Dahlseid et al. 2003; He et al. 2003). Moreover, Tel1p belongs to a KOG that includes *Drosophila* ATM, which is also an NMD target; both proteins are involved in telomere length regulation and DNA repair (Pandita 2002). These observations, together with recent reports implicating human SMG5 and SMG6 in telomerase function (Reichenbach et al. 2003; Snow et al. 2003), suggest that the role of NMD in regulating the expression of genes involved in telomere maintenance is conserved.

Interestingly, smg5 and smg6 were also found to be upregulated in response to starvation stress in two independent studies performed in embryos and adult flies, respectively (Zinke et al. 2002; Harbison et al. 2005). In addition to smg5 and smg6, 11 transcripts commonly identified in the two studies are present in the list of *Drosophila* NMD targets. Thus it is possible that the NMD pathway has been integrated into metabolic circuits activated in response to nutrient deprivation, as suggested by Mendell et al. (2004).

Finally, mRNAs encoding AGO2 and Dicer-2 are upregulated in NMD-deficient cells. However, RNAi efficiency in these cells is not apparently altered (Rehwinkel et al. 2005), suggesting that these proteins may not be limiting in wild-type cells. In *C. elegans,* UPF1 (i.e., SMG-2), SMG5 and SMG6 are required for persistence of RNAi, but not for the establishment of silencing, suggesting that these NMD factors act downstream of RISC (Domeier et al. 2000; Kim et al. 2005a). In contrast, UPF2, UPF3, and SMG-1, which are also essential for NMD, are not required to maintain silencing, and thus persistence of RNAi does not depend on the NMD pathway per se (Domeier et al. 2000). Maintenance of silencing involves the amplification of the RNA trigger by a RNA-dependent RNA polymerase, a process that does not occur in *Drosophila* (Roignant et al. 2003). It would therefore be of interest to determine whether *C. elegans* UPF1, SMG-5, and SMG-6 affect RNAi indirectly, e.g., by altering the expression levels of genes encoding components of the RNA silencing machinery or whether these NMD factors have acquired a specialized role in RNAi in this organism.

Evolutionary diversification of the physiological role of NMD

The different phenotypes observed upon inhibition of the NMD pathway across species could be explained by the acquisition of novel functions by NMD components during evolution. Along these lines, recent reports have implicated a subset of NMD factors in other cellular processes including telomere maintenance and DNA repair (Reichenbach et al. 2003; Snow et al. 2003; Brumbaugh et al. 2004). These additional functions may not result in changes in mRNA expression levels and cannot be revealed by profiling experiments. Therefore, we cannot rule out the possibility that NMD factors have acquired specialized roles in cellular processes distinct from mRNA decay. Furthermore, it has been reported that human UPF1 can be recruited to the 3′ UTR of specific transcripts via interactions with Staufen1 (Kim et al. 2005b). In this way, UPF1 elicits mRNA decay by a mechanism not requiring UPF2 or UPF3 (Kim et al. 2005b).

In principle, recruitment of any of the core components of the NMD machinery to a transcript by heterotypic interactions could lead to mRNA decay, in a process that may or may not require additional NMD components. Our genome-wide analysis shows, however, that only a few transcripts are specifically regulated by individual NMD components in *Drosophila*. For instance, transcripts exclusively regulated by UPF3, SMG1, SMG5, or SMG6 are unlikely to represent authentic NMD targets, and may be regulated by a mechanism unrelated to NMD. Whether the regulation of these transcripts reflects specialized functions of these proteins, indirect effects of the depletion or of the specific dsRNAs remain to be established.

Another mechanism that can lead to phenotypic differences is changes in selected targets. Evidence already exists that this is indeed the case. For instance, rearrangements of the immunoglobulin and T-cell receptor genes in vertebrates result in frame-shifted genes at high frequency (ca. 66% of the recombination events), and transcripts from these genes are degraded by NMD (Holbrook et al. 2004). In addition, differences in the mechanism by which premature stop codons are recognized across species are likely to contribute to changes in selected targets. For instance, human transcripts containing introns in the 3′ UTR are also targeted by NMD if the intron is located at least 50 nt downstream of the natural stop (Mendell et al. 2004). In this study we provide evidence that NMD regulates heterologous genes across species.

In conclusion, although we cannot rule out that individual NMD factors regulate the expression of specific transcripts or have acquired specialized functions that do not affect steady-state mRNA levels, a conserved role of these proteins is to regulate in concert the expression of a common set of genes. This set differs between species. In a given organism, target-genes which are ubiquitously expressed are expected to be regulated in all cell types; nevertheless, some targets may have temporal and/or cell-specific expression patterns, so that the constellation of regulated genes may vary in different developmental stages as well as in a tissue specific manner. In this way, changes in the physiological role of NMD are largely driven by target selection in addition to a potential functional diversification of its components.

MATERIALS AND METHODS

RNA interference and RNA isolation

RNA interference in cultured *Drosophila* Schneider cells was performed essentially as described in Herold et al. (2001). dsRNAs used in this study correspond to fragments encompassing about 700 nt of the coding sequences and have been described before (Gatfield et al. 2003; Gatfield and Izaurralde 2004). All depletions were analyzed on day 8 after a reknockdown on day 4, except for POLO kinase, which was analyzed without reknockdown on day 4. Total RNA was isolated using TRIzol Reagent (Life Technologies).

Flow cytometric analysis

Cells were washed once in PBS, fixed in 70% ethanol for 2 h at 4°C, washed again in PBS, and stained for 2 h at 37°C using 0.1 mg/mL propidium-iodide in PBS. The staining solution was supplemented with 0.2 mg/mL RNaseA and 0.2% Triton X-100. Cells were analyzed on a FACScan (Becton-Dickinson).

Northern blots and RT-PCR

RNA samples were analyzed by Northern blot as described in Gatfield et al. (2003). Unless indicated otherwise, 1% agarose gels were used. To detect 3′-decay intermediates resulting from endonucleolytic cleavage, probes corresponding to the following nucleotide positions on the transcripts were used: oda-RA 168–898, transformer-RA 1–594, CG13900-RA 2351–2840, pgi-RA 1900–2476, CG30035-RA 2672–3016, smg5-RA 3735–4229, smg 6-RA 2701–3202, eIF-5C-RE 1296–1697, and vsg-RA 1622–2198. For dcr-2 and ago2, probes corresponding to the 3′-end of the coding sequences were used. In order to detect smg6, ago2, and dcr-2 transcripts by Northern blot, total RNA was enriched for poly(A)$^+$ RNA using oligo-dT cellulose (Ambion, small-scale mRNA purification kit). RT-PCR analysis was performed as described in Herold et al. (2001). PCR-primers amplifying the region targeted by the probe sets on the microarray were used to detect upf1, upf2, smg1, smg5, and smg6. For upf3, primers corresponding to positions 997 (sense) and 1515 (antisense) of the transcript were used to avoid overlap with the dsRNA.

Genome-wide expression analysis

High-density oligonucleotide microarrays covering more than 13,500 genes from *Drosophila* were used in this study (Affymetrix-chip "DrosGenome1"). The microarray results have been submitted to the ArrayExpress database at EBI under accession number E-MEXP-202. Biotinylated targets were prepared from 5 μg of total RNA according to standard Affymetrix procedures. Standard Affymetrix protocols were used for hybridization, washing, and data acquisition (Fluidics station 400, GeneArray 2500 scanner, Affymetrix Microarray suite version 5.1). Control parameters were within recommended limits. Data were imported into GeneSpring 6 (Silicon Genetics) (control sample = control channel, knockdown experiment = signal channel). All experiments were normalized using an intensity-dependent normalization scheme (Lowess). In a control experiment in which two independent replicates were compared all spots after intensity-dependent normalization had an average ratio of 1.03 ± 0.33. We therefore judged a change of at least 1.5-fold to be a meaningful cutoff.

To exclude mRNAs regulated unspecifically in response to the treatment with dsRNAs, we compared mock-treated cells and cells treated with GFP dsRNA. In two independent experiments, 87 and 83 mRNAs were consistently up- and downregulated, respectively, in cells treated with GFP dsRNA. These mRNAs were excluded from further analysis.

Characterization of NMD targets

Chromosomal positions of regulated transcripts were retrieved using analysis tools provided by Affymetrix (www.affymetrix.com). We analyzed transcript abundance using signal intensities after normalization in GeneSpring. Transcript lengths and GC content were analyzed using the BioMART tool available from the EBI (http://www.ebi.ac.uk/biomart/index.html). Intron and isoform counts, and 5′ UTRs were obtained from Ensembl (drosophila_melanogaster_core_30_3d, ftp.ensembl.org). For each annotated gene, the length, number of introns, and number of predicted isoforms were noted. To reduce the gene length bias, intron counts were calculated as introns per 500 nt. Upstream ORFs in each 5′ UTR were detected using a set of perl scripts. ORFs were defined as consecutive in-frame codons starting with ATG and ending with a stop codon (TAA, TGA, or TAG). The gene strand was used as the reference for the uORF strand. Clusters of orthologous groups of proteins (KOGs) (Tatusov et al. 1997) represented in the list of human, yeast, and *Drosophila* NMD targets were identified using the STRING database (von Mering et al. 2005).

Gene ontology analysis

The gene ontology (GO) nomenclature (Ashburner et al. 2000) was used to characterize protein products of genes exhibiting altered expression patterns. GO terms associated with regulated genes were identified using the GO mining tool (www.affymetrix.com) and exported to Excel. The over- or underrepresentation of GO-terms among regulated genes was assessed by the probability (*P* value) that an equally high or higher enrichment could be obtained by chance given the frequency of the GO-terms among detectable genes.

SUPPLEMENTARY MATERIAL

Supplementary materials are available at http://www-db.embl.de/jss/EmblGroupsHD/g_127?sP=4.

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