REPORT

Drosophila mutants show NMD pathway activity is reduced, but not eliminated, in the absence of *Smg6*

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ABSTRACT

The nonsense-mediated mRNA decay (NMD) pathway is best known for targeting mutant mRNAs containing premature termination codons for rapid degradation, but it is also required for regulation of many endogenous transcripts. Components of the NMD pathway were originally identified by forward genetic screens in yeast and *Caenorhabditis elegans*. In other organisms, the NMD pathway has been investigated by studying the homologs of these genes. We present here the first unbiased genetic screen in *Drosophila* designed specifically to identify genes involved in NMD. By using a highly efficient genetic mosaic approach, we have screened ~40% of the *Drosophila* genome and isolated more than 40 alleles of genes required for NMD. We focus on alleles we have obtained in two known NMD components: *Upf2* and *Smg6*. Our analysis of multiple alleles of the core NMD component *Upf2* reveals that the *Upf2* requirement in NMD may be separate from its requirement for viability, indicating additional critical cellular roles for this protein. Our alleles of *Smg6* are the first point mutations obtained in *Drosophila*, and we find that *Smg6* has both endonucleolytic and nonendonucleolytic roles in NMD. Thus, our genetic screens have revealed that *Drosophila* NMD factors play distinct roles in target regulation, similar to what is found in mammals, but distinct from the relatively similar requirements for NMD genes observed in *C. elegans* and yeast.

Keywords: *Drosophila*; NMD; *Smg6*; *Upf2*; nonsense-mediated decay

INTRODUCTION

Nonsense-mediated mRNA decay (NMD) is a cellular pathway that selectively targets mRNAs containing premature termination codons (PTCs) for rapid degradation (Chang et al. 2007) and is thought to function to prevent the translation and build-up of potentially harmful truncated protein products (Cali and Anderson 1998). *Trans*-acting factors involved in NMD were originally identified in suppressor screens performed in yeast (Leeds et al. 1991, 1992) and *Caenorhabditis elegans* (Hodgkin et al. 1989; Cali et al. 1999). These screens took advantage of PTC-containing alleles of genes whose truncated protein products, rather than being toxic, possess residual function. Since NMD targets these transcripts, the truncated proteins cannot be made, effectively resulting in a complete loss-of-function phenotype. Loss of function of genes required for NMD results in stabilization of such PTC-containing transcripts, allowing for production of the truncated protein products and

amelioration of the mutant phenotype. These screens identified three genes required for NMD in yeast—*UPF1*, *UPF2* (also called NMD2), and *UPF3* (*Upf*, *up-frameshift suppressor*) (Leeds et al. 1991, 1992)—and seven in *C. elegans*—*smg-1* through *smg-7* (*smg*, *suppressor with morphogenetic effect on genitalia*) (Hodgkin et al. 1989; Cali et al. 1999). Cloning of these genes has revealed that *UPF1*, *UPF2*, and *UPF3* are orthologous to *smg-2*, *smg-3*, and *smg-4*, respectively, thus defining an evolutionarily conserved core machinery for NMD (Cui et al. 1995; He and Jacobson 1995; Lee and Culbertson 1995; Cali et al. 1999; Page et al. 1999; Aronoff et al. 2001; Serin et al. 2001; Chiu et al. 2003; Grimson et al. 2004). All eukaryotes analyzed contain orthologs of these core genes, and, in all cases examined, they are known to be required for NMD (Kerényi et al. 2008). The four other genes identified in *C. elegans* (*smg-1*, *smg-5*, *smg-6*, *smg-7*) are generally conserved only in metazoa, although there is evidence that a homolog of *Smg7* is also required for NMD in plants (Luke et al. 2007). *Smg5* and *Smg7* are structurally similar (Fukuhara et al. 2005), although not redundant for their function in NMD, and some organisms, including *Drosophila*, appear only to possess *Smg5* (Gatfield et al. 2003).

¹Corresponding authorE-mail markm@genetics.utah.eduArticle published online ahead of print. Article and publication date are at <http://www.rnajournal.org/cgi/doi/10.1261/rna.032821.112>.

The design of the suppressor screens used to identify NMD genes demanded that compromising NMD function would not in itself result in lethality. Indeed, this is the case in yeast and *C. elegans*: Complete absence of core NMD components and NMD activity is compatible with viability (Leeds et al. 1991, 1992; Cui et al. 1995; He and Jacobson 1995; Lee and Culbertson 1995; Cali et al. 1999; Page et al. 1999; Aronoff et al. 2001; Grimson et al. 2004). However, for most eukaryotes, disruption of NMD gene function is lethal (Hwang and Maquat 2011), and this essential role has limited the ability to identify NMD genes as suppressors of PTC-containing alleles in these organisms.

Since the initial screens, the only unbiased forward genetic screen that has been performed to identify genes required for NMD has been an RNAi-based approach in *C. elegans*. This screen did not rely on suppression, but instead used an NMD-sensitive reporter whose expression increased upon disruption of NMD activity (Longman et al. 2007). This screen identified two new NMD genes, *smgl-1* and *smgl-2* (*smg lethal*), whose knockdown leads to defective NMD and also organismal lethality. Knockdown of *smgl-1* and *smgl-2* in human cells (Longman et al. 2007) and zebrafish (Anastasaki et al. 2011) also leads to impairment of NMD. However, no mutations in *smgl-1* or *smgl-2* have been reported in any organism.

The primary mechanism that distinguishes premature from normal termination codons seems to involve detection of 3'-UTR length (Amrani et al. 2004; Longman et al. 2007; Eberle et al. 2008). Such a mechanism also plays a role in mammalian cells and plant cells, but there is also an important contribution from interactions between NMD components and splicing-deposited exon junction complexes (Lejeune and Maquat 2005; Kertész et al. 2006; Eberle et al. 2008). Recognition of a PTC is thought to result in the recruitment of the core NMD protein Upf1 (SMG-2 in *C. elegans*), an ATP-dependent RNA-helicase, to the targeted mRNA, triggering degradation (Kashima et al. 2006). In both yeast and mammalian cells, degradation is known to occur through deadenylation-independent decapping, dependent on the recruitment of the decapping proteins DCP1 and DCP2 by Upf1 (Lejeune et al. 2003; Swisher and Parker 2011). This is followed by degradation by 5'-3' exonucleases, such as XRN1 (He et al. 2003). However, there is now considerable evidence that in metazoan cells, degradation can also occur through endonucleolytic cleavage in the vicinity of the PTC (Gatfield and Izaurralde 2004; Huntzinger et al. 2008; Eberle et al. 2009). The endonuclease responsible for this cleavage is likely to be the NMD component Smg6. Smg6 contains a C-terminally located PIN-endonuclease domain that is required for cleavage of target mRNAs (Glavan et al. 2006; Huntzinger et al. 2008; Eberle et al. 2009). Smg6 is thought to be recruited to target mRNAs by virtue of its 14-3-3-like domain, which can bind phosphorylated Upf1 (Fukuhara et al. 2005). In mammalian cells, Smg6 is also

recruited to target mRNAs through direct recruitment to the EJC (Kashima et al. 2010). Smg6 function has primarily been analyzed in cell culture, and while some genetic analysis has been performed in *C. elegans* (Hodgkin et al. 1989; Page et al. 1999), a role for SMG-6 endonuclease function has not yet been analyzed in this organism.

Roles of *Drosophila* NMD factors have been well established in cell culture experiments (Gatfield et al. 2003; Rehwinkel et al. 2005; Huntzinger et al. 2008). These experiments have shown that all the *Drosophila* homologs of known NMD genes are required for NMD and that loss of any of these genes leads to similar defects in NMD. Less is known about how NMD genes function in intact animals. Previous genetic analysis has identified alleles of *Upf1-3* and *Smg1* (Chen et al. 2005; Metzstein and Krasnow 2006; Avery et al. 2011). All of these have been shown to have function in NMD, although, interestingly, some of these roles differ. For instance, while *Upf1* and *Upf2* seem to be absolutely required for NMD and organismal viability, loss of *Smg1* or *Upf3* leads to viable animals with a reduction, but not a complete loss, of NMD pathway activity. Why intact animals and cell culture show these differences is not understood, but probably indicate additional layers of pathway regulation.

Here, we present a novel forward genetic screen in *Drosophila* that uses an NMD-sensitive reporter combined with a genetic mosaic approach. This has allowed us to isolate more than 30 mutations in the known NMD genes *Upf1*, *Upf2*, *Smg1*, and *Smg6*. Our alleles of *Upf2* have revealed that differential interactions of Upf2 with other core NMD components are correlated with whether these alleles are required for viability. The *Smg6* mutations are the first obtained in *Drosophila*, and we have used genetic and molecular criteria to show that several of these are complete loss of function. We find that mutants carrying these null alleles still show considerable NMD pathway function, thus revealing that Smg6 is not absolutely required for NMD in vivo.

RESULTS AND DISCUSSION

Tissue-directed mosaic screen for mutations affecting NMD

To identify novel genes required for NMD as well as alleles of known NMD genes, we performed a large-scale forward genetic screen in *Drosophila*. The screen uses the simian virus 40 small t antigen intron and polyadenylation signal (SV40 3' UTR), which is targeted for degradation by the NMD pathway in *Drosophila* (Metzstein and Krasnow 2006). Transgenic reporter constructs that use the SV40 3' UTR as their termination signal result in a low level of expression in wild-type backgrounds; however, the signal increases dramatically when genes involved in NMD are inactivated (Fig. 1A,B). Fluorescent protein reporters with

FIGURE 1. Mosaic approach used to detect animals with mutations in genes affecting NMD. (A) $y w FRT^{19A}/Y; e22c-GAL4 UAS-nlsDsRed2::SV40\ 3'UTR/+$ L3 (nonmosaic) larva shows uniform, low-level DsRed expression in epidermal nuclei. (B) $y w Upf2^{25G} FRT^{19A}/Y; e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR/+$ (nonmosaic) L3 larva, taken at the same exposure as A, shows uniform, high-level DsRed expression in epidermal nuclei, due to stabilization of the reporter mRNA. (C) $UAS-FLP$ expression in the early embryonic ectoderm driven by $e22c-GAL4$ catalyzes recombination in heterozygous mitotic cells, resulting in homozygous mutant and wild-type sister cells. Cells that have not undergone recombination remain heterozygous. Expression of an NMD-sensitive reporter $UAS-nlsDsRed2::SV40\ 3'UTR$, which encodes a nuclear-localized red-fluorescent protein, is driven by $e22c-GAL4$ in the larval epidermis. In heterozygous and the wild-type sister cells, the reporter mRNA is targeted for degradation by NMD and shows a low level of DsRed fluorescence (light red). When a gene required for NMD is mutated, the homozygous mutant cell is recognized by an increase in fluorescence (increased red color). (Circles) Centromeres; (rectangles) FRT sites; (*) EMS-generated mutations. (D) $y w FRT^{19A}; e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR, UAS-FLP/+$ mosaic L3 larva shows uniform DsRed expression in epidermal nuclei. (Yellow asterisks) Underlying tracheal nuclei that also express DsRed. These are easily distinguished from epidermal cells under the microscope. (E) $y w Upf1^{26A} FRT^{19A}/y w FRT^{19A}; e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR, UAS-FLP/+$ L3 larva shows mosaic increased DsRed expression in epidermal nuclei: Low-level DsRed expression is punctuated with brighter, homozygous mutant nuclei (yellow arrowheads). (F) $y w Smg1^{32AP} FRT^{19A}/y w FRT^{19A}; e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR, UAS-FLP/+$ L3 larva shows mosaic DsRed expression in epidermal nuclei: Low-level DsRed expression is punctuated with brighter, homozygous mutant nuclei (yellow arrowheads). Scale bars, (A,B) 1 mm; (D–F) 0.5 mm.

the SV40 3' UTR can thus be used to monitor NMD pathway activity on a single-cell level, and this allowed us to perform a screen for genes involved in NMD using a genetic mosaic approach. Importantly, this mosaic approach allowed us to bypass the organismal lethality associated with strong loss of NMD function. Furthermore, by using a live-cell marker, we could score for NMD defects in F_1 generation animals, greatly increasing the screen throughput.

Our screen used the $e22c-GAL4$ driver, which is expressed in the early embryonic ectoderm and later is restricted to ectodermally derived epithelium (Duffy et al. 1998), including the larval epidermis. This driver served two purposes in our screen (Fig. 1C). Expression of FLP recombinase driven by $e22c-GAL4$ early in development produces animals with mosaicism in ectodermal derivatives. Later in development, $e22c-GAL4$ drives expression of an NMD-sensitive fluorescent reporter in larval epidermal cells. Importantly, we found that this expression in epidermal cells is uniform (Fig. 1D), so differences in reporter levels caused by mutations in NMD genes could be readily identified.

To test our mosaic strategy, we crossed our mosaic generating reporter line to animals carrying known mutations in the core NMD components *Upf1* and *Upf2* (Fig. 1E; data

not shown). Animals entirely mutant for null mutations in either of these genes show 100% early larval lethality and have strong defects in NMD (Metzstein and Krasnow 2006; Avery et al. 2011). We found that animals with tissue-directed mosaics of *Upf1* or *Upf2* were viable to the L3 larval stage, and we could easily detect increased expression in homozygous epidermal cells, confirming the feasibility of our approach for identifying organismal lethal mutations affecting NMD. We also found that mutants of the NMD auxiliary factor *Smg1*, which is viable and has weak defects in NMD (Chen et al. 2005; Metzstein and Krasnow 2006; Avery et al. 2011), produced an obvious mosaic phenotype (Fig. 1F). Thus, our strategy should allow us to isolate alleles of both essential and nonessential NMD genes.

We screened for mutations affecting NMD on the X chromosome and the right arm of the third chromosome using chemical mutagenesis and our mosaic approach (Fig. 2). While we identified our new mutants in mosaic animals, for characterization of their roles in the NMD pathway, we studied the alleles in nonmosaic, homozygous animals. In this way, we were able to determine the degree to which the new mutation is required for NMD. We were also able to assess whether the new mutation was lethal or viable in

FIGURE 2. Screens to identify genes required for NMD on X and 3R. In the P₀, males carrying the appropriate FRT are mutagenized by treatment with EMS and mated *en masse* to reporter-containing females. In the F₁ generation, candidate NMD-mutant L3 larvae are identified by mosaic epidermal reporter expression, collected, and individually mated to flies containing marked FRTs. F₂ larvae are scored for transmission of the mosaic-expression phenotype, collected, and crossed to balancer-containing animals to establish stocks. (Middle column) Numbers of animals identified in each generation. (*e22RF*) Genotype *e22c-GAL4*, *UAS-nlsDsRed2::SV40 3'UTR*, *UAS-FLP*.

homozygous animals, giving insight into the requirement of the NMD pathway into organismal survival.

X-chromosome screen has isolated numerous alleles of known NMD genes

We identified 27 X-chromosome mutants, including both lethal and viable alleles (Table 1). Complementation tests revealed that all of these are alleles of the known NMD genes *Upf1*, *Upf2*, and *Smg1*, in which mutations had been previously isolated (Chen et al. 2005; Metzstein and Krasnow 2006). The most interesting phenotypic diversity we found was in our alleles of *Upf2*. When tested in homozygous animals, we found that six of these alleles were lethal and six were viable. This phenotypic difference provided us an opportunity to study the effects of *Upf2* mutations in vivo. *Upf2* is thought to function primarily as a bridge between the other two core components of the NMD complex, *Upf1* and *Upf3*. Binding of *Upf2* to *Upf3* has been mapped to a conserved MIF4G domain (Fig. 3A), while binding of *Upf2* to *Upf1* occurs primarily through a C-terminally localized domain, with a contribution from a domain close to the N terminus (He et al. 1997; Kadlec et al. 2004; Clerici et al. 2009). *Upf2* also contains two other MIF4G domains of unknown function (Fig. 3A). To identify which domains of the *Upf2* protein are necessary for viability, we sequenced the coding region of *Upf2* in eight of our alleles (four lethal and four viable) (Fig. 3A). Our analysis of these *Upf2* alleles revealed an interesting pattern: Lethal mutations are predicted to disrupt the second MIF4G domain but leave the other protein domains intact. Two previously characterized lethal alleles of *Upf2* are also predicted to disrupt the second MIF4G domain (Metzstein

and Krasnow 2006); although these are also protein truncations, this disruption is not very specific. We did recover mutations that are predicted to disrupt the *Upf2* interaction with *Upf1* and that were not lethal. When we assayed NMD pathway activity in these viable mutants, we found that there was always residual pathway activity (Fig. 3B). These results suggest that pathway activity correlates with viability. This agrees with previous results showing that complete loss of the *Upf1/Upf2* interaction is not compatible with viability (Avery et al. 2011). However, we cannot currently rule out that the second MIF4G domain has a cellular function independent of its role in NMD and disruption of this function also results in organismal

TABLE 1. 27 alleles recovered on the X chromosome

<i>Upf1</i>		<i>Upf2</i>		<i>Smg1</i>	
Allele	Viability	Allele	Viability	Allele	Viability
42	Lethal	14-01-3M	Lethal	7-24-3	Viable
A92A	Lethal	3-01-3R	Lethal	8-9A	Viable
8-7H	Lethal	7-5A	Lethal	7-10-2	Viable
14D	Lethal	C41	Lethal	11-29-3-1	Viable
		C51	Lethal	C9	Viable
		C2	Lethal	C73	Viable
		2-8A	Viable	8D2	Viable
		4-5F	Viable	A6	Viable
		C72	Viable	A91	Viable
		3D	Viable	6B	Viable
		C52	Viable	11-2-10B	Viable
		13D	Viable		

X-chromosome screen mutants. All alleles of *Upf1* are lethal, and all alleles of *Smg1* are viable, while alleles of *Upf2* can be either lethal or viable.

FIGURE 3. Characterization of *Upf2* alleles. (A) Mutations in *Upf2*. Alleles shown above the gene model are hemizygous lethal; those below the gene are hemizygous viable. 2-8A contains two mutations: a late glutamine to stop (black), as well as a serine-to-phenylalanine missense mutation (gray). This latter change is in a nonconserved domain of *Upf2*, and we suspect it does not contribute to the *Upf2* defect. (Filled boxes) Coding sequences; (open boxes) 5' and 3' UTRs; (blue) bipartite *Upf1* binding domain; (green) *Upf3* binding domain; (purple) MIF4G domains (the third one of which overlaps the *Upf3* binding domain); (open arrow) direction of transcription. (B) Effect of viable *Upf2* mutations on levels of the PTC-containing transcript *Adh^{m4}* (Q83Stop). Values are the proportion of *Adh^{m4}* transcript relative to *Adh⁺* transcript normalized to *Upf2^{25G}*, in which *Adh^{m4}* is completely stabilized.

lethality. Finally, we noted that three of the four viable *Upf2* alleles were male sterile (data not shown), suggesting a role for *Upf2* in the male germline (Table 2). Whether this role is directly related to its role in NMD has not yet been determined.

We obtained four alleles of *Upf1* but did not find that any of them are compatible with viability (Table 1), suggesting that even subtle perturbations in *Upf1* structure are disruptive to protein function. In addition to *Upf1* and *Upf2*, we isolated 11 alleles of *Smg1*. It has previously been observed that complete loss of *Smg1* leads to no effect on viability and only a slight decrease in NMD function (Chen et al. 2005; Metzstein and Krasnow 2006). Indeed, we found that all 11 alleles were viable (Table 1).

Based on the large number of *Upf1*, *Upf2*, and *Smg1* alleles, we believe that we have saturated the X chromosome for genes required for NMD and that can be mutated by EMS, the chemical mutagen we used. Notably, we did not isolate any alleles of CG32533, the *Drosophila* homolog of the *C. elegans* gene *smgl-2* (Longman et al. 2007), which is located on the X chromosome. In addition to not identifying mutations in CG32533 in our screen, we also tested its function using an RNAi approach (A Chapin and MM Metzstein, unpubl.) and did not detect an NMD defect. Combined, these data suggest that *smgl-2* does not play a significant role in NMD in *Drosophila*. However, since EMS mutagenesis and RNAi knockdown do not affect all genes equally, it will be necessary to obtain mutations in *smgl-2* by some other means to definitively test the role of this gene in NMD in *Drosophila*.

Third chromosome screen has isolated first *Drosophila* alleles of *Smg6*

We screened the right arm of the third chromosome and identified 13 mutants. Similar to the X chromosome, after identifying the mutants with our mosaic approach, we studied

the role of the genes affected in wholly mutant animals. By a complementation test with a chromosomal deficiency, we found that six of our 13 lines were likely to be mutations in *Smg6* (Supplemental Fig. S1). In all cases, the *Smg6* mutation was viable in *trans* to the deficiency. These six alleles also failed to complement each other (using the assay of reporter expression) (Supplemental Fig. S1). Three of the lines were viable as homozygotes. However, the remaining three lines were homozygous lethal, suggesting that extraneous lethal mutations are present on these chromosomes. The other seven lines complemented the deficiency for both viability and reporter enhancement and will be characterized further in a later study.

To identify the molecular changes in our candidate *Smg6* alleles, we sequenced the *Smg6* genomic locus in each of the six mutant lines. A single mutation affecting the *Smg6* gene was identified in each line (Fig. 4A; Table 3). The identified lesions are spread throughout the length of the *Smg6* gene and include premature termination codons, splice mutations, and a single missense change. This missense change,

TABLE 2. Molecular analysis of *Upf2* alleles

Allele	Viability	Mutation	Residue change	Male fertile
14-01-3M	Lethal	T1723A	C575S	N.A.
3-01-3R	Lethal	G1627A	Q543Stop	N.A.
7-5A	Lethal	A1694T	K324Stop	N.A.
C41	Lethal	G1924A	Q642Stop	N.A.
2-8A	Viable	C1876T	S384F	No
		C3298T	Q1100Stop	
4-5F	Viable	T494A	L165Q	No
C52	Viable	T3065C	L1022P	No
13D	Viable	G175A	A59T	Yes

For mutation positions, bases are numbered with the A of the start codon as 1, and introns are included. 7-5A appears to be identical to the previously isolated *Upf2* allele 29AA (Metzstein and Krasnow 2006).

FIGURE 4. Molecular and phenotypic characterization of *Smg6* alleles. (A) Mutations in *Smg6*. (Filled boxes) Coding sequences; (open boxes) 5' and 3' UTRs; (blue) 14-3-3-like protein binding domain; (green) PIN endonucleolytic cleavage domain; (open arrow) direction of transcription. (B) Alignment of *Smg6* PIN domains. These highly conserved domains are representative of PIN domains found in *Smg6* and other PIN-domain-containing proteins (Takeshita et al. 2007). (Dm) *D. melanogaster*; (Hs) *Homo sapiens*; (Ce) *C. elegans*. (*) Invariant catalytic residues; (gray shading) residues conserved in all three species; (red shading) aspartic acid residue altered to a valine in *Smg6^{2a}*. (C) Effect of *Smg6* mutations on levels of the PTC-containing transcript *Adhⁿ⁴* (Q83Stop). Values are the proportion of *Adhⁿ⁴* transcript relative to *Adh⁺* transcript normalized to *Upf2^{25G}*, in which the *Adhⁿ⁴* is completely stabilized. Error bars indicate SD, *n* = 2. (D) Effect of *Smg6* mutations on levels of the endogenous NMD target *tra* in males. Levels for *Smg6* alleles are normalized to *FRT^{82B}/Df* animals; *Smg1^{32AP}/Y* and *Upf2^{25G}/Y* are normalized to *FRT^{19A}/Y*. Error bars indicate SEM, *n* = 3. (E) Percentage survival to adulthood of *Smg6* mutants. Each of the alleles are in *trans* to an *Smg6* deficiency. The percentage is calculated relative to balancer-containing siblings. Error bars: 95% confidence interval of the binomial distribution. (F) Microarray analysis of relative magnitude of NMD defects observed in *Upf2^{25G}*, *Smg1^{32AP}*, and two *Smg6* alleles, *Smg6^{2a}* and *Smg6²⁹²*. For each mutant genotype, we calculated transcript expression relative to its respective wild-type control. To compare the effect of different NMD genes with transcript regulation, we first extracted targets that increased more than twofold in all four of our NMD-mutant conditions, giving a list of 87 genes (Supplemental Table S1). We then compared the magnitude of the increase observed in *Smg1^{32AP}*, *Smg6^{2a}*, and *Smg6²⁹²* with that observed in *Upf2^{25G}*. (Black bars) Transcripts that increased more in *Upf2^{25G}* relative to transcripts in *Smg1* or *Smg6*; (gray bars) transcripts that increase more in *Smg1* or *Smg6* alleles relative to *Upf2^{25G}*.

found in the allele *Smg6^{2a}*, is of particular interest, because it alters an invariantly conserved aspartate residue found in all PIN domains (Fig. 4B) and is known from structural studies to be part of the endonucleolytic catalytic core (Glavan et al. 2006; Takeshita et al. 2007).

Complete loss of *Smg6* still shows NMD-pathway activity in *Drosophila*

To characterize the role of *Drosophila Smg6* in NMD, we analyzed mRNA levels of the well-characterized NMD

TABLE 3. Molecular analysis of *Smg6* alleles

Allele	Base change	Residue change
52a	A535T	K124Stop
70e	GAgtagtatat465Δ	N.A.
292	g558a	N.A.
91a	C2775T	Q748Stop
21a	T2794A	L754Stop
2a	A3580T	D917V

For mutation positions, bases are numbered with the A of the start codon as 1, and introns are included. 70e is a 12-bp deletion that removes the last two bases of the fourth exon (uppercase) and 10 bp of the fourth intron (lowercase). 292 is a point mutation that converts the G of the sixth intron splice donor to an A.

target gene *Adhⁿ⁴*, which contains a nonsense mutation (Chia et al. 1987; Brogna 1999). *Adhⁿ⁴* has been shown to be a bona fide NMD target in both cell culture and whole animals (Brogna 1999; Gatfield et al. 2003; Chen et al. 2005; Metzstein and Krasnow 2006). Furthermore, *Adh* is expressed in most, if not all *Drosophila* tissues (Chintapalli et al. 2007), so by analyzing this gene we can assess the role played by NMD in diverse cell types.

Steady-state mRNA levels depend on mRNA stability and are thus a proxy for decay rates, and in *Adhⁿ⁴/Adh⁺* animals, *Adhⁿ⁴* mRNA is expressed at ~10% of the amount of the *Adh⁺* transcript, due to NMD-dependent destabilization of the mutant transcript (Brogna 1999; Chen et al. 2005; Metzstein and Krasnow 2006). We found that loss of *Smg6* function results in stabilization of the *Adhⁿ⁴* transcript to ~30% of the amount of *Adh⁺* transcript (Fig. 4C). A similar degree of stabilization was observed in each of the six *Smg6* alleles in *trans* to a deficiency (Fig. 4C). In comparison, as previously reported, we found that *Smg1* mutants had only a very slight effect on *Adhⁿ⁴* levels (Chen et al. 2005; Metzstein and Krasnow 2006), whereas *Upf2^{25G}*, a strong hypomorphic allele of the core NMD component *Upf2* that essentially abolishes NMD, leads to complete stabilization of *Adhⁿ⁴* (Metzstein and Krasnow 2006). Loss of *Smg6* leads to considerably higher levels of *Adhⁿ⁴* than loss of *Smg1*, but significantly, stabilization of *Adhⁿ⁴* in *Smg6* is not nearly as strong as that observed in *Upf2^{25G}*. This suggests that *Smg6* is not absolutely required for NMD in vivo. We found similar results analyzing a PTC-containing allele of the developmental gene *eyes absent* (*eya*) (Supplemental Fig. S2A).

To determine if any of our *Smg6* alleles are definitively null, we used the classic genetic test of comparing homozygotes to the allele in *trans* to a deficiency. For this, we used quantitative reverse transcription coupled PCR (qRT-PCR) to measure the levels of the endogenous NMD-target gene *transformer* (*tra*) in *Smg6* mutant males (Fig. 4D) (this analysis was possible using the alleles 292, 91a, and 2a, since these were not associated with any linked lethal mutation). Like *Adh*, *tra* is broadly expressed in *Drosophila* tissues and

has been shown to be a bona fide NMD target in both intact animals (Metzstein and Krasnow 2006; Avery et al. 2011) and S2 cell culture (Rehwinkel et al. 2005).

Similar to what we observed for *Adhⁿ⁴*, we found that loss of *Smg6* function results in increased expression of *tra* to a level intermediate between *Smg1* and *Upf2^{25G}*. Of particular note, we observed that the degree of stabilization of *tra* was the same in *Smg6²⁹²* homozygotes and *Smg6²⁹²/Df* hemizygotes, defining this as a null allele. Additionally, one of our other alleles, *Smg6^{52a}*, is predicted to be a molecular null, because it contains an early stop codon truncating the coding region of *Smg6* before any known functional domains. Although we could not examine *Smg6^{52a}* homozygotes due to a linked lethal mutation, we did find that *Smg6^{52a}* displays a similar degree of *tra* stabilization in *trans* to a deficiency, as does *Smg6²⁹²*. These results were confirmed using two other endogenous NMD targets, *Smg5* and *Oda* (Supplemental Fig. S2B; Gatfield and Izaurralde 2004; Rehwinkel et al. 2005; Avery et al. 2011). Thus, by both molecular and genetic criteria, we have isolated the first definitively null alleles of *Smg6*.

Interestingly, two of the *Smg6* alleles that specifically disrupt the PIN domain seem to retain residual activity. *Smg6^{91a}*, a nonsense mutation just preceding the PIN domain, and *Smg6^{2a}*, the missense mutation in the PIN domain, have lower levels of *tra* stabilization than null alleles, and this stabilization is increased when these alleles are placed in *trans* to a deficiency (Fig. 4D; Supplemental Fig. S2B). These results suggest that *Smg6* has some activity independent of the PIN domain and thus presumably independent of its role as an endonuclease.

In general, the severity of NMD mutations and organ-ismal viability are correlated in *Drosophila*. The NMD genes *Upf1* and *Upf2* are lethal as null alleles in *Drosophila*. The strong hypomorphic allele of *Upf2^{25G}* is strongly disrupted in NMD function, and mutant animals are only partially viable, while a deletion of *Upf3* has moderate effects on NMD and is viable (Avery et al. 2011). Null alleles of *Smg1* have mild effects on NMD and are fully viable (Chen et al. 2005; Metzstein and Krasnow 2006). We therefore investigated the requirement for *Smg6* for viability by analyzing each of the *Smg6* alleles in *trans* to a deficiency for their survival to adulthood. In each case, loss of *Smg6* leads to only a moderate reduction in viability (Fig. 4E), further demonstrating that *Smg6* is not absolutely required for NMD in *Drosophila*.

To determine on a more global level whether loss of *Smg6* activity had less effect on the NMD pathway than loss of *Upf2*, we performed a microarray analysis. RNA was extracted from *Upf2^{25G}*, *Smg6^{2a}*, and *Smg6²⁹²* mutant animals and hybridized to microarrays representing the complete *Drosophila* transcriptome. We then tabulated genes that increased twofold over matched wild-type controls in all of our mutant conditions (Supplemental Table S1). We found that the large majority of

such genes had increased expression levels in *Upf2*^{25G} mutants higher than the increase observed in *Smg6* mutants (Fig. 4F). From this, we conclude that, for the majority of endogenous NMD targets, *Smg6* mutants still possess residual NMD-pathway activity. When we tested *Smg1*^{32AP} by the same assay, we found that it led to generally lower levels of transcript stabilization compared with *Smg6* (Fig. 4F). These results are consistent with our analysis of candidate transcripts, described above.

Another phenotype associated with loss of NMD function in *Drosophila* is impairment of cell proliferation, both in S2 cell culture as well as in vivo (Rehwinkel et al. 2005; Metzstein and Krasnow 2006; Avery et al. 2011). We examined cell proliferation mosaic analysis in the eye, a technique that permits comparison of homozygous mutant cell growth in the presence or absence of wild-type cells (Stowers and Schwarz 1999). We find that, similar to other NMD mutants (Metzstein and Krasnow 2006; Avery et al. 2011), *Smg6* mutant cells are defective in cell proliferation when in competition with wild-type cells, as assayed by examination of mitotic clones within the eye (Supplemental Fig. S4A–D). However, when competing cells are removed, *Smg6* mutant cells can essentially proliferate as well as wild-type cells (Supplemental Fig. S4E–H). These data show that while *Smg6* is dispensable for viability, it is still required for aspects of cell function in vivo.

Conclusions

We have isolated point mutations in *Drosophila Smg6*, which are the first mutant alleles of *Smg6* characterized in any organism since the initial identification of this gene in *C. elegans* (Hodgkin et al. 1989). Since a number of our *Smg6* alleles clearly lack all function, this has allowed us to assess the contribution of this gene to the NMD process in vivo. We find that in *Drosophila*, *Smg6* has only a moderate role in targeting PTC-containing transcripts for degradation. In comparison, mutations in core NMD components, such as *Upf2*, lead to complete stabilization of NMD targets in vivo (Metzstein and Krasnow 2006; Avery et al. 2011). Our results contrast with previous analysis of *Smg6* in *Drosophila* S2 cells using RNAi-mediated knockdown, in which inactivation of *Smg6* appeared to result in as strong an effect on NMD targets as inactivation of other components of the NMD pathway (Gatfield et al. 2003; Rehwinkel et al. 2005). One explanation for this contradiction is that it is not possible to assess the exact degree of gene inactivation in RNAi knockdown experiments, making comparisons of the requirements of different genes in a process difficult to assess.

It is interesting to compare our results of *Smg6* mutations in vivo with the study of this gene in other species. In *C. elegans*, the organism in which *Smg6* was first identified,

the NMD process seems to be as dependent on *Smg6* as any of the other NMD genes (Hodgkin et al. 1989). However, because no description of the molecular nature of *C. elegans Smg6* alleles has been published, it is unknown if any of the known alleles are null. Indeed, there is some evidence that null alleles of *C. elegans smg-6* are not viable, thus the alleles allowing viable NMD-defective animals may indeed possess residual function (Cali and Anderson 1998). In zebrafish, *Smg6* has been analyzed by morpholino knockdown in intact fish and by expression of an *Smg6* dominant-negative construct in fish cell lines (Wittkopp et al. 2009). The morpholino knockdown behaves very similarly to knockdown of other NMD components, leading to early lethality with distinct patterning and neural defects, suggesting that *Smg6* has similar roles to these other components in vivo. However, because direct measurement of NMD-pathway activity was not performed in the intact *Smg6* morphants, it is possible that there is still residual pathway activity, as observed in *Drosophila*. Measurements of NMD-pathway activity were analyzed in fish cell culture and clearly show a defect in NMD. Whether this defect is equivalent to complete NMD-pathway loss was not analyzed.

Smg6 is an endonuclease that cleaves target mRNAs around the site of the premature termination codon (Huntzinger et al. 2008), and our data support this as an important *Smg6* function. All of our alleles of *Smg6* are predicted to disrupt the C-terminal endonucleolytic PIN domain, including one that is a point mutation affecting a key catalytic residue. However, since *Smg6*-null mutants still show considerable NMD-pathway activity, this suggests that in intact *Drosophila* there exists an *Smg6*-independent degradation pathway. In yeast, NMD targets are thought to be degraded by decapping followed by 5'–3' exonucleolytic degradation (Muhlrad and Parker 1994; He et al. 2003). Inactivation of this pathway was shown not to have an effect on NMD-mediated degradation in S2 cells (Gatfield et al. 2003), but one possibility is that this mechanism is only used in the absence of *Smg6*-mediated cleavage. Indeed, mammalian cells are known to use multiple decay pathways (Chen and Shyu 2003; Lejeune et al. 2003; Huang et al. 2011). *Drosophila* genetics may provide a powerful system for identifying such alternative degradation pathways, because mutations in such genes would be predicted to act as enhancers of the *Smg6* phenotype.

Like *Smg6*, *Smg1* is not absolutely required for NMD in *Drosophila* (Chen et al. 2005; Metzstein and Krasnow 2006). *Smg1* is a PIKK-family kinase that phosphorylates the core NMD component *Upf1* to provide a binding platform for *Smg6* (Kashima et al. 2006; Okada-Katsuhata et al. 2012). If such binding were absolutely necessary for *Smg6* activity, we would expect that *Smg1* mutations would have at least as strong an NMD defect as *Smg6* mutations. However, we find just the opposite: Loss of *Smg6* activity leads to a significantly stronger NMD defect than loss of *Smg1*, suggesting that *Smg6* has activity independent of

Smg1-mediated phosphorylation of Upf1. In mammalian cells, Smg6 can interact with the NMD complex independent of binding to phosphorylated Upf1 through interactions between two N-terminal exon junction complex binding domains (Kashima et al. 2010). *Drosophila* is thought to lack these domains, but it remains possible that Upf1-phosphorylation-independent interactions can still occur. Alternatively, the *Drosophila* genome may contain an alternative kinase redundant with Smg1 that can phosphorylate Upf1. This could explain why Smg1 has a relatively weak effect on NMD in intact *Drosophila* and provide an alternative mechanism of Smg6 entry into the NMD complex. It should be possible to identify such a kinase by testing other kinases in the *Drosophila* genome for synergistic effects with *Smg1* mutants on the NMD pathway.

Analysis of NMD in mammalian cells has suggested that NMD is not a linear pathway, but potentially different branches act in parallel to robustly regulate the abundance of target mRNAs. For instance, there exist Upf3-dependent and Upf3-independent NMD pathways targeting certain nonsense transcripts in mammalian cells (Chan et al. 2007; Yepiskoposyan et al. 2011). Our genetic screen and molecular analysis presented here has also revealed that multiple mechanisms function to regulate NMD in *Drosophila*. Finally, the efficient screening method for NMD genes presented here should be applicable to the entire *Drosophila* genome, providing the opportunity to identify all of the components contributing to these parallel mechanisms.

MATERIALS AND METHODS

Fly stocks

Drosophila melanogaster lines were raised at 25°C on cornmeal/dextrose medium using standard conditions. Control chromosomes used for the screens were *y w FRT^{19A}* and *FRT^{82B}* (Xu and Rubin 1993); the *e22c-GAL4* and *UAS-FLP* stocks (Duffy et al. 1998) were used in all screen stocks to induce epidermal mosaics using the *GAL4/UAS* system (Brand and Perrimon 1993) and *FLP/FRT* systems (Xu and Rubin 1993). The *nlsDsRed2::SV40 3'UTR* reporter transgene was provided by M. Galko (M.D. Anderson Cancer Center). *e22c-GAL4*, *UAS-nlsDsRed2::SV40 3'UTR*, *UAS-FLP* was built using standard recombination methods (MM Metzstein, unpubl.). NMD alleles *Upf2^{25G}*, *Smg1^{32AP}*, and *Upf1^{26A}* have been described previously (Metzstein and Krasnow 2006). The *breathless* promoter *btl-Gal4* was used in combination with *UAS-GFP* as the reporter in the X-chromosome complementation tests (Shiga et al. 1996). For the balancer chromosomes, we used *w^{*}*; *ry⁵⁰⁶ Dr¹/TM6B*, *P{Dfd-EYFP}* *Sb¹ ca¹* (Le et al. 2006) and *FM7i*, *P{ActGFP}JMR3* (Reichhart and Ferrandon 1998). Complementation tests and deficiency experiments for *Smg6* were performed using *Df(3R)ED6220/TM6c Tb*, *Sb* (Ryder et al. 2007). Eye clones were generated using *w*; *ey-FLP*; *FRT^{82B} P{w+ ry+}90E* and *y w*; *ey-GAL4*, *UAS-FLP*; *FRT^{82B} GMR-hid/TM6* (Stowers and Schwarz 1999). For NMD tests, we used the PTC-containing alleles *Adhⁿ⁴* (Chia et al. 1987) and *eya^{cti-III}* (Bui et al. 2000).

Mutagenesis and screen scheme

For mutagenesis, isogenized males were aged 1–3 d, starved overnight, and fed 25 mM ethane methylsulfonate in 1% sucrose for 24 h (Newsome et al. 2000). For the X-chromosome screen, *y w FRT^{19A}/Y* mutagenized males were mated *en masse* to *y w FRT^{19A}*; *e22c-GAL4*, *UAS-nlsDsRed2::SV40 3'UTR*, *UAS-FLP/CyO* virgins. F₁ wandering third-instar larvae were collected in glycerol and analyzed using a Leica MZ16 fluorescence stereomicroscope. NMD mutants were identified by mosaic enhancement of the DsRed reporter in the larval epidermis. Candidate mutant F₁ larvae were collected, rinsed in 1× PBS, placed in individual vials, and allowed to complete development. Surviving F₁'s were mated to *sc cv ct v g f FRT^{19A}/Y*, and F₂ progeny were retested for mosaic enhancement of DsRed expression; mosaic animals were collected and mated to *FM7c/Y* males to establish balanced mutant lines. We screened about 38,000 DsRed-expressing F₁ animals. Of these, we expected 50% to be females, which could exhibit mosaicism (the others are hemizygous males and thus would not be expected to undergo FLP-mediated recombination). We identified 122 mosaic larvae from which we recovered 27 balanced mutant lines, representing a recovery rate of ~22%.

For the third chromosome, mutagenized *w/Y*; *FRT^{82B}* males were mated *en masse* to *e22c-GAL4*, *UAS-nlsDsRed2::SV40 3'UTR*, *UAS-FLP/CyO*; *FRT^{82B}* virgins, and F₁ animals were scored and collected as described above. Candidate mutants were retested by mating to *y w*; *FRT^{82B} Sb¹/TM6B ry Tb⁺* animals of the opposite sex, and male progeny that retested were collected and mated to *w*; *Kr/CyO*; *D/TM6c Tb*, *Sb* or to *w^{*}*; *ry⁵⁰⁶ Dr¹/TM6B*, *P{Dfd-EYFP}*, *Sb¹ ca¹* to establish mutant lines. We scored about 17,000 F₁'s (both males and females would be expected to be mosaics), and we identified 81 mosaic larvae. Of these we were able to establish 13 balanced mutant lines, representing a recovery rate of ~16%.

Complementation tests

X

There are three known NMD genes on the X chromosome with previously isolated alleles (Metzstein and Krasnow 2006). Null alleles of *Upf1* and *Upf2* are lethal, whereas null alleles of *Smg1* are viable (Chen et al. 2005; Metzstein and Krasnow 2006). Complementation tests were performed using these alleles. For *Upf1* complementation tests, *y w FRT^{19A} */FM7c* (where * indicates EMS-induced mutation) virgins were mated to *y w Upf1^{13D} FRT^{19A}/Dp(1;Y)BSC1[Upf1⁺]* males, and F₁ progeny were scored for the absence of B⁺ females, which indicates failure to complement *Upf1* for lethality. In cases in which complementation for lethality occurred, the mutant lines were then crossed to *y w Upf1^{13D} FRT^{19A}/Dp(1;Y)BSC1[Upf1⁺]*; *Btl-Gal4*, *UAS-GFP::SV40 3'UTR/+* males, and F₁ female third-instar larvae were scored for enhancement of GFP. Failure to complement this phenotype would have indicated that the candidate gene is a viable *Upf1* hypomorph. However, we did not find any alleles of this class.

For *Upf2* complementation tests, *y w FRT^{19A}/FM7c* virgins were crossed to *y w Upf2^{14J} v g f FRT^{19A}/Y*; *P{w⁺, Upf2⁺}/+* males (Metzstein and Krasnow 2006), and F₁ animals were scored for the absence of w B⁺ females, which indicates failure to complement *Upf2* for lethality. In cases in which complementation occurred,

the mutant lines were crossed to $y w Upf2^{14j} v g f FRT^{19A}/Y; P\{w^+, Upf2^+\}/Btl-Gal4, UAS-GFP::SV40\ 3'UTR$ males, and F₁ female third-instar larvae were scored for GFP enhancement. Failure to complement indicates that the *Upf2* allele is a viable hypomorph. For *Smg1* complementation tests, females from mutant lines were crossed to $y w Smg1^{32AP} FRT^{19A}/Y; Btl-Gal4, UAS-GFP::SV40\ 3'UTR/+$ male larvae and were scored for GFP enhancement, indicating failure to complement *Smg1*.

3R

Complementation tests were performed by crossing *e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR, UAS-FLP/CyO; FRT^{82B}*/TM6c Tb, Sb* females to *Df(3R)ED6220/TM6c Tb, Sb* animals, and third-instar larvae were scored for DsRed enhancement. Lines that failed to complement *Smg6* were intercrossed to determine if the *Smg6* mutations were lethal or had additional linked lethals.

RNA isolation and quantitative RT-PCR

Total RNA was isolated from five to 10 flash-frozen samples of adult males using TRIzol reagent (Invitrogen) and phase-lock tubes (5-prime), and the RNeasy mini kit (QIAGEN). Genomic contamination was reduced by on-column DNase treatment (Ambion). RNA concentration was determined using a spectrophotometer and normalized for reverse transcription using the MuMuv reverse transcriptase Retroscript Kit (Ambion). Quantitative RT-PCR was performed on a Bio-Rad iCycler thermocycler using SYBR Green Supermix (Bio-Rad). All experimental reactions were performed in triplicate with a minimum of three biological replicates. For quantitation, samples were normalized to *RpL32*.

Analysis of *Adhⁿ⁴*

Adhⁿ⁴/CyO animals were mated to *Df(3R)ED6220/TM6B, P{Dfd-EYFP} Sb¹ ca¹*. Males in the F₁ generation *Adhⁿ⁴/Adh⁺; Df(3R)ED6220/+* were collected and mated to virgin *FRT^{82B}/TM6B, P{Dfd-EYFP} Sb¹ ca¹* or *FRT^{82B} Smg6*/TM6B, P{Dfd-EYFP} Sb¹ ca¹* flies. Animals of genotype *Adhⁿ⁴/Adh⁺; FRT^{82B} Smg6*/Df(3R)ED6220* and *Adhⁿ⁴/Adh⁺; FRT^{82B}/Df(3R)ED6220* were collected by genotyping for the presence of the *Adhⁿ⁴*. Animals of genotypes *Smg1^{32AP}/Y; Adhⁿ⁴/Adh⁺* and *Upf2^{25G}/Y; Adhⁿ⁴/Adh⁺* were collected in parallel. Two biological replicates were collected for each genotype, and RNA isolation of five to eight adult males was performed as described above. Samples were normalized for RNA concentration and reverse-transcribed. The cDNA from each sample was used as template DNA in a PCR amplification of the *Adh* transcript. To distinguish the *Adh⁺* and *Adhⁿ⁴* alleles, the PCR product was digested using PvuII and run on a 1% agarose gel (Supplemental Fig. S3). The gels were imaged using an Alpha Innotech Gel Doc System and quantitated using ImageJ (Abramoff et al. 2004).

Analysis of *eya^{cli-IID}*

eya^{cli-IID} cn¹ bw¹ sp¹/CyO males were mated to *e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR/CyO; FRT^{82B}*, where * represents Smg6⁺, Smg6^{2a}, or Smg6²⁹²* virgins. Male progeny of genotype *eya^{cli-IID} cn¹ bw¹ sp¹/e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR; FRT^{82B}*/+* were mated to virgins of genotype *Btl-Gal4, UAS-GFP::SV40\ 3'UTR/+; Df(3R)ED6220/+*. In the next generation, we collected

larvae of genotype *eya^{cli-IID} cn¹ bw¹ sp¹/Btl-Gal4, UAS-GFP::SV40\ 3'UTR; FRT^{82B}*/Df(3R)ED6220* based on increased GFP expression (due to the *Smg6* mutation) and a lack of DsRed expression. A similar strategy was used on the X chromosome to collect larvae that were either *y w FRT^{19A}* or *y w Upf2^{25G} FRT^{19A}/Y; eya^{cli-IID} cn¹ bw¹ sp¹/e22c-GAL4, UAS-nlsDsRed2::SV40\ 3'UTR*. RNA was isolated as described above, reverse-transcribed, and amplified using primers surrounding the *eya^{cli-IID}* PTC mutation. We determined the sequence of the RT-PCR product. *eya^{cli-IID}* stabilization was determined by measuring heights of the *eya⁺* and *eya^{cli-IID}* sequencing peaks.

Microarray analysis

RNA was isolated from male larvae collected from 0 to 4 h after the molt to third instar. To identify genotypes at this stage, we used balancers marked with fluorescent reporters: *ActGFP* for the X (Reichhart and Ferrandon 1998) and *Dfd-EYFP* for the third (Le et al. 2006). For *Smg6* experiments, we crossed *FM7i, P{ActGFP}JMR3/Y; Df(3R)ED6220/TM6B, P{Dfd-EYFP} Sb¹ ca¹* males to either *w; FRT^{82B}/TM6B, P{Dfd-EYFP} Sb¹ ca¹* or *w; Smg6^{2a}/TM6B, P{Dfd-EYFP} Sb¹ ca¹*, or *w; Smg6²⁹²/TM6B, P{Dfd-EYFP} Sb¹ ca¹* virgins. For the X-chromosome NMD genes, we crossed *FM7i, P{ActGFP}JMR3/Y* males to either *y w FRT^{19A}* or *y w Smg1^{32AP} FRT^{19A}*, or *y w Upf2^{25G} FRT^{19A}/FM7i, P{ActGFP}JMR3* virgins. Progeny from these crosses were staged, and the appropriate genotypes were collected by scoring for the absence of the fluorescent balancers. RNA from two biological replicates of each genotype was reverse-transcribed, and the cDNA was labeled with either Cy3 or Cy5 and hybridized to Agilent 44K 2-color arrays. Data files were produced using Agilent's feature extraction software version 10.5. We normalized each array to total signal.

Smg6 viability analysis

To test the viability of *Smg6* alleles, virgin *FRT^{82B}* or *FRT^{82B} Smg6*/TM6B, P{Dfd-EYFP} Sb¹ ca¹* flies were mated to *Df(3R)ED6220/TM6B, P{Dfd-EYFP} Sb¹ ca¹* males, and progeny adults were analyzed for the presence and absence of the *Sb* marker associated with the balancer chromosome.

Eye clones

Mosaic eyes were generated by crossing *w; ey-FLP; FRT^{82B} P{w+ry+}90E* virgins to *FRT^{82B}* or *FRT^{82B} Smg6** (where * is EMS-generated mutation) males. Images at multiple planes were taken on an Axioskop2 plus (Zeiss) microscope and analyzed using an ImageJ stack analyzer and a stack focuser plug-in. Entirely mutant eyes were generated by the *GMR-hid* technique (Stowers and Schwarz 1999).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

ACKNOWLEDGMENTS

We thank Michael Galko for *UAS-nls:DsRed* transgenes; Alex Chapin and Jon Nelson for unpublished data; Shanhong Cheng,

Sarah Elliott, and Deborah Johnson for assistance in screening; Barry Moore and Mark Yandell for help with array bioinformatics; the Metzstein and Thummel laboratories for useful discussions; and Gillian Stanfield, Carl Thummel, and members of the Metzstein laboratory for their comments on the manuscript. Fly stocks were obtained from the Bloomington *Drosophila* Stock Center. This work was supported by National Institutes of Health (NIH) University of Utah Developmental Biology Training Grant 5T32-HD07491 (to K.A.F.) and NIH 1R01GM084011 and a March of Dimes Award 5-FY07-664 (to M.M.M.).

Received February 20, 2012; accepted June 7, 2012.

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RNA 2012 18: 1475-1486 originally published online June 27, 2012

Access the most recent version at doi:[10.1261/rna.032821.112](https://doi.org/10.1261/rna.032821.112)

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