Functions of the Nonsense-Mediated mRNA Decay Pathway in *Drosophila* Development

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Nonsense-mediated mRNA decay (NMD) is a cellular surveillance mechanism that degrades transcripts containing premature translation termination codons, and it also influences expression of certain wild-type transcripts. Although the biochemical mechanisms of NMD have been studied intensively, its developmental functions and importance are less clear. Here, we describe the isolation and characterization of *Drosophila* “photoshop” mutations, which increase expression of green fluorescent protein and other transgenes. Mapping and molecular analyses show that photoshop mutations are loss-of-function mutations in the *Drosophila* homologs of NMD genes *Upf1*, *Upf2*, and *Smg1*. We find that *Upf1* and *Upf2* are broadly active during development, and they are required for NMD as well as for proper expression of dozens of wild-type genes during development and for larval viability. Genetic mosaic analysis shows that *Upf1* and *Upf2* are required for growth and/or survival of imaginal cell clones, but this defect can be overcome if surrounding wild-type cells are eliminated. By contrast, we find that the PI3K-related kinase *Smg1* potentiates but is not required for NMD or for viability, implying that the *Upf1* phosphorylation cycle that is required for mammalian and *Caenorhabditis elegans* NMD has a more limited role during *Drosophila* development. Finally, we show that the SV40 3’ UTR, present in many *Drosophila* transgenes, targets the transgenes for regulation by the NMD pathway. The results establish that the *Drosophila* NMD pathway is broadly active and essential for development, and one critical function of the pathway is to endow proliferating imaginal cells with a competitive growth advantage that prevents them from being overtaken by other proliferating cells.

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Introduction

Nonsense-mediated mRNA decay (NMD) is a cellular surveillance pathway in eukaryotes that recognizes and degrades transcripts with premature termination codons (PTCs). Such transcripts arise as a consequence of genomic mutation, as in numerous human genetic diseases [1,2], and from errors in transcription and aberrant RNA splicing. Destruction of PTC-containing transcripts by NMD prevents production of truncated, potentially harmful proteins that can interfere with normal cellular processes (e.g., [3]). The NMD pathway has also been found to influence expression of a variety of wild-type transcripts (reviewed in [4]), implying that the pathway has regulatory roles beyond its surveillance function. In this paper, we describe *Drosophila* mutants that affect NMD.

NMD pathway genes were discovered by genetic studies in yeast (up-frameshift suppressor [Upf] genes; [5]) and *Caenorhabditis elegans* (suppressor with morphogenetic effect on genitalia [smg] genes; [6]), and their functions and mechanisms of action have been characterized by molecular genetic and biochemical analysis of the proteins and target RNAs in yeast [7] and cultured mammalian and *Drosophila* cells [8–10]. There are three conserved core components of the pathway, *Upf1* (smg-2), *Upf2* (smg-3), and *Upf3* (smg-4) (reviewed in [11]). *Upf1* is an RNA helicase that associates with the translation termination complex at PTCs and, at least in yeast, targets the RNA to cytoplasmic RNA processing centers called P bodies [12]. *Upf1* is proposed to recruit *Upf2* and *Upf3* to these termination complexes, which leads to activation of decapping enzymes and nucleases that degrade the target RNA.

Additionally, in metazoans, *Upf1* undergoes a phosphor-ylation cycle (reviewed in [13]). *Upf1* is phosphorylated on serine residues by Smg1, a PI3K-related kinase. The phosphates are subsequently removed by complex(es) containing Smg5, Smg6, and/or Smg7, three similar proteins that are thought to recruit the phosphatase PPA2. The *Upf1* phosphorylation cycle is apparently necessary for *Upf1* and NMD activity at least in some organisms, because NMD function is abrogated when Smg1, Smg5, Smg6, or Smg7 activity is reduced [6,9,10,14].

One intriguing mechanistic question is how the NMD machinery distinguishes a PTC from a normal termination codon. In mammals, an important feature appears to be the...
Synopsis

Cells possess a variety of surveillance mechanisms that detect and dispose of defective gene products. One such system is the nonsense-mediated mRNA decay (NMD) pathway, which degrades aberrant mRNAs containing nonsense mutations or other premature translation stop signals. In a genetic screen in *Drosophila*, the authors identified a set of mutations they call "photoshop" mutations because they increase expression of green fluorescent protein transgenes such that cells expressing green fluorescent protein are more easily visualized. They found that the photoshop mutations are mutations in three different genes implicated in NMD. Using these mutations, they show that the NMD pathway not only degrades mutant mRNAs but also influences expression of many transgenes and dozens of endogenous genes during development and is essential for development beyond the larval stage. One important function of the pathway is to provide proliferating cells with a competitive growth advantage that prevents them from being overtaken by other proliferating cells during development. Thus, the *Drosophila* NMD pathway has critical cellular and developmental roles beyond the classical surveillance function of eliminating mutant transcripts.

Results

Identification of Mutations That Increase Transgene Expression in *Drosophila*

We conducted a genetic mosaic screen of ethane methyl sulfonate–induced mutations on the X chromosome for tracheal (respiratory) system mutants. Details of the screen will be described elsewhere. In the screen, we used the *S. cerevisiae* FLP1 recombinase (FLP)/FLP1 recombinase target (FRT) system [22] to generate homozygous mutant cell clones in otherwise heterozygous animals. Tracheal clones were identified by labeling them with GFP using the MARCM system [23] in which a *btl-GAL4* transgene drives tracheal expression of a *UAS-GFP* transgene but is kept off in heterozygous cells by a *btl-GAL80* transgene present on the wild-type X chromosome (Figure 1A). In a screen of 749 ethane methyl sulfonate–induced X-linked lethal lines, we identified six mutations (*13D, 14J, 25G, 26A, 29AA, and 32AP*) that caused markedly increased GFP signal in homogygous mutant tracheal clones compared to control wild-type clones examined as third instar (L3) larvae (Figure 1B). We named this the "photoshop" phenotype because it increased visualization of clones like that achieved by digital enhancement with Photoshop software (Adobe, http://www.adobe.com). The photoshop phenotype was not dependent on the *btl-GAL80* repressor in the MARCM system: homogygous photoshop tracheal cell clones in *btl-GAL4, UAS-GFP* larvae showed a similar enhancement of GFP signal, and viable hemizygous 25G and 32AP larvae and adults (see below) carrying the same transgenes showed increased GFP signal throughout the tracheal system (Figure 1C and 1D and data not shown). The photoshop phenotype was not specific to GFP, because mutant clones showed similar enhancement of DsRed1 signal (Figure 1E). All tracheal cells examined showed the photoshop phenotype, and all six mutations gave similar GFP enhancement.

All but two of the mutations (*14J, 29AA, 13D, and 26A*) lead to hemizygous male lethality before L3. One exception was 25G: 25G/T hemizygous males and 25G homogygous females developed to L3 larvae at approximately normal frequencies.
and produced a few percent of escaper adults. These adults appeared morphologically normal but had greatly reduced fertility. The other exception was 32AP, which after removal of extraneous linked lethal loci (see Materials and Methods) was found to be hemizygous-male and homozygous-female viable and fertile. We generated a deficiency uncovering 32AP and found that 32AP/Df showed the same GFP enhancement as 32AP/Y (Figure 1D), implying that 32AP is an amorphic (null) allele.

To study the effect of the mutations on GFP signal in other tissues and other stages of development, we used the viable alleles 25G and 32AP along with da-GAL4 and UAS-CD8:GFP transgenes, which give ubiquitous expression of GFP [24]. Compared to control animals, 25G/Y male and homozygous 25G female larvae and adults had greatly increased GFP in all tissues examined, including epidermis, salivary glands, fat body, and eyes (Figure 1F and data not shown). 32AP mutants also showed enhanced GFP signal in all tissues examined, although enhancement appeared slightly weaker than in 25G mutants. 32AP also enhanced levels of a nuclear localized DsRed2 construct expressed in the larval epidermis (Figure 1G). Thus, photoshopped mutations increase transgene signal in many, if not all, larval and adult tissues.

To test whether the increased signal seen in the photoshopped mutants was due to increased expression of the transgene, we performed quantitative RT-PCR experiments on RNA...
Figure 2. Photoshop Mutations Are Alleles of Upf2, Upf1, and Smg1

(A) Mapping of photoshop mutations 14J and 29AA. Shown are genetic maps of the X chromosome (top) and ct–v interval (middle) with visible markers (top) and SNP markers (middle) used to map lethality associated with 14J and 29AA mutations. Lines beneath maps show regions of 14J and 29AA X chromosomes (top) or 14J chromosome (middle) that were hemizygous-male viable, localizing 14J between XC35 and XC40 markers. Bottom panel shows predicted genes in mapped interval. Genes are alternately shaded black and white for clarity.

(B) Mapping of photoshop mutations in photoshop mutants 14J, 29AA, and 25G. 29AA (AAG to TAG) and 25G (TGA to AGA) are point mutations that disrupt Upf2 protein sequence as indicated, whereas 14J is a deletion of nucleotides 1682–1695 (TCTGCCCTATCTC) that disrupts the coding sequence at codon 562. Filled boxes, coding sequence; open boxes, UTRs; arrow, direction of transcription; bracket, extent of Upf2 genomic fragment in rescue transgenes, extending from ~600 bp upstream of Upf2 mRNA to 78 bp downstream of polyadenylation site.

(C) End of Upf2 coding sequence showing effect of 25G mutation, which converts stop to arginine codon and extends coding sequence 15 residues. Below is an alignment of C-termini of Drosophila melanogaster (D.m.) Upf2 and homologs from human (H.s.), C. elegans (C.e.), and Saccharomyces cerevisiae (S.c.). Similar residues shaded grey.

(D) Mapping of photoshop mutations 13D and 26A. Lethality associated with 13D and 26A mutations mapped between markers XA46 and XA48, an interval that includes Upf1.

(E) Mutations in Upf1 in 13D and 26A. Grey box, region homologous to domain in S. cerevisiae Upf1p required for interaction with Upf2p.

(F) Mapping of photoshop mutation 32AP. Lines beneath each map indicate regions of 32AP chromosome that did not enhance GFP expression in hemizygous males, showing that 32AP maps between XA24 and XB9, an interval containing Smg1. Df(Smg1)exe2B is an ~46-kb deficiency that uncovers Smg1 and the photoshop phenotype of 32AP.

(G) Mutation in Smg1 in 32AP. 32AP also contains a silent mutation (not shown) in codon 1755 (GCC to GCT). Grey box, Smg1 kinase domain.

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derived from hemizygous 25G, hemizygous 32AP, or control wild-type male L3 larvae, each also containing da-GAL4 and UAS-CD8:GFP. GFP RNA levels were increased ~5-fold in 25G mutants and ~2.5-fold in 32AP mutants compared to the wild type (Figure 1H). Hence, the increased GFP signal in photoshop mutants is due at least in part to increased GFP RNA levels.

Photoshop Mutations Are Loss-of-Function Alleles of Three NMD Pathway Genes

We used meiotic recombination to map the lethality and the mosaic tracheal GFP enhancement phenotype of the photoshop mutations. 14J and 29AA mapped between ct and v (Figure 2A). Complementation analysis (see below) showed that 14J and 29AA and the semi-lethal allele 25G formed a single complementation group. 13D and 26A mapped between v and g (Figure 2D), and complemented 14J for lethality. 32AP was mapped by its enhancement of GFP expression in hemizygous males, and localized between cv and ct (Figure 2F). Thus, the six photoshop mutations define at least three loci.

We further refined the position of mutation 14J by single nucleotide polymorphism (SNP) mapping and localized it to an ~200-kb interval (Figure 2A). DNA sequencing of predicted genes in this interval identified changes in the Drosophila homolog of the NMD pathway gene Upf2 in all three alleles of the 14J complementation group. The 14J mutation is a 14-bp deletion approximately halfway through the 1,241-residue coding sequence of Upf2 (Figure 2B), which causes a frame shift at codon 562 followed by a stop 40 codons later. 29AA is a nonsense mutation at codon 562. Both 14J and 29AA truncate the Upf2 coding sequence and are likely to be null alleles. A transgene comprising the Upf2 gene (Figure 2B) rescued 14J hemizygous males and homozygous females to viability and fertility; 29AA could not be tested because of a linked lethal mutation. We conclude that 14J and 29AA are loss-of-function mutations in Upf2.

25G hemizygous males and homozygous females survived to L3 and beyond (see above), whereas 25G/14J trans hetero-
zygotes were not viable after L2, suggesting that 25G is a haploinsufficient allele of Upf2. The Upf2<sup>2</sup><sup>transgene</sup> rescued 25G hemizygous males and homozygous females to viability and fertility, confirming this assignment. The 25G allele is a curious mutation that alters the natural Upf2 stop codon to an arginine codon (TGA to AGA). The next in-frame termination codon is 45 bp downstream, so 25G encodes a Upf2 protein with a 15-residue C-terminal extension (Figure 2C). The C-terminus of yeast Upf2p is required for its interaction with Upf1p [25], so the Upf2 extension might interfere with this function. The 25G mutation also creates a consensus 3' splice site, but RT-PCR experiments on 25G mutant RNA did not detect any novel splice forms involving this site.

The map positions of 13D and 26A were refined using SNP markers, and the lethality associated with each allele localized to an ~400-kb interval that contained the Drosophila homolog of Upf1 (Figure 2D). Sequencing of the Upf1 gene in 26A revealed a nonsense mutation (CAG to TAG; Q637stop) in the middle of the 1,180-residue coding sequence (Figure 2E), and sequencing of 13D identified a missense mutation (TGC to AGC; C186Y) in a domain required for interaction between yeast Upf1p and Upf2p [25], so the Upf2 extension might interfere with this function. The 25G mutation also creates a consensus 3' splice site, but RT-PCR experiments on 25G mutant RNA did not detect any novel splice forms involving this site.

Table 1. Transgenes Tested for Enhanced Expression in Photoshop Mutants

| Transgene [Reference]<sup>a</sup> | Promoter<sup>b</sup> | Reporter 3' UTR | Enhanced<sup>c</sup> |
|------------------------------------|------------------|-----------------|-------------------|
| pUAST-GFP<sup>+</sup> | US5/hsp70 | GFP | SV40 | (+, a, b, c) |
| pUAST-CD8-GFP, [23] | US5/hsp70 | CD8-GFP | SV40 | (+, b, c) |
| pUAST-DiRed<sup>+</sup> | US5/hsp70 | DiRed1 | SV40 | (+, b, c) |
| pUAST-clsDiRed<sup>+</sup> | US5/hsp70 | clsDiRed2 | SV40 | (+, b, c) |
| pUAST-EGFP | US5/hsp70 | eGFP | SV40 | (+, a, b) |
| pUAST-EGFP<sup>+</sup> | US5/hsp70 | eGFP | SV40 | (+, a, b) |
| ACT-GFP<sup>+</sup> | Actin | GFP | dis | (b) |
| His-GFP<sup>+</sup> | His2AvD | his.GFP | His2AvD | (b) |
| Ir-GFP<sup>+</sup> | US5/hsp70 | src.GFP | hsp70 | (c) |
| pUASP-GFP<sup>+</sup> | US5/P transposase | GFP | K10 | (b) |
| pUAST-h-eGFP<sup>+</sup> | US5/hsp70 | eGFP | hsp70 | (b) |
| pUAST-h-eGFP<sup>+</sup> | US5/hsp70 | eGFP<sup>+</sup> | hsp70 | (b) |
| pUAST-nls:eGFP<sup>+</sup> | US5/hsp70 | eGFP | SV40 | (a, b) |
| pUAST-nls:eGFP<sup>+</sup> | US5/hsp70 | eGFP<sup>+</sup> | SV40 | (a, b) |

<sup>a</sup> All transgenes are described in this work except as indicated.
<sup>b</sup> For UAS constructs, expression of the transgene was driven with da-GAL4, btl-GAL4, or e22c-GAL4.
<sup>c</sup> Transgene expression was examined in Upf2<sup>32AP</sup> or Upf2<sup>26A</sup> genetic mosaics (a), in hemizygous Upf2<sup>26A</sup> L3 male larvae (b), and/or in hemizygous Smg1<sup>13D</sup> L3 male larvae (c), +, visibly increased expression in a photoshop mutant; −, not increased in a photoshop mutant.

Photoshop Mutations Abolish or Reduce NMD of a Mutant Transcript In Vivo

Upf1, Upf2, and Smg1 are required for NMD in yeast and C. elegans and have been shown to be involved in NMD in cultured Drosophila cells [10], although recent data question the in vivo role of Smg1 in Drosophila [21]. To determine whether photoshop genes are required for NMD in vivo, we tested the effects of photoshop mutations on mRNA levels of Adh<sup>26A</sup>, a nonsense mutation in Adh [26] that subjects the mRNA to NMD in S2 cells [10]. We isolated RNA from adult wild-type males (y w FRT10A/Y; Adh<sup>26A</sup>/Adh<sup>+</sup>) and from Upf2 (Upf2<sup>26A</sup>/Y; Adh<sup>26A</sup>/Adh<sup>+</sup>) and Smg1 (Smg1<sup>13D</sup>/Y; Adh<sup>26A</sup>/Adh<sup>+</sup>) mutant males, amplified Adh mRNA by RT-PCR, and quantitated the Adh<sup>+</sup> and Adh<sup>26A</sup> products to assess the relative levels of the two transcripts (Figure 3). In wild-type animals, steady state transcript levels from the Adh<sup>26A</sup> allele were reduced 13-fold relative to Adh<sup>+</sup> transcripts, presumably because of increased turnover of the mutant transcript by the NMD pathway. In Upf2<sup>26A</sup> animals, Adh<sup>26A</sup> levels increased 13-fold, such that Adh<sup>26A</sup> and Adh<sup>+</sup> transcript levels were equivalent, implying that the 25G mutation abolishes NMD pathway function. By contrast, Smg1<sup>13D</sup> had only a modest effect, increasing Adh<sup>26A</sup> levels by 30%.

The NMD Pathway Targets the SV40 3' UTR of Drosophila Transgenes

To investigate how the NMD genes influence transgene expression, we examined the effect of photoshop mutations on transgenes containing different reporter genes, promoters, and 3' UTRs (Table 1; Figure 4). All of the transgenes whose steady state expression levels were found to be upregulated in photoshop mutants were constructed in the...
Effect of a Photoshop Mutation on Expression of GFP Transgenes

Pairs of larvae of genotypes Upf2<sup>25G/y</sup>; e22c-GAL4, UAS-nls:DsRed2/UAS-GFP (Upf2<sup>25G</sup> mutant, left in each panel) and w/Y; e22c-GAL4, UAS-nls:DsRed2/UAS-GFP (Upf2<sup>+</sup> control, right in each panel). The DsRed2 (internal control) transgene contains an SV40 3' UTR and was the same in all larvae, whereas the GFP test transgene differed in reporter and 3' UTR sequences as indicated. DsRed channel (A–G) shows effect of Upf2<sup>25G</sup> on the internal control DsRed2 transgene. GFP channel (A'–G') shows effect of Upf2<sup>25G</sup> on the GFP test transgenes indicated. Photographic exposures were the same and images were processed identically to facilitate comparison. Similar results were obtained for at least two independent insertions of each eGFP-variant transgene. The ratio of GFP expression for each transgene in Upf2<sup>25G</sup> versus Upf2<sup>+</sup> larvae was quantitated by GFP fluorescence measurements of micrographs of pairs of Upf2<sup>25G</sup> and Upf2<sup>+</sup> larvae, and the fluorescence ratio (average ± standard deviation, n = 2 independent insertions for each eGFP transgene) is shown. (The expression ratio of the DsRed2 internal control construct in Upf2<sup>25G</sup> versus Upf2<sup>+</sup> larvae was 4.7 ± 0.7). Only GFP transgenes with an SV40 3' UTR were enhanced in Upf2<sup>25G</sup> mutants, independent of the intron in this UTR. Transgenes with a 3' UTR derived from hsp70 were not enhanced. eGFP, enhanced GFP reporter; +, wild-type, left in each panel; 1, with added synthetic intron; Δ, SV40 intron deleted; pUAST.h, pUAST with hsp70 3' UTR replacing SV40 3' UTR. Bar, 1 mm.

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pUAST vector, the standard vector for gene misexpression in <i>Drosophila</i> [27]. This vector contains multiple binding sites for the yeast transcription factor GAL4 followed by a core promoter derived from the hsp70 gene upstream (5') of the reporter insertion site, and the SV40 3' small t antigen intron and polyadenylation signal (henceforth referred to as the SV40 3' UTR) downstream (3') of the insertion site [28]. Hence, it was possible that the photoshop phenotype was due to increased GAL4 expression or activity, increased hsp70 promoter activity, an interaction with the SV40 3' UTR, or some special feature of the GFP and DsRed coding sequences. Upf2<sup>25G</sup> enhanced expression of the pUAST-eGFP transgene (Figure 4B), which has a metazoan optimized codon bias, suggesting that the unusual codon bias of native GFP and DsRed genes is not critical for the photoshop effect. The GFP and DsRed reporters do not contain introns in their coding sequences, so we inserted a 61-bp intron from <i>Drosophila</i> gene CG3585 into pUAST-eGFP to make pUAST-eGFP<sup>+</sup>I. This transgene did not show significantly increased eGFP signal in wild type and was still sensitive to photoshop mutations (Figure 4D and 4E), confirming the importance of the 3' UTR. Interestingly, expression levels of GFP from the constructs containing the hsp70 3' UTR were somewhat lower than those of the corresponding pUAST-eGFP or pUAST-eGFP<sup>+</sup>I transgenes in a wild-type background and much lower than those of pUAST-eGFP or pUAST-eGFP<sup>+</sup>I transgenes in a photoshop mutant background. The significance of this finding is discussed below.

A small intron present in the SV40 3' UTR was an appealing candidate for sensitizing transcripts to the photoshop effect. A model for recognition of PTCs in vertebrates is that they occur 5' of the site of an intron, the same arrangement of the termination codons of GFP and DsRed with respect to the SV40 intron. RT-PCR experiments confirmed that the SV40 intron was indeed recognized and spliced in <i>Drosophila</i> larvae. However, deletion of this intron in the pUAST-eGFP and pUAST-eGFP<sup>+</sup>I constructs (to make pUASTΔI-eGFP and pUASTΔI-eGFP<sup>+</sup>) did not eliminate the photoshop effect (Figure 4F and 4G). Thus, targeting of the 3' UTR by the NMD pathway does not require splicing of this intron, the only known intron in the UTR.

Targets of the NMD Pathway during Development

To identify candidate endogenous targets of the NMD pathway, we compared steady state RNA levels in hemizygous Upf2<sup>25G</sup> male larvae to those in wild-type male larvae using a whole genome microarray. To avoid confounding effects of the Upf2 mutation on developmental progression, we focused our analysis on a set of 954 genes that do not undergo significant changes in expression levels during development or differ in expression between male and female larvae (see
Materials and Methods). Among this set, we found 14 genes whose expression was upregulated 2-fold or more, and 26 genes that were downregulated 2-fold or more in the mutant compared to wild type (Tables S1 and S2). Genes whose expression was downregulated could be novel targets whose expression is paradoxically enhanced by the NMD pathway, or they could be indirect effects of NMD pathway inactivation. The affected genes encode proteins of diverse classes, including signal transduction molecules, proteases, and proteins involved in cell metabolism. Most of the affected genes differed from ones identified recently in Drosophila S2 cells depleted of NMD-gene function by RNA interference [19], suggesting tissue-specific regulation.

Two well characterized genes upregulated in the photoshop mutant were analyzed further. The mRNA of orthinine decarboxylase antizyme (oda, also called gut feeling) contains a naturally occurring coding sequence frame shift [30] that causes the transcript to contain early termination codons, and is an NMD target in cultured Drosophila S2 cells [19]. oda transcript is also a target of the NMD pathway during development: oda RNA levels were increased in hemizygous Upf2^{225G} larvae as determined by microarray analysis (3-fold) and by quantitative RT-PCR (2-fold; Figure 5A). However, oda transcript levels were not significantly increased in Smg^{123AP} mutants (Figure 5A).

The sex determination gene transformer (tra) [31] is also an NMD pathway target during development, as in S2 cells [19]. tra transcript levels were increased 3- to 4-fold in hemizygous Upf2^{225G} larvae, as determined by microarray analysis and quantitative RT-PCR (Figure 5A). Smg^{123AP} also increased tra levels, but to a lesser extent (Figure 5A). tra might be expected to be an NMD pathway target because the primary transcript is spliced in males to produce a long mRNA (traL) that contains an early termination codon that would likely be recognized as premature, whereas in females some of the primary transcript is alternatively spliced to produce a shorter transcript (traS) lacking the early termination codon and encoding full-length protein (Figure 5B). Indeed, RT-PCR experiments indicate that the increase in tra levels in Upf2^{225G} mutant males and females was due to selective stabilization of traL (Figure 5B and data not shown).

Drosophila NMD Genes Are Dispensable for Many Developmental Processes but Provide Cells a Competitive Growth or Survival Advantage

The above results show that the core NMD gene Upf2 is broadly active during development and influences expression of dozens of genes, including a key sex determination gene, and is required for larval viability. To identify specific cellular and developmental functions of the NMD pathway, we analyzed the effect of photoshop mutations on sex determination and cell growth and differentiation. We did not detect any defects in sex determination in adult males hemizygous for Upf2^{225G}, despite the observed increase in traL levels: sex-specific splicing of downstream gene dsx was normal, as was that of the sex determination genes Sex-lethal (Sxl) and male sterile-2; sex combs and genitalia appeared normal; and males made sperm and were capable of mating. Homozygous Upf2^{225G} females also appeared normal. We also did not detect any defects in larval cell differentiation. Larval tracheal cell clones lacking Upf1 or Upf2 displayed wild-type morphology at all levels of branching, including tracheal terminal cell clones, which showed normal branching patterns and luminal (imaginal) structures. Whereas homozygous clones of all photoshop mutants were readily obtained in the larval tracheal system, we did not recover clones in the adult tracheal system, which is generated by proliferation of imaginal tracheal precursor cells during metamorphosis. We also did not recover large clones in adult epidermis or eyes.
although small peripheral eye clones were occasionally observed (Figure 6E and 6F). However, when the GMR-hid technique [33] was used to eliminate all heterozygous and wild-type cells in the developing eye, we found that the remaining photomosaic mutant cells could proliferate, differentiate, and form an eye, although the eyes were smaller and more disorganized (rougner) than those of controls (Figure 6G and 6H). These results suggest that Drosophila NMD genes are not required for cell proliferation, survival, or differentiation, but provide proliferating cells with a competitive growth or survival advantage during development.

Discussion

We have isolated to our knowledge the first mutations affecting NMD in Drosophila based on their ability to enhance expression of a GFP transgene, an effect we call the photomosaic phenotype. Mapping of the mutations, complementation tests, and molecular analysis demonstrate that the photomosaic mutations identify three genes, the Drosophila orthologs of NMD pathway genes Upf1, Upf2, and Smg1. The results show that Upf1 and Upf2 are essential genes, required for NMD and, at least in the case of Upf2, for proper expression of dozens of native mRNAs during development, including oda and the sex-nonspecific form of tra that contain early termination codons. By contrast, Smg1 is dispensable and only potentiates the NMD pathway. Genetic mosaic analysis of the Upf genes showed that they are not required for cell proliferation, survival, or complex cell differentiation events such as tracheal and neuronal growth and sprouting, but they provide proliferating imaginal cells with a competitive growth or survival advantage during development. We also mapped the cis-acting signal that confers sensitivity to the NMD pathway in the transgenic reporter assay, and discovered that it resides in the heterologous 3’ UTR present in the reporter construct. Below, we discuss the implications of these results for our understanding of the functions and mechanism of the Drosophila NMD pathway during development, and compare and contrast them with what has been found for NMD pathway function in other organisms.

Roles of NMD Genes in Drosophila Development

The finding that mutations in Drosophila NMD genes Upf1 and Upf2 cause lethality during larval development contrasts with the minor effect of mutations in the homologous genes in yeast (mutations have almost no discernable effect on growth or survival [5]) and in C. elegans (mutants are viable and have only morphogenetic defects late in development [6]). Why are Upf1 and Upf2 essential in Drosophila? One possibility is that they are required to eliminate mutant transcripts with PTCs that encode truncated, deleterious protein products. Such PTC-containing alleles could be present in the background of our Upf1 and Upf2 mutants, and in the absence of NMD activity, these mutations become lethal. However, all our Upf1 and Upf2 mutations were independently isolated, and the lethality in these lines segregates with the Upf mutations, not with other genomic regions. Thus, if this explanation is correct, there would have to be multiple, potentially lethal PTC mutations distributed throughout the genome.

A second possibility is that the NMD pathway has a more general surveillance function that also eliminates naturally occurring transcripts resulting from aberrant splicing events or repetitive DNA elements, and accumulation of such transcripts is toxic. However, many aspects of cell biology and development appear normal in Upf mutants. Indeed,
sensitive assays examining individual tracheal cells and neurons show that loss of Upf1 function does not lead to cell death or impairment of complex cell morphogenesis events, implying that NMD inactivation does not cause general cellular toxicity.

A third possibility, which we favor, is that the NMD pathway modulates the activity of specific native transcripts, whose misregulation leads to lethality. An initial microarray survey identified several dozen genes of diverse functional classes whose expression was altered in NMD mutant larvae (Tables S1 and S2). Some of the affected transcripts contain early stop codons that are interpreted as bona fide PTCs, as for tra and oda genes (Figure 5A); other affected transcripts could harbor other cis-acting signals that are interpreted as aberrant by the NMD machinery, like the SV40 3’ UTR discussed below. In the absence of the NMD pathway, overexpression of such transcripts would perturb the development or function of select cells or tissues and lead to lethality. Indeed, although many cells appear to develop normally in photoshop mutants, we never observed Upf1− and Upf2− clones in adult tracheae or epidermis, and only small clones were found in the eye, implying that the NMD pathway promotes growth or survival of the proliferating imaginal cells that give rise to these tissues. Because Upf genes are broadly expressed ([34] and unpublished data) throughout development, identification of the tissue focus of Upf lethality will be an important first step towards identifying the critical cellular targets of NMD gene regulation.

The sole cellular defect we identified in NMD mutants was the absence or small size of mutant clones in the adult tissues described above, which is reminiscent of the cell cycle arrest observed in cultured Drosophila S2 cells depleted of Upf function [19]. Although these results suggest a function for the NMD pathway in cell proliferation or survival, the requirement for the pathway in these processes is not absolute: Upf1− and Upf2− cells were able to proliferate and form an eye when competing wild-type eye progenitor cells were eliminated (Figure 6). This implies that the NMD pathway provides proliferating imaginal cells with a competitive growth advantage that prevents them from being overtaken by other proliferating cells during development. This could be a cell autonomous effect, like Minute mutations [35], or a cell nonautonomous effect, e.g., if the pathway influences expression of a signal secreted from proliferating cells that affects growth of neighboring cells (e.g., [36]). The NMD pathway may play a similar, or more extreme, role in mice, because a mouse Upf1 knockout is lethal and attempts at establishing UPF1− embryonic stem cells were unsuccessful [18].

Recently, it has been suggested that Upf1 and Upf2 participate in other aspects of gene regulation besides NMD, such as stimulating translation [37] and in translational termination [38] (reviewed by [39]). It is important to note that for neither mouse nor our Drosophila mutants is it established that the lethality associated with Upf1 and Upf2 mutations derives from their roles in NMD. Indeed, our analysis of Upf225G suggests the opposite possibility. This allele appears completely compromised for NMD, as assessed by expression of an Adh mRNA carrying a PTC (Figure 3), yet unlike Upf2 null alleles, some Upf225G mutants survived to adulthood. Thus, either the essential function of Upf2 is in a process other than NMD, or this allele retains residual NMD function sufficient for regulation of its essential target genes but not of others like the mutant Adh mRNA.

The differing molecular, cellular, and developmental requirements for NMD pathway genes in yeast, C. elegans, Drosophila, and mice make clear that the function of this pathway has diversified during evolution. Perhaps the ancestral function of the pathway was in some general process like translation termination, and only later did the pathway evolve roles in monitoring transcripts for PTCs and more specialized regulatory roles. Alternatively, the ancestral function could have been regulation of RNAs involved in a specific cellular process such as cell growth regulation, and only later did the pathway acquire a more general role in RNA surveillance.

**Smg1 Potentiates the Drosophila NMD Pathway**

Our genetic analysis demonstrated a striking difference in the developmental requirements of Smg1 compared to those of Upf1 and Upf2. First, Upf1 and Upf2 are essential genes, whereas an amorphic Smg1 allele resulted in viable and fertile animals. Second, a Upf2 mutation abolished NMD of an Adh PTC allele, whereas the amorphic Smg1 mutation only modestly reduced NMD efficiency. Third, the magnitude of the Smg1 mutant effect differed at different targets. At some targets, such as oda, there was little or no effect of the Smg1 mutation, whereas at other targets, such as tra and a GFP transgene, the Smg1 mutant effect was up to half that of the Upf2 mutant. The small and gene-selective effect of Smg1 could explain why a recent genetic analysis failed to detect a role for Drosophila Smg1 in NMD [21], whereas earlier Drosophila cell culture studies suggested an important role for the gene [10]. The small and gene-selective function of Drosophila Smg1 contrasts with genetic results in C. elegans, which did not identify differences in the requirements of smg-1 and the Upf1 and Upf2 homologs smg-2 and smg-3 [6]. One possibility is that Drosophila has another protein with activity similar to that of Smg1. This seems unlikely because Smg1 is the only sequence ortholog of Smg1-family genes in the Drosophila genome, although there are other genes that encode proteins with PI3K-related kinase domains. Another possibility is that phosphorylation of Upf1 by Smg1 is not absolutely required for Upf1 activity in Drosophila but only enhances its activity or reactivates spent protein after a catalytic cycle. This would be more similar to the NMD pathway in yeast, which lacks a Smg1 ortholog and is thought to function without a Upf1 phosphorylation cycle, than to the NMD pathways in C. elegans and vertebrates, where the Upf1 phosphorylation cycle is thought to be essential for pathway activity.

**Targeting of a Specific 3’ UTR by the Drosophila NMD Pathway**

We found that a variety of reporter constructs in the pUAST transformation vector were upregulated when NMD pathway function was abrogated (Table 1), implying that transcripts derived from this vector are recognized as aberrant by the RNA surveillance machinery. Strictly speaking, this is not an NMD process, because all of the transgenic constructs contain full-length coding sequences with no PTCs. However, because multiple NMD genes are involved in this regulation, and because the observed increase in
reporter activity is associated with increased reporter mRNA, it suggests that an NMD-related RNA decay process normally limits expression of pUAST transgenes in *Drosophila*. The signal that targets transcripts for regulation by the NMD pathway appears to lie in the SV40 3’ UTR of the pUAST vector: all reporter constructs that were sensitive to the NMD pathway contain this UTR, and swapping it for one derived from the hsp70 gene rendered the transcript insensitive to NMD, the first example to our knowledge of a change in NMD signal for regulation by the NMD pathway in *Drosophila* (17, 40). In wild-type (WT), SV40 3’ UTR enhances transgene expression (arrow). However, effect is partially offset by transcript degradation by NMD machinery, giving an intermediate level of expression (+ + +). In photoshopped (NMD) mutants, transcript degradation is abrogated, resulting in strong enhancement by SV40 3’ UTR (thick arrow) and high level of expression (+ + + +). The hsp70 3’ UTR does not enhance expression (or alternatively promotes degradation) but is also not a target of the NMD pathway, giving a low level of expression (+) insensitive to photoshopped mutants.

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**Figure 7.** Model for *Drosophila* NMD Pathway Action on SV40 3’ UTR

In wild-type (WT), SV40 3’ UTR enhances transgene expression (arrow). However, effect is partially offset by transcript degradation by NMD machinery, giving an intermediate level of expression (+ + +). In photoshopped (NMD) mutants, transcript degradation is abrogated, resulting in strong enhancement by SV40 3’ UTR (thick arrow) and high level of expression (+ + + +). The hsp70 3’ UTR does not enhance expression (or alternatively promotes degradation) but is also not a target of the NMD pathway, giving a low level of expression (+) insensitive to photoshopped mutants.

Because expression of UAS-GFP and other reporters is affected by mutations in homologs of all three NMD genes on the chromosome we screened, the assay can likely be used to identify and characterize additional NMD pathway genes on other chromosomes. The assay has two important features. First, because it can be carried out in single cells in genetically mosaic animals, a requirement of candidate NMD genes for organismal viability can be bypassed. Second, the assay is very sensitive to perturbations in the NMD pathway. For example, loss of *Smg1* activity leads to a modest increase in stability of PTC-containing transcripts but a readily detectable enhancement of GFP reporter expression. Together these features suggest that the transgenic assay system can be used to test requirements in vivo of candidate NMD genes and drugs that influence pathway activity, which could be useful in modulating expression of human disease genes carrying PTCs (42, 43).

**Materials and Methods**

*Fly stocks and genetics.* GAL4/UAS system (27) drivers used were *btl-GAL4* (44), *e22-GAL4* (45), *ppk-GAL4* (32), and *da-GAL4* (24). GFP and *DsRed* transgenes are referenced in Table 1. *Df(3L)* w* ey* 2b/2 was generated by using FLP-mediated recombination between the FRTs in P[VP16]*G31* (42) and *P{BAC}16* (32) as described in Thibault et al. [46]. Marker mutations and balancer chromosomes are described at http://www.flybase.org. Flies reared at 25 °C on cornmeal-dextrose medium.

The photoshopped mutations were obtained by mutagenesis of an isogenic y w *FRT04* chromosome (92) with 25 mM ethane methyl sulfonate overnight (47) in a tracheal mutant screen (to be described elsewhere). The mutations used were on this chromosome unless otherwise noted. To generate homozygous mutant clones, 2- to 6-h-old embryos were collected at 25 °C from a cross of y w *FRT04*/Cy*s* females to *gal80 FRT04*/Cy*s* males. After a 45-min heat shock at 38 °C to induce FLP expression, embryos were returned to 25 °C to continue development. L3 larvae of genotype y w *FRT04*/*gal80 FRT04*; *cyp12A1*; *UAS-GFP*, *UAS-GFP*-labeled larvae were sought, and GFP mosaicism within the tracheal system and scored for the photoshopped phenotype.

The original *Smg1*22AP chromosome (designated 32AP) carried lethal mutations not associated with the photoshopped phenotype. Lethals were removed by crossing 32AP+/y w *FRT04* females to w/Y; *btl-GAL4*, UAS-GFP males and identifying L3 larvae with enhanced GFP throughout their tracheal system. These *Smg1*22AP*, *btl-GAL4*, UAS-GFP larvae developed into viable adult males. We also found a viable wing morphology mutation on 32AP that is allelic to *wany*. Existing *wany* alleles do not show a photoshopped phenotype, and the wing phenotype is separable from the photoshopped phenotype, so *wany* does not seem to contribute to the photoshopped phenotype.

The *Upf2*04A* chromosome carries a linked lethal mutation. When recombined away from *Upf2*04A, the mutation had no effect on tracheal development or reporter expression. However, we have not obtained a recombinant containing *Upf2*04A without the extraneous mutation.

For complementation tests of *Upf2*, we used genomic rescue transgenes located on the autosomes to generate males of genotype y w *Upf1*21A* v g f *FRT04*/Cy*s*; P[w*, *Upf2*2+] and crossed these to y w *FRT04*/Cy*s* females, where the asterisk indicates the tested mutation. Absence of Bar*, white-eyed female progeny indicated failure to complement. For complementation tests of *Upf1*, we used the Y-linked duplication *Df(1)YBNCl*, y*, which covers the *Upf1* locus. Males of genotype *Upf1*21A* y Df(1)YBNCl* were crossed to *Upf1*21A* y FRT04*/Cy*s* females, and the absence of female Bar* progeny indicated a failure to complement.

**Mapping of photoshopped mutations.** Identification of SNPs, construction of the SNP map of the X chromosome, and details of their use in mapping X chromosome mutations will be described elsewhere. Briefly, the location of the lethality associated with a photoshopped mutation was mapped by crossing y w *FRT04*/Cy*s* cv v g f *FRT04* females (where the asterisk indicates the lethal mutation) to F7/S7 males and scoring the viable male progeny for the visible markers to determine the lethal interval. To refine the map position,
we collected male progeny in which a recombination event had occurred within the mapped interval and scored them for SNPs. For each mutation we typically scored 300–400 males for SNPs. For Upf225G and Upf225G;Pf, which map between v and g, we crossed the v * w * FRT19A (sc 2 v g f) females to males of genotype Df(1)1418, g3/sd'Id(y1)2v w* to distinguish v * w * FRT19A from recombinants, which we could identify because of the absence of v g. We also crossed the w * FRT19A (sc 2 v g f) females to sc 2 v g FRT19A males to identify recombinant females, which were then tested for the phenotypic signature in genetic mosaics to confirm that the lethality and phenotype phenotype was mapped to the same interval. For the viable mutation 32AP, we followed a similar strategy as for the lethals, except we scored recombinant males for the presence or absence of 32AP by testing the enhancement of GFP in bl-GAL4, UAS-GFP transgenic animals.

Transgene construction. For the Upf2 genomic rescue construct, Drosophila genomic DNA BAC 24A2, which contains Upf2, was transformed into Escherichia coli strain E250, which harbors heat-shock-inducible, homologous recombination machinery [49]. We then cloned a 200-bp fragment located upstream, and a 300-bp fragment located downstream, of Upf2 coding sequence and UTRs based on the cDNA RP04031 (rather than the canonical Upf2 cDNA SD07292, which appears to be defective as it lacks a preserved portion of Upf2 coding sequence) tandemly into the Drosophila transformation vector pCaSper4 [50] with a unique NotI site between the fragments to give pMMP200 in which the 200-bp fragment was linearized with NotI and cloned into E250 [24,42], in which the recombination machinery was constitutively induced. Transfomers were plated onto LB plates containing carbenicillin to select for gap repair of pMMP200, which can occur by homologous recombination with the BAC and result in transfer of Upf2 into pMMP200. The resultant plasmids were analyzed by restriction enzyme digestion and one with the correct structure was used to establish transgenic lines on the second and third chromosomes by P-element insertion. Six lines were tested and restriction digestion, and one with the expected pattern (pMM#201) of rescue varied based on insertion site, but for the strongest lines Upf2

...performed on an ABI 3730x1 (Applied Biosystems; http://www.appliedbiosystems.com) and analyzed using GeneMapper v3.0 software (Applied Biosystems).

Microarray analysis. RNA was isolated from Upf225G/Y; bl-GAL4, UAS-GFP +/- and w FRT19A/Y; bl-GAL4, UAS-GFP +/- L3 larvae using Trizol as described above. cDNA labeled with Cy5 or Cy3 was prepared from each RNA sample and hybridized to microarrays containing ~14,000 gene probes [32,53]. Hybridizations were performed with two independently isolated and labeled RNA samples. Analysis was carried out using the Stanford Microarray Database (http://genomewww.stanford.edu). During analysis we noted that the Upf225G mutants were delayed in development. To avoid confounding effects of changes in gene expression that result from developmental regulation rather than direct effects of Upf2 loss of function, we used the available wild-type developmental gene expression time course [51] to filter out genes whose transcription changed more than 25% from their maximal value during hours 72–96 of larval development. The wild-type dataset includes ~35% of genes, and we used this subset for our analysis. Furthermore, the wild-type dataset is for mixed sex populations, while our microarray was performed on only males. To compensate for sex differences, we also excluded from analysis genes that differed between males and females by more than 50% based on data from male and female larvae (E. Johnson and M. A. K., unpublished data). Our analysis of genes regulated by NMD is therefore conservative, covering only non-developmentally regulated and non-sex-regulated genes whose expression was affected by a hypomorphic Upf2 allele, and thus provides only a lower estimate of genes regulated by NMD.

Supporting Information

Table S1. Genes Upregulated More than 2-Fold in Microarray Analysis of Upf225G Larval RNA

Table S2. Genes Downregulated More than 2-Fold in Microarray Analysis of Upf225G Larval RNA

Table S3. Sequences of DNA Primers Used

Accession Numbers

The FlyBase (http://flybase.net) and Entrez Gene (http://ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene) accession numbers for the genes described in the text are Adh (CG3481 [Gene ID: 37718577]), aoda (CG16747), Smgl1 (CG29743 [Gene ID: 31625]), Upf2 (CG1559 [Gene ID: 32153]), and Upf2 (CG2255 [Gene ID: 31724]). Microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) and are accessible through accession number GSE5585.

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