GENETIC DISSECTION OF NONSENSE-MEDIATED MRNA DECAY IN *DROSOPHILA*

by

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ABSTRACT

The nonsense-mediated mRNA decay (NMD) pathway functions as a quality control mechanism and a feature of post-transcriptional gene regulation. NMD degrades mRNAs containing premature termination codons (PTCs) to prevent the production of potentially harmful truncated proteins, and it also destroys many error-free endogenous mRNAs to limit the expression of these genes. NMD is critical for viability in most complex organisms, highlighting the importance of this pathway; however, it is unknown which of the two NMD functions is the feature essential for viability. Understanding how NMD recognizes and degrades targets may provide insight to uncover the requirement of this pathway for viability, but the molecular mechanisms of NMD target recognition and destruction also remain unclear. Work presented in this dissertation describes genetic analysis in *Drosophila* to reveal the feature of the NMD pathway that is critical for viability, and refines the model describing the mechanism of NMD target degradation. We screened a collection of heterozygous deficiencies for suppression of the incomplete lethality of a hypomorphic allele of the core NMD factor *Upf2*. This screen identified three autosomal regions that partially suppress *Upf2* mutant lethality when deleted. The endogenous NMD target *Gadd45* is located within one suppressing region, and we found that elimination of *Gadd45* restores viability to multiple null NMD mutants. *Mekk1*, a factor that acts downstream of *Gadd45*, resides in another suppressing region, and loss of *Mekk1* also restores viability to NMD mutants. The third suppressing region contains

Arc2, and we found that *Arc2* and the closely related *Arc1* may also contribute to the lethality of *Drosophila* lacking NMD activity. In addition, this dissertation describes the first genetic analysis of *Drosophila Smg5* mutants, and determines that *Smg5* is a critical NMD factor required for viability and all NMD function. Further analysis of the first characterization of double mutants for multiple NMD factors reveals there are multiple mechanisms for NMD target degradation. These findings provide a new foundation for understanding the crucial NMD gene regulatory function and reshape the model of the NMD pathway.

This dissertation is dedicated to my wife, Sarah.

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CHAPTER 1

INTRODUCTION

The precise temporal and spatial control of gene expression in response to genetic and environmental cues is a fundamental aspect of development. Indeed, the mis-expression of genes is the driving force behind many diseases. Factors that impact mRNA **transcription** are usually focused on as the key aspect of gene expression, however post-transcriptional regulation of mRNA **stability** also has a major influence. The importance of post-transcriptional gene regulation is highlighted by the diseases that can occur when many of the factors that regulate this process are disrupted (Cooper et al., 2009). Thus, understanding mRNA degradation is critical to comprehending the regulation of gene expression and the detrimental effects caused by loss of this activity.

There are a variety of mRNA decay mechanisms, which can broadly be classified as general or specific. General mRNA decay occurs after deadenylation or removal of the 5' m^7G-cap. These events allow for 5'-to-3' and 3'-to-5' exonucleases, respectively XRN1 and the Exosome complex, to have access to the mRNA (Chen and Shyu, 2011). Specific decay mechanisms require the presence of *cis*-acting features for mRNA to be recognized by such pathways. These specific decay pathways have two main features: regulatory mechanisms that maintain proper levels of gene expression, and surveillance mechanisms that ensure mRNA quality (Adjibade and Mazroui, 2014). The regulatory mechanisms include pathways that use proteins to recognize sequence- or structure-

specific features of targets, such as ARE-mediated, GU-mediated, CDE-mediated, and IRE1-dependent decay, and pathways that require non-coding RNAs to recognize targets, such as miRNA-mediated decay (Adjibade and Mazroui, 2014; Maurel et al., 2014). Surveillance mechanisms do not require recognition of any specific sequence or structure, but instead recognize errors occurring during translation and destroy the defective mRNAs. These surveillance pathways include nonstop decay, which destroys mRNAs with no stop codon when the ribosome reaches the poly-A tail (Vasudevan et al., 2002), and no-go decay, which initiates the destruction of mRNAs with stalled translation elongation complexes (Harigaya and Parker, 2010).

One mRNA decay pathway, nonsense-mediated mRNA decay (NMD), straddles the classification of regulatory or surveillance mechanism (Celik et al., 2015; Peccarelli and Kebaara, 2014). The NMD pathway was first identified as a mechanism that degrades mRNAs containing premature termination codons (PTCs) (Celik et al., 2015). A PTC is an erroneous stop codon that occurs in the open reading frame prior to the endogenous stop, usually caused by a nonsense mutation or error in mRNA splicing or transcription (Kurosaki and Maquat, 2016). By degrading PTC-containing mRNAs, NMD prevents the translation of truncated polypeptides that can have dominant detrimental effects in the cell (Miller and Pearce, 2014). NMD was later discovered to also degrade many wild-type mRNAs as a mechanism of post-transcriptional gene regulation, possibly because these mRNAs contain features that simulate PTCs (Peccarelli and Kebaara, 2014). Both the quality control and gene-regulatory activities of NMD are conserved in all eukaryotes (Conti and Izaurralde, 2005), indicating that these functions are critical features of the NMD pathway. These dual roles of NMD suggest it

is a crucial aspect of cellular mRNA metabolism. The modulation of NMD activity is a potential therapy for a wide range of genetic diseases (Keeling and Bedwell, 2011). Some alleles produce PTC-containing mRNAs that manage to evade NMD and can cause dominantly-inherited disease. In these cases, increasing NMD activity may serve to alleviate the severity of such diseases (Bhuvanagiri et al., 2010). Additionally, many recessive genetic diseases are caused by loss of protein function due to the PTC-containing mRNAs being degraded by NMD. In these cases, suppression of NMD activity may allow for translation of partially functional proteins and restore some gene activity, reducing disease symptom severity (Keeling and Bedwell, 2011). When considering potentially altering NMD activity, it is particularly important to understand the mechanisms of target recognition and degradation to identify how to manipulate these functions. In addition, it is important to understand the biological processes regulated by the NMD pathway to recognize possible side-effects that may result from enhancing or inhibiting NMD activity. This dissertation will describe work to understand the mechanisms of NMD targeting and decay, as well as the requirement for the gene regulatory NMD function.

Components of the NMD Complex

The phenomenon of the instability of PTC-containing mRNAs has been well known since the 1970s (Losson and Lacroute, 1979). However, it was not until the late 1980s and early 1990s that the first molecular factors required for NMD were identified through forward genetic screens in yeast and *C. elegans* (Hodgkin et al., 1989; Leeds et al., 1991; 1992). Many separate groups identified mutants that suppressed defects caused by PTC-containing alleles of diverse genes. The suppressor mutations identified in *S.*

cerevisiae were mapped to three genes and named *up frameshift* (*upf*) 1-3, because they suppressed a +1 frameshift mutation of *his4* that created a PTC (Leeds et al., 1991; 1992). The *C. elegans* alleles mapped to six loci and were termed *suppressor with morphological effect on genitalia* (*smg*) 1-6 because they suppressed the paralysis defect of *unc-54* and other mutants, and showed morphological defects of both male and female genitals (Hodgkin et al., 1989). A seventh *C. elegans* NMD factor, *smg-7,* was later identified through similar analysis (Cali et al., 1999). *upf1, upf2,* and *upf3* are orthologues of *smg-2, smg-3*, and *smg-4*, respectively (Aronoff et al., 2001; Johns et al., 2007; Page et al., 1999), and all seven worm factors are highly conserved throughout metazoans.

While these screens were the first to identify the NMD factors, they were not a comprehensive identification of all of the components contributing to NMD activity. One major limitation to these screens was that they could only identify viable mutants. Future screens in *C. elegans* using an RNAi approach identified two other genes, called *smg lethal*-1 (*smgl-1*) and *smgl*-2, which are required for both NMD and viability in these animals (Longman et al., 2007). Two additional factors, called *smg-8* and *smg-9*, were later identified to bind *smg-1* in HeLa cells, and homologues of these genes are found in *C. elegans* (Yamashita et al., 2009), however their contribution to NMD function remains unclear (Rosains and Mango, 2012). Although potential NMD factors continue to be identified (Cho et al., 2013; Melero et al., 2016), *Upf1, Upf2, Upf3, Smg1, Smg5, Smg6,* and *Smg7* are generally focused on as the major factors contributing to NMD activity due to their high conservation and the strong NMD defects that occur in any eukaryote when their function is lost.

A series of studies characterizing the molecular interactions between NMD factors, combined with mutant analysis in yeast and *C. elegans*, have provided a working model of the protein interactions that occur during NMD after target recognition **(Figure 1.1)**. Characterization of Upf1 protein function found it has RNA-dependent ATPase and RNA helicase activities, in addition to an ability to bind mRNA, all of which are required for NMD targets to be degraded (Czaplinski et al., 1995; Weng et al., 1996b; 1996a). The ATPase activity stimulates Upf1 to dissociate from mRNAs, and Upf1 that cannot bind or hydrolyze ATP promiscuously binds both target and non-target mRNAs (Franks et al., 2010; Lee et al., 2015), suggesting that Upf1 may bind all mRNAs transiently. The inhibition of Upf1 ATPase activity when bound to PTC-containing mRNAs is then likely a critical feature of NMD target recognition. The specificity of Upf1 binding to PTC-containing mRNAs may be due to the interaction between Upf1 and the translation termination factor eRF3 at PTCs, which blocks Upf1 ATPase activity (Czaplinski et al., 1998). During normal translation termination, Poly-A Binding Protein 1 (PABP1) binds eRF3, promoting a stable, continuously translated messenger ribonucleoprotein (Cosson et al., 2002; Hoshino et al., 1999), and this interaction antagonizes Upf1 binding to eRF3 (Singh et al., 2008). The probability of Upf1 binding eRF3 is likely determined by the proximity of PABP1 to eRF3 during translation termination, since tethering PABP1 near a PTC is sufficient to block Upf1 binding (Lee et al., 2015). Thus, it is likely that the increased distance between the site of translation termination and the poly-A tail caused by a PTC allows for Upf1 to bind eRF3, inhibiting ATP hydrolysis, and stabilizing Upf1 binding to the PTC-containing mRNA **(Figure 1.1A)**.

Upf1 protein was found to stably interact in a complex with Upf2 and Upf3, via directly binding Upf2, which bridges the interaction between Upf1 and Upf3 (He et al., 1997; Lykke-Andersen et al., 2000). This interaction with Upf2 stimulates Upf1 ATPase and helicase function (Chakrabarti et al., 2011; Chamieh et al., 2007) and is required for the degradation of NMD targets (He et al., 1996), indicating the assembly of this complex on a target mRNA is a critical step in the NMD pathway. Upf3 promiscuously binds mRNAs through direct interaction with the exon-exon junction complex (EJC) (Gehring et al., 2003), and Upf2 also associates with EJC factors, likely thorough its binding with Upf3 (Le Hir et al., 2001). Tethering of EJC factors or any one of the Upf proteins to an mRNA downstream of a stop codon is sufficient to induce degradation (Lykke-Andersen et al., 2000; 2001), suggesting that an EJC downstream of a PTC can promote NMD activity. These findings support a model where Upf1 interacts with eRF3 at PTCs, blocking ATPase activity and maintaining binding to the mRNA, which cannot be released until Upf2 binds Upf1. When an EJC is located downstream of this PTC, Upf3 bound to the EJC increases the likelihood for Upf2 to interact with Upf1, forming a stable complex of these three proteins bound to the mRNA through Upf1 and the EJC **(Figure 1.1B)**. Upf2 then activates the Upf1 ATPase and helicase, initiating NMD activity.

The characterization of the binding activities of the Upf proteins can explain how the NMD complex is recruited to PTC-containing mRNAs, but it does not solve the mechanisms of mRNA degradation. Upf1 has been shown to interact with members of the Dcp decapping complex in both yeast and human cells (Lykke-Andersen, 2002; Tarassov et al., 2008), suggesting it may promote decapping and subsequent 5'-to-3'

exonucleolytic degradation of target mRNAs. In fact, the 5'-to-3' exonuclease *xrn1* is required for degradation of NMD target mRNAs in yeast (He and Jacobson, 2001), suggesting decapping is the major mechanisms for target degradation in yeast. NMD also initiates decapping of target mRNAs in human cells (Lejeune et al., 2003); however, 5'-to-3' exonuclease only appears to be a minor factor in the degradation of human NMD targets (Lykke-Andersen et al., 2014), indicating most NMD activity occurs through a different mechanism in human cells. It is likely that this difference in the degradation mechanism between yeast and human cells is due to the additional NMD factors that are present in metazoans.

The first clue as to the function of the metazoan-specific NMD genes was revealed when SMG-2 (Upf1) was discovered to be phosphorylated in *C. elegans* (Page et al., 1999). The phosphorylation of SMG-2 was dependent on *smg-1*, *smg-3* (*Upf2*), and *smg-4* (*Upf3*), and SMG-2 was hyper-phosphorylated in *smg-5, smg-6,* and *smg-7* mutants (Page et al., 1999). Smg1 was then discovered to be a phosphatidylinositol kinase (PIK)-related kinase that is required for phosphorylation of Upf1 in both human cells and *C. elegans* (Grimson et al., 2004; Yamashita et al., 2001). Upf1 is phosphorylated at multiple residues in the N-terminal and C-terminal regions (Okada-Katsuhata et al., 2012); however, it is unclear if Smg1 contributes to the phosphorylation of all of these residues, and if all phosphorylation occurs after target recognition or if some occurs prior to complex assembly. However, since *smg-3* and *smg-4* are also required for SMG-2 phosphorylation in *C. elegans* (Page et al., 1999), it is likely that SMG-1 cannot phosphorylate SMG-2 until after complex assembly. Interestingly, the Upf1 orthologue in *S. cerevisiae*, which do not express a *Smg1* orthologue, is

phosphorylated at 11 residues, some of which are potentially phosphorylated in other organisms (Lasalde et al., 2014), suggesting Smg1-independent Upf1 phosphorylation also occurs. Importantly, loss of Smg1 causes defects in NMD activity in human cells and *C. elegans* (Hodgkin et al., 1989; Yamashita et al., 2001), indicating that the phosphorylation of Upf1 by Smg1 is a key step in the NMD mechanism **(Figure 1.1C)**.

The phosphorylation of Upf1 alone does not explain how an NMD target is degraded, so some other mechanisms is likely activated by this modification. The mechanism of degradation first began to become clear when it was identified that NMD targets in *Drosophila* cells are cleaved by an endonuclease near the PTC site, producing two fragments: one with a 5' cap and an exposed 3' end, and a second with an exposed 5' end and a poly-A tail (Gatfield and Izaurralde, 2004). The exposed ends of these fragments would then be accessible by general exonucleases to be destroyed (Gatfield and Izaurralde, 2004). Analysis of the *Drosophila* Smg6 protein sequence uncovered a Pilt N-terminus (PIN) domain, a domain that is capable of having endonuclease activity (Glavan et al., 2006). The Smg6 PIN domain was later shown to be both necessary and sufficient for endonucleolytic cleavage of NMD targets in *Drosophila* and human cells (Huntzinger et al., 2008), revealing Smg6 as the endonuclease that cleaves NMD targets. Smg6 sequence analysis also discovered a 14-3-3-like domain, which is capable of binding phosphorylated proteins (Fukuhara et al., 2005), and Smg6 was found to bind phosphorylated Upf1 (Okada-Katsuhata et al., 2012), suggesting Upf1 phosphorylation by Smg1 is required for recruitment of Smg6 endonuclease. Smg6 has been shown to be capable of binding Upf1 in the absence of Smg1 kinase function, and is required for NMD even when Smg1 is depleted (Chakrabarti et al., 2014; Nicholson et al., 2014),

suggesting that Smg1 may not be needed to recruit Smg6. However, it has remained unclear if Smg1-independnent Smg6 activity occurs during native NMD conditions, or if Smg1 serves to enhance Smg6 binding to Upf1, even if Smg6 can bind on its own. These findings all support the model that Smg6 is recruited to phosphorylated Upf1, likely after Smg1 kinase activity, **(Figure 1.1D)**, and then cleaves target mRNAs near the stop codon, exposing free 5' and 3' ends, which are then degraded by exonucleases without the removal of the 5' cap or deadenylation **(Figure 1.1E)**.

Smg5 and Smg7 both have 14-3-3-like domains, and form a heterodimer to also bind phosphorylated Upf1, which is required for NMD activity (Anders et al., 2003; Fukuhara et al., 2005; Jonas et al., 2013; Ohnishi et al., 2003) **(Figure 1.1D)**. The contribution of Smg5 and Smg7 to NMD is the least clear, but there is evidence to suggest a few different possibilities. The Smg5/Smg7 complex binds a subunit of the PP2A phosphatase (Anders et al., 2003; Ohnishi et al., 2003), and *C. elegans* and human cells lacking Smg5 or Smg7 have hyper-phosphorylated Upf1 (Ohnishi et al., 2003; Page et al., 1999), suggesting Smg5 and Smg7 are required for Upf1 to be dephosphorylated after target degradation is initiated. Interestingly, Smg6 is also required for dephosphorylation of Upf1 (Chiu et al., 2003; Page et al., 1999), suggesting endonuclease activity may be required before Upf1 can be dephosphorylated, or Smg6 binding Upf1 is necessary for the Smg5/Smg7 dimer to also bind. It is unclear how this potential role in Upf1-dephosphorylation contributes to NMD activity, but it has been suggested to be required for NMD complex disassembly and recycling (Ohnishi et al., 2003) **(Figure 1.1E)**, although this model has not been further substantiated. There is evidence suggesting Smg5 is also capable of recruiting the Dcp decapping complex via

an interaction with PRNC2 to potentially allow for 5'-to-3' transcript degradation (Cho et al., 2013; 2009). Additionally, Smg7 has been shown to recruit a deadenylase complex through interaction with POP2 (Loh et al., 2013) to potentially allow for 3'-to-5' degradation. However, the frequency of these interactions and if they contribute to NMD in native conditions remains unclear. Thus, Smg5 and Smg7 potentially have multiple roles in NMD, but their requirement for Upf1 dephosphorylation is the most well described, and thus generally referred to as their function. However, the necessity and relative contribution of each of the potential functions of these genes to NMD activity *in vivo* remains very unclear.

Piecing all of these steps together creates a canonical model of NMD pathway function. This model suggests the majority of NMD activity occurs through a single linear pathway, initiated by a complex of Upf1-3, then recruiting Smg1, which allows for Smg6 and Smg5/7 to be recruited, initiating decay and promoting complex recycling **(Figure 1.1)**. Additional alternative decay mechanisms may potentially exist, such as Smg1-independent Smg6 activity, or decapping and deadenylation complex recruitment via Smg5 or Smg7, but the evidence for these activities to occur during natural NMD conditions remains limited. Genetic analysis of model organisms with mutations in individual NMD factors may be able to distinguish between the possible single or multiple decay mechanisms contributing to NMD. If NMD proceeds through a single pathway, loss of any individual NMD factor would cause the entire pathway to fail, producing similar defects when different individual NMD factors are inhibited. Alternatively, if multiple NMD mechanisms exist, then loss of any individual NMD factor will have differing effects on NMD activity. Interestingly, siRNA knockdown of

any of the known NMD factors in *Drosophila* S2 cells produces a similar profile of genome wide changes (Rehwinkel, 2005), suggesting all of these genes are equivalently required for NMD function. Additionally, mutations in any NMD gene generally have the same effect on viability within a given model organism. For example, all NMD loss-of-function conditions tested in mouse, zebrafish, and *Arabidopsis* cause lethality (Kerényi et al., 2008; McIlwain et al., 2010; Medghalchi et al., 2001; Weischenfeldt et al., 2008; Wittkopp et al., 2009), while no NMD factors are essential for viability in *C. elegans*, *S. cerevisiae*, or *S. pombe* (Hodgkin et al., 1989; Leeds et al., 1991; Mendell et al., 2000). These genetic studies so far are consistent with the idea that all NMD factors are required for all NMD function.

An exception to this trend is *Drosophila*, in which different NMD factors have varied requirements for viability. Loss of *Upf1* or *Upf2* activity causes lethality in *Drosophila*, but animals are capable of living without *Upf3*, *Smg1*, or *Smg6* (Avery et al., 2011; Chen et al., 2005; Frizzell et al., 2012; Metzstein and Krasnow, 2006). Upf1 and Upf2 are known to function in NMD-independent processes, such as staufen-mediated decay and possibly in translation initiation (Park and Maquat, 2013; Wilkinson, 2005). It is possible that *Drosophila* are capable of living without any NMD activity and that *Upf1* and *Upf2* mutants are dying due to loss of the NMD-independent functions of these genes. However, there is a correlation between the viability of an NMD allele and the expression of endogenous NMD targets with these alleles (Avery et al., 2011; Frizzell et al., 2012; Metzstein and Krasnow, 2006), indicating that the lethal alleles have more severe defects in NMD function than the viable alleles. Since there are differences in the severity of the NMD defects between *Drosophila* mutants lacking different NMD genes,

it suggests that the contribution to NMD function may not be equal between all NMD factors, as predicted by the canonical model of NMD activity. It is possible that those organisms which require all NMD genes for viability are very sensitive to any loss in NMD function, while those organisms that do not require NMD are not sensitive to any perturbation in NMD activity. *Drosophila* may reside at a pivotal point in the tree of life where some NMD activity is permissive for viability, but complete loss of NMD function causes lethality. For this reason, genetic analysis of *Drosophila* NMD mutants may be a powerful tool to understand the individual contribution of each factor to the NMD pathway.

Analysis of *Drosophila Smg6* mutants is an example of the novel understandings of the NMD pathway that can be gained through investigating *Drosophila* NMD mutants (Frizzell et al., 2012). The canonical model of NMD activity proposes that Smg1 is required for any Smg6 to bind Upf1, and thus NMD activity **(Figure 1.1C, D)**. *Drosophila Smg6* mutants are semi-viable, with approximately 50% of mutants surviving to adulthood, while 100% of *Smg1* mutants survive (Chen et al., 2005; Frizzell et al., 2012; Metzstein and Krasnow, 2006), and *Smg6* mutants have much higher expression of endogenous NMD targets than *Smg1* mutants (Frizzell et al., 2012), indicating that *Smg6* mutants have a stronger defect in NMD activity than *Smg1* mutants. This finding is consistent with the alternative model that Smg6 can bind Upf1 independent of Smg1 phosphorylation (Chakrabarti et al., 2014; Nicholson et al., 2014), suggesting that Smg1-independent binding occurs *in vivo* during native NMD activity. By continuing to use the benefits of dissecting the function of individual NMD factors through *Drosophila* genetics, Chapter 3 of this dissertation will describe how analysis of *Smg5* mutants and

double mutants of NMD genes revealed novel features of the NMD pathway.

Endogenous NMD Targets: How Are They Recognized?

The NMD pathway was first identified as a mechanism to remove PTC-containing mRNAs from the cell. However, this quality-control feature is not the only NMD function. NMD also functions as a mechanisms of post-transcriptional gene regulation, degrading many wild-type mRNAs to reduce their expression. The regulation of these endogenous NMD targets is an important aspect of the overall landscape of gene expression, since loss of NMD activity in yeast, *C. elegans*, *Drosophila*, or mammalian cells can lead to changes in expression of approximately 10% of the genome (Barberan-Soler et al., 2009; Chapin et al., 2014; Hansen et al., 2009; He et al., 2003; Lewis et al., 2003; Nguyen et al., 2014; Ramani et al., 2009; Rehwinkel, 2005; Tani et al., 2012a). While this post-transcriptional gene regulatory feature is clearly important, it is still unclear what *cis*-regulatory features of endogenous NMD targets influence their recognition by the NMD pathway.

It is well established that the 3' untranslated region (UTR) can have a major influence on the targeting of an endogenous mRNA by NMD. The 3' UTR of an endogenous NMD target attached to a reporter coding sequence is sufficient to render that reporter sensitive to NMD activity (Nelson et al., 2016), indicating that the 3' UTR is a major determining factor in NMD target recognition. Most studies to identify mRNA characteristics recognized by the NMD machinery have focused on the 3' UTR. One potential feature of endogenous NMD targets may be long 3' UTRs. An mRNA with a PTC would have an artificially long 3' UTR due to the early termination of translation, and this long "faux" 3' UTR may influence the recognition of PTC-containing mRNAs

as NMD targets (Amrani et al., 2004). For this reason, mRNAs encoded with endogenous long 3' UTRs may appear as PTC-containing mRNAs to the NMD pathway **(Figure 1.2A)**. Indeed, endogenous NMD targets tend to have longer than average 3' UTRs in both human and *Drosophila* cells (Chapin et al., 2014; Tani et al., 2012a); however, a long 3' UTR is not sufficient to induce NMD, and some endogenous NMD targets have a 3' UTR that is shorter than average (Chapin et al., 2014; Tani et al., 2012a). Sequence-specific enhancer and suppressor features have been identified in 3' UTRs of yeast and human mRNAs that can influence the efficiency of NMD targeting (Ge et al., 2016; Toma et al., 2015; Zhang et al., 1995). It is likely that these enhancer and suppressor elements combined with 3' UTR length regulate the targeting of endogenous NMD targets **(Figure 1.2B)**.

Many of the same features that cause NMD to target PTC-containing mRNAs may also be present in endogenous NMD target 3' UTRs. One such feature may be the presence of an exon-exon junction downstream of the stop site **(Figure 1.2C)**. It is well characterized that EJC factors contribute to NMD activity in mammalian cells (Palacios et al., 2004); however, the EJC is not required for degradation of all mammalian NMD targets (Buhler et al., 2006), and EJC factors are dispensable for NMD in *Drosophila* cells (Gatfield et al., 2003). Although 3' UTR EJCs may contribute to the recognition of endogenous NMD targets, this does not appear to be the only mechanism influencing targeting.

Splice isoforms that introduce features that mimic a PTC is another mechanism that produces endogenous NMD targets **(Figure 1.2D)**. In *Drosophila*, the long isoform of the sex determinant gene *transformer* is a well-characterized endogenous NMD target

(Metzstein and Krasnow, 2006; Rehwinkel, 2005). There are two isoforms of *transformer*: a short isoform (*traS*), which encodes the Transformer protein, and a long isoform (*traL*), which includes an early exon that introduces an early termination codon (Inoue et al., 1990). The early termination codon in the *traL* mRNA resembles a PTC, and the expression of this mRNA is restricted by the NMD pathway, while *traS* expression is not influenced by NMD (Metzstein and Krasnow, 2006). Similar NMD-dependent degradation of specific alternative splice isoforms has been characterized in many human genes as well (Drummond and Friderici, 2013; Panelli et al., 2012; Yan et al., 2015). Other NMD targets contain upstream open reading frames (uORFs) as their likely source of NMD targeting **(Figure 1.2E)**. Translation termination at an uORF would similarly simulate a PTC, causing recognition by the NMD pathway. Up to a half of all uORF-containing transcripts in *S. cerevisiae* and humans are regulated by NMD (Malabat et al., 2015; Somers et al., 2013), suggesting this is a common feature of NMD targeting. Although these features that simulate PTCs exist in many endogenous NMD targets, many other NMD targeted mRNAs do not contain these features, so a more general understanding of the RNA characteristics that cause NMD target recognition is unknown.

One approach to understanding the *cis*-acting features that influence NMD target recognition is to first identify all of the endogenous NMD targets in an organism and discovery their commonality. The most frequent method to identify these genes has been to use transcriptome profiling (through microarray or RNA-sequencing analysis) of NMD mutants or in cells with reduced expression of NMD factors. As previously mentioned, inhibition of NMD causes increased expression of a large percentage of the

genome in many model systems, including *S. cerevisiae*, *C. elegans*, *Drosophila*, and mammalian cells (Barberan-Soler et al., 2009; Chapin et al., 2014; Guan et al., 2006; Hansen et al., 2009; He et al., 2003; Lewis et al., 2003; Nguyen et al., 2014; Ramani et al., 2009; Rehwinkel, 2005; Tani et al., 2012a). However, not all of the genes that have increased expression during NMD inhibition are necessarily endogenous NMD targets, as the increased stability of any given endogenous NMD target is likely to have downstream effects that lead to the transcriptional increase of other secondary genes. This possibility makes it impossible to distinguish direct endogenous targets from those secondary targets by simply detecting the genes with increased expression in cells lacking NMD function.

Several efforts have been made to parse the direct endogenous targets from those secondary targets with increased expression during NMD inhibition. One approach has been to use RNA cross-linking immunoprecipitation to detect mRNAs that bind NMD factors. The difficulty in this approach has been that the interactions between most NMD factors and their targets are too transient for cross-linking to pull down RNAs bound to these proteins. RNAs that bind Upf1 in mouse cells have been successfully detected using this technique (Hurt et al., 2013). This study found Upf1 bound approximately 200 mRNAs, most of which have increased expression during loss of NMD activity (Hurt et al., 2013). These NMD targets again tended to have 3' UTRs that were longer than average, but some short 3' UTR targets were identified (Hurt et al., 2013).

An alternative approach to identify endogenous NMD targets was to characterize the stability of mRNAs when NMD is either active or inactive at a genome-wide scale. BRIC-seq is a method to pulse label mRNAs with 5'-bromo-uridine (BrU) followed by

immunoprecipitation of BrU-incorperated mRNAs at time points following pulse labeling, and sequencing of these mRNAs to measure the decrease in their abundance over time without the use of transcriptional inhibitors (Tani et al., 2012b). Using this technique in HeLa cells with active NMD and Upf1 inhibited cells identified over 700 mRNAs that are degraded in a UPF1-dependent manner (Tani et al., 2012a). Interestingly, only around 10% of these mRNAs had increased expression in the Upf1 inhibited cells (Tani et al., 2012a). Additionally, only a small portion of the mRNAs with increased expression in Upf1 inhibited cells had Upf1-dependent decay (Tani et al., 2012a), indicating that the majority of genes whose expression is influenced by NMD are not endogenous NMD targets. Another study that measured genome-wide Upf2-dependent mRNA decay in *Drosophila* found a similar phenomenon. Measuring the abundance of mRNAs on a time course after expression of wild-type *Upf2* in *Upf2* mutant *Drosophila* discovered a set of mRNAs that quickly reduced cellular abundance following expression of wild-type *Upf2* (Chapin et al., 2014). These mRNAs with quickly reduced expression are likely direct endogenous NMD targets, explaining why they are the first set of mRNAs to have their expression reduced. Interestingly, only a small subset of the mRNAs with increased expression in *Upf2* mutants had quickly reduced abundance upon expression of wild-type *Upf2* (Chapin et al., 2014). Additionally, a similarly small amount of those genes that are likely endogenous NMD targets have increased expression in *Upf2* mutants (Chapin et al., 2014). Transcriptional inhibition in $Upf1^{+}$ and *Upf1*Δ yeast strains also identified that only about a third of genes with increased expression in the *Upf1*Δ strain had Upf1-dependent decay (Guan et al., 2006), indicating that a minority of genes repressed by NMD are actually NMD

targets in general. These similar findings in multiple organisms suggest that many of the endogenous NMD targets have additional regulatory factors that maintain their expression during loss of NMD activity. This discovery may explain the mRNAs bound by Upf1 that do not have increased expression during NMD inhibition.

Both the *Drosophila* and human cell line studies to identify endogenous NMD targets discovered that these mRNAs tend to have longer than average 3' UTRs (Chapin et al., 2014; Tani et al., 2012a). While average 3' UTR length was the only difference between endogenous NMD targets and non-targeted mRNAs identified in these studies, 3' UTR length did not completely account for the targeting of these mRNAs. Many endogenous targets had UTRs shorter than average, and many mRNAs with long UTRs were not targeted, consistent with the notion that there are other modifiers that can influence NMD targeting that were not accounted for in these studies.

While NMD has widespread effects on gene expression, many questions about endogenous NMD targets still remain. Although the approaches described here discovered many endogenous NMD targets, these methods have a low sensitivity and likely failed to detect many other endogenous targets. Additionally, these studies did not reveal an insight into the function of the regulation of endogenous mRNAs by NMD. Understanding both the targets regulated by NMD and the biological features of that regulation may reveal important features of NMD as a gene regulatory mechanism.

Endogenous NMD Targets: Why Are They Regulated by NMD?

While much is known about how the NMD pathway distinguishes endogenous NMD targets from non-target mRNAs, very little is understood about the purpose of this

mechanism of post-transcriptional gene regulation. In many organisms, NMD is required for viability, suggesting that the NMD pathway is regulating some biological process with critical influence on development (Frizzell et al., 2012; Kerényi et al., 2008; Li et al., 2015; McIlwain et al., 2010; Medghalchi et al., 2001; Metzstein and Krasnow, 2006; Thoren et al., 2010; Weischenfeldt et al., 2008; Wittkopp et al., 2009). However, less complex organisms such as *C. elegans* and yeast do not require NMD activity for viability, indicating that the process required to be regulated by NMD in more complex organisms is not regulated by NMD in these animals (Hodgkin et al., 1989; Leeds et al., 1991). Since NMD represses the expression of both PTC-containing mRNAs and endogenous NMD targets, it is unclear which class of NMD target is the cause of this lethality (Hwang and Maquat, 2011).

This difference in the requirement for NMD may reveal some feature of the NMD pathway that is different between the two groups of species. One possibility may be that the more complex organisms have greater opportunity for PTC-containing mRNAs to be produced. However, the *C. elegans* genome contains more protein coding genes than the *Drosophila* genome (Hillier et al., 2005; Lin et al., 2007), so a potential difference in PTC-abundance could not be explained by differences in number of loci alone. It may be possible that those organisms that require NMD function are more sensitive to the existence of spontaneously produced PTCs than those that do not require NMD. While there is no direct evidence that PTC-containing mRNAs are the cause of lethality in NMD mutants, loss of the surveillance feature of NMD may contribute to its requirement for viability.

Alternatively, the death of animals lacking NMD function may be due to

increased stability, and thus subsequent increased protein expression, of endogenous NMD targets. It is possible that some endogenous NMD targets may have functions that could influence development and viability, and the stabilization and subsequent increased expression of these mRNAs would be lethal. These targets causing lethality may not be expressed, or may not be regulated by NMD in *C. elegans* and yeast, which would explain why NMD is not required for viability in these animals. In *Arabidopsis*, NMD degrades the mRNAs encoding a subset of immune-related intracellular nucleotide-binding leucine-rich repeat receptors that can promote cell death, and blocking the transcription of these receptors restores viability to NMD mutants (Gloggnitzer et al., 2014), suggesting they contribute to lethality in plants lacking NMD activity. However, no endogenous target in any animal has yet to be shown to influence viability.

While it is unclear how NMD is required for viability, the function of the degradation of endogenous NMD targets has been well characterized in a few cases. *MAC1* is a transcription factor that regulates the expression of genes involved in copper transport in *S. cerevisiae*, which is targeted by NMD in rich media, but is resistant to NMD in low copper media, leading to increased expression (Peccarelli et al., 2016). It is unclear how the *MAC1* transcript becomes resistant to NMD in low copper, but it is likely due to changes in RNA structure that may occur under this different cellular condition. Additionally, two NMD factors themselves, *Smg5* and *Smg6,* are endogenous NMD targets in both *Drosophila* and mammalian cells (Chapin et al., 2014; Tani et al., 2012a), suggesting a potential negative feedback loop to regulate NMD activity itself.

While NMD-mediated gene regulation can be used to produce feedback loops

like those described above, targeting of mRNAs by NMD could also be used to limit the number of proteins produced by any given mRNA. It may be beneficial for the cell to tightly correlate the number of proteins made to the number of mRNAs of a given gene in some cases. For example, the mRNA for the mammalian post-synaptic regulator *Activity-regulated cytoskeletal-associated protein* (*arc*) is an endogenous NMD target, due to having two exon-exon junctions in its 3' UTR (Giorgi et al., 2007). *Arc* mRNA localizes to dendritic segments and is translated in response to induction of long-term potentiation (Rodríguez et al., 2005; Steward et al., 1998). Given the precise spatial and temporal regulation of *arc* translation, it is likely that degradation of *arc* mRNA by NMD quickly after translation helps maintain this tight regulation (Bramham et al., 2008).

One long-standing question has been whether NMD is differentially required in different tissues or during different stages in development. Loss of *Upf1* or *Upf2* causes early lethality during mouse embryogenesis, so NMD appears to be critical for these early developmental stages (Medghalchi et al., 2001; Weischenfeldt et al., 2008). Tissue-specific ablation of *Upf2* in the liver, hematopoietic precursors, or Sertoli cells in the testis leads to almost complete loss of these tissues (Bao et al., 2015; Thoren et al., 2010; Weischenfeldt et al., 2008). Although NMD is required for development of all of these tissues tested, reduction in NMD activity has varying effects on the compensatory increase of NMD factors in different tissues (Huang et al., 2011), indicating there may be varying requirements for NMD between these cell types. Interestingly, NMD is actually inhibited in the nervous system during mouse development. *miRNA-128* is expressed in the brain beginning at embryonic day 9.5, increases during postnatal development, and

persists during adulthood (Bruno et al., 2011). *miRNA-128* targets the *Upf1* mRNA and inhibits UPF1 expression (Bruno et al., 2011), promoting expression of genes required for neural differentiation. This study suggests that inhibition of NMD may be an important aspect of development in some contexts, and indicates that NMD has varying requirements in tissue specific context.

Understanding the requirement of NMD for viability will be necessary to understand the potential tissue specific functions of NMD. Determining if the surveillance or gene regulatory NMD function is the feature required for viability is also important to understand the contributions of this pathway. This dissertation will approach the understanding of the requirement of NMD for viability and other biological functions in *Drosophila*.

Clinical Importance of NMD

NMD activity can have a very strong influence on disease. Although NMD serves to degrade PTC-containing mRNAs, there are many alleles in the population that produce PTCs that evade NMD. These NMD evading alleles can uniquely influence disease in one of three ways: (1) create a dominant disease inheritance, (2) produce more severe disease symptoms, or (3) have reduced disease severity (Bhuvanagiri et al., 2010; Khajavi et al., 2006; Miller and Pearce, 2014). These types of alterations to disease inheritance and severity are defined by the differences that occur between PTC alleles located in the 5' region of a gene that are degraded by NMD and PTC alleles in the 3' region of the same gene that evade NMD. In the case of alleles that produce dominant disease inheritance, the 5' alleles degraded by NMD have no effect as a heterozygote, but cause disease in a recessive manner when inherited as a homozygote. 3' PTC alleles

in the same gene that are not degraded by NMD instead cause the same disease as a heterozygote, and thus are inherited in a dominant way **(Figure 1.3A)**. Mutations in the ß-Globin encoding gene *HBB* have these varied inheritance patterns due to differences in NMD targeting (Hall and Thein, 1994).

In addition to influencing the inheritance pattern of a disease, PTC-inducing alleles that evade NMD can also cause more severe disease symptoms than PTC alleles in the same gene that are degraded by NMD. This phenomenon usually occurs with genes where haploinsufficiency during heterozygous loss of function leads to disease. In these cases, individuals heterozygous for a PTC-inducing allele that is degraded by NMD have disease, but individuals for a PTC allele in the same gene not degraded by NMD have more severe disease symptoms **(Figure 1.3B)**. These more severe symptoms can manifest either as a more intense condition of disease symptoms, such as with mutations in *COL1A1* (Körkkö et al., 1998; Willing et al., 1996), or they can manifest as completely different symptoms, like with mutations in *SOX10* or *ELN* (Inoue et al., 2004; Tassabehji et al., 1997). In these conditions where more severe disease symptoms or dominant inheritance occur due to alleles evading NMD, it may be beneficial to bolster NMD activity to degrade these NMD-insensitive alleles. Understanding the mechanisms of NMD targeting and decay can help provide tools to increase NMD activity in these patients.

Although enhancing NMD activity may be a therapy for the diseases described in these first two ways that NMD can alter disease severity and inheritance, NMD may also degrade PTC-containing mRNAs that would produce proteins that are not harmful. In these cases, alleles that evade NMD could potentially produce a truncated protein that

may maintain some function. Individuals homozygous for PTC-inducing alleles that are degraded by NMD would have complete loss of function, which may cause disease, but those homozygous for NMD-evading alleles may have reduced disease severity because of the partially functional protein **(Figure 1.3C)**. In fact, this phenomenon occurs in cases of muscular dystrophy with alleles of *DMD* (Kerr et al., 2001) and cystic fibrosis with *CFTR* alleles (Rowntree and Harris, 2003). It is likely that there are many other genes in which NMD-evading PTC alleles have reduced disease severity compared to alleles of the same gene that are degraded by NMD, but they are uncharacterized because they do not have any symptoms.

Since PTC-alleles that evade NMD may have suppressed disease symptoms compared to those alleles degraded by NMD, NMD inhibition may be an effective therapy for individuals suffering from loss-of-function genetic diseases. NMD inhibition would stabilize PTC-containing mRNAs normally degraded by NMD, allowing them to produce partially functional proteins that could suppress, and potentially eliminate, disease symptoms **(Figure 1.3D)**. Additionally, combining NMD-inhibition with stop codon read-through could potentially not only stabilize these mRNAs, but also produce full-length proteins, which would be very likely to eliminate disease symptoms completely (Keeling and Bedwell, 2011). Considering that up to a third of genetic diseases are due to loss-of-function PTC alleles (Khajavi et al., 2006), a general therapy for these diseases would be an effective treatment for a wide range of patients suffering from both common diseases and rare diseases for which therapies might not otherwise be developed. However, given that loss of NMD activity causes lethality in most model systems, NMD inhibition has not been heavily pursued as a therapy option given the

high likelihood of harmful side effects, and no NMD inhibitors have been tested in humans (Bhuvanagiri et al., 2010). Discovering the cause of the lethality in animals lacking NMD activity may allow for a way to subvert the potential harmful effects from NMD inhibition and the development of this therapy.

Conclusions

While there are many different mechanisms to regulate mRNA stability, the nonsense-mediated mRNA decay pathway functions both as a regulator of gene expression and as a quality-control mechanism in the cell. Understanding how the NMD pathway identifies and degrades its targets is important for understanding both the maintenance of mRNA quality in the cell, and for understanding the regulation of gene expression. Although many of the molecular interactions of NMD have been well characterized, genetic understanding of the relative contribution of each NMD factor is lacking. By using genetics to uncover the function of each NMD factor, we can better model the role of the complex as a whole. Making these discoveries also has important clinical implications, as modifying NMD activity could serve as a therapy for a wide range of both rare and common genetic diseases. This dissertation presents my work to uncover the biological role NMD gene regulation has in development and maintaining viability and reshapes the long-standing canonical model of the mechanisms of NMD function.

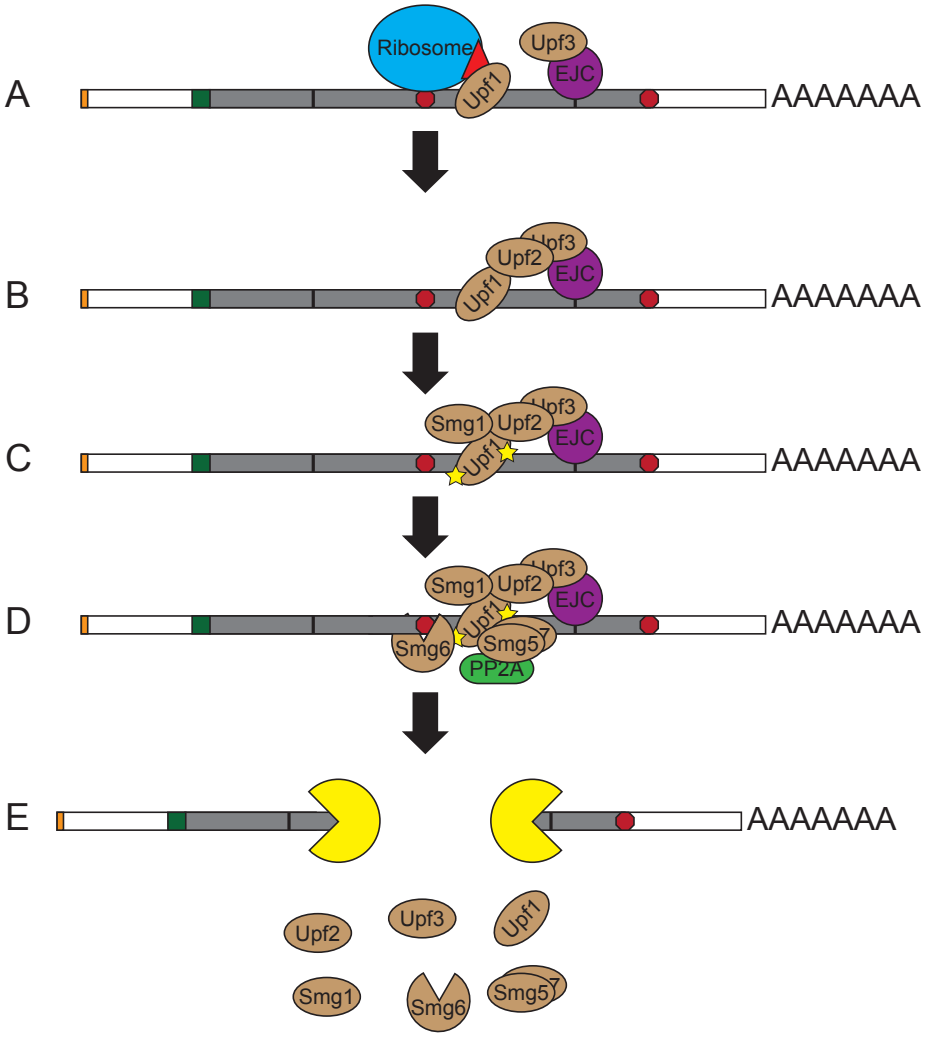

Figure 1.1. The canonical model of the NMD mechanism. (**A**) Translation initiates at the start codon and terminates at a premature termination codon (PTC). Upon translation termination, eRF3 (red triangle) binds the ribosome. When translation terminates at a PTC, Upf1 binds eRF3, stabilizing Upf1 binding target mRNA. The NMD factor Upf3 is bound to exon junction complexes (EJCs) at exon-exon junctions (black line). EJCs bound to exon-exon junctions upstream of the PTC are removed during translation, but those downstream of the PTC remain. (**B**) Upf2 binds both Upf1 and Upf3, bridging these factors and stabilizing the NMD complex on the target mRNA. (**C**) Smg1 binds Upf1 and phosphorylates (yellow star) it at multiple residues in the C-terminal and N-terminal ends. (**D**) The endonuclease Smg6 and a heterodimer of Smg5 and Smg7 binds phosphorylated Upf1 residues. The Smg5/7 heterodimer also binds the phosphatase PP2A to recruit it to the NMD complex. (**E**) Smg6 cleaves the target mRNA near the PTC. PP2A dephosphorylates Upf1, which allows for complex disassembly. The general 5'-to-3' and 3'-to-5' exonucleases can degrade the mRNA at the now exposed 5' end that does not have a m^7G-cap (orange) and the exposed 3' end that does not have a poly-A tail.

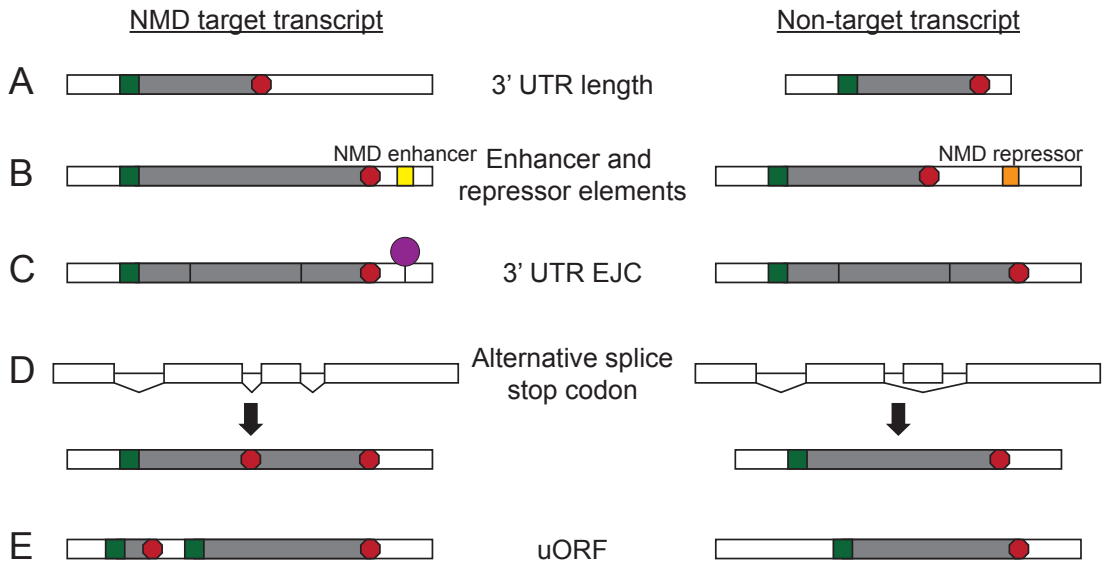

Figure 1.2. *cis*-acting features of endogenous NMD targets. (**A**) Many endogenous NMD targets contain a long 3' untranslated region (UTR), while a short 3' UTR is usually unrecognized by NMD. (**B**) The presence of sequence-specific NMD enhancer elements (yellow) in the 3' UTR can stimulate NMD, even when in a short 3' UTR that might otherwise not be recognized by NMD. Sequence-specific NMD repressor elements (orange) in the 3' UTR can reduce targeting by NMD, even when in a long 3' UTR that may otherwise be recognized by NMD. (**C**) An exon junction complex (EJC, purple circle) found on an exon-exon junction (black bar) in the 3' UTR can induce NMD targeting. EJCs are removed from exon-exon junctions in the 5' UTR and open reading frame during translation, and thus those junctions do not stimulate NMD targeting. (**D**) Alternative splice sites that incorporate an early termination codon can induce NMD when exclusion of the termination-codon-containing exon may cause no NMD targeting. (**E**) An upstream open reading frames (uORF) located in the 5' UTR is frequently targeted by NMD, while 5' UTRs lacking uORFs are not necessarily targeted.

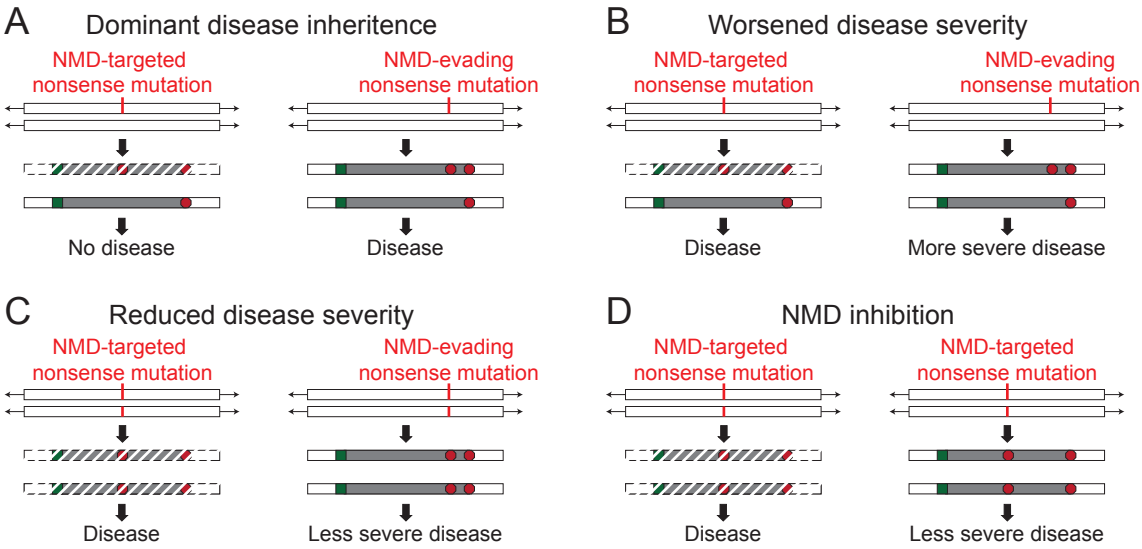

Figure 1.3. NMD can modulate disease inheritance and severity. (**A**) NMD-evading PTC alleles can cause dominant disease inheritance. Recessive disease-causing PTC alleles degraded by NMD do not produce disease as a heterozygote, but only when both copies are lost. NMD-evading alleles of the same gene produce truncated proteins that cause disease as a heterozygote, making a normally recessively inherited disease dominantly inherited.. (**B**) NMD-evading PTC alleles can cause more severe disease symptoms than alleles degraded by NMD. Haploinsufficiency of a gene with a single PTC allele causes dominantly inherited disease. NMD-evading alleles of the same gene produce truncated proteins that can cause more severe, or additional, symptoms than those resulting from haploinsufficiency. (**C**) NMD-evading alleles can have less severe disease symptoms than complete loss of function due to homozygous PTC mutations that are degraded by NMD. Homozygous PTC alleles can cause recessive loss-of-function disease. NMD-evading alleles can produce truncated proteins that maintain some protein function, which has less severe symptoms than complete loss of function. (**D**) Inhibition of NMD may restore stability to PTC containing mRNAs in loss-of-function genetic diseases, producing truncated proteins that have partial function, and suppressing disease symptoms.

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CHAPTER 2

DEGRADATION OF *GADD45* MRNA BY NONSENSE-MEDIATED DECAY IS ESSENTIAL FOR VIABILITY

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SHORT REPORT

Degradation of *Gadd45* mRNA by nonsense-mediated decay is essential for viability

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Abstract The nonsense-mediated mRNA decay (NMD) pathway functions to degrade both abnormal and wild-type mRNAs. NMD is essential for viability in most organisms, but the molecular basis for this requirement is unknown. Here we show that a single, conserved NMD target, the mRNA coding for the stress response factor growth arrest and DNA-damage inducible 45 (GADD45) can account for lethality in *Drosophila* lacking core NMD genes. Moreover, depletion of *Gadd45* in mammalian cells rescues the cell survival defects associated with NMD knockdown. Our findings demonstrate that degradation of *Gadd45* mRNA is the essential NMD function and, surprisingly, that the surveillance of abnormal mRNAs by this pathway is not necessarily required for viability.

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Introduction

Maintaining proper gene expression is critical for normal development and physiology. In addition to *de novo* transcription, mRNA stability substantially contributes to forming the landscape of expression in a cell. The nonsense-mediated mRNA decay (NMD) pathway is a *trans*-acting mechanism that destabilizes mRNAs, and is best known for its well-described role as a quality control system, degrading abnormal mRNAs containing premature termination codons (PTCs) (***Celik et al., 2015***). NMD also degrades many wild-type endogenous mRNAs and thus is an important aspect of their post-transcriptional (***Peccarelli and Kebaara, 2014***). Loss of either of the core NMD genes *Upf1* (*Rent1*) or *Upf2* causes lethality in most eukaryotes (***Kerényi et al., 2008***; ***Medghalchi et al., 2001***; ***Metzstein and Krasnow, 2006***; ***Weischenfeldt et al., 2008***; ***Wittkopp et al., 2009***), indicating regulation of mRNA stability by NMD is critical for viability. However, the relative contributions to lethality from ectopic stabilization of PTC-containing mRNAs or endogenous NMD targets in NMD mutants remains unclear (***Hwang and Maquat, 2011***).

To identify which ectopically stabilized mRNAs are responsible for inducing lethality in NMD mutants, we performed an unbiased genetic suppressor screen seeking to restore viability in a *Drosophila* NMD mutant. To detect subtle increases in survival, we screened to suppress the lethality of animals mutant for the partially viable, hypomorphic $Upf2^{25G}$ allele, of which 10% survive to adulthood (***Chapin et al., 2014***; ***Metzstein and Krasnow, 2006***). We crossed this allele to heterozygous deficiencies to simultaneously reduce the mRNA abundance of several loci (***Figure 1A***). Of the 376 deficiencies tested, covering more than half the genome, ~10% suppressed NMD mutant lethality (***Figure 1B***, ***Figure 1—figure supplement 1A***). The suppression effect could not be explained by a reduction in overall mRNA load, as there was only a weak correlation between the increase in mRNAs expressed from a genomic region upon loss of NMD function and the strength of

eLife digest Messenger RNA (mRNA) molecules act as the templates from which proteins are made, and so control the amount of protein in a cell. Having too much of certain proteins can harm cells. Additionally, some mRNAs contain errors, and so can create faulty proteins that may also harm the cell.

Cells have therefore developed ways to destroy excess or error-ridden mRNAs to avoid a deadly build up of proteins. One such quality control mechanism is called nonsense-mediated decay (NMD). This mechanism is so important that cells that cannot perform nonsense-mediated decay die, although it is not clear exactly what kills the cells.

Now, Nelson et al. have found that fruit flies whose cells are unable to perform nonsense-mediated decay die because a harmful protein called Gadd45 builds up in the cells. In normal cells, nonsense-mediated decay destroys the mRNA that relays the instructions for making Gadd45, which keeps the amount of the Gadd45 protein in the cell low. Further experiments show that removing Gadd45 from cells that lack nonsense-mediated decay saves the flies. Removing Gadd45 from human and mouse cells that are unable to perform nonsense-mediated decay also allows these cells to survive.

These findings imply that the only nonsense-mediated decay function needed for cells to live is the destruction of *Gadd45* mRNA. This further implies that most faulty and normal mRNAs that are normally destroyed by nonsense-mediated decay do not cause the cells to die when nonsense-mediated decay is lost.

Learning that creating faulty proteins when nonsense-mediated decay is lost is not necessarily harmful to cells opens new possibilities to treating numerous genetic diseases. In some diseases, cells can only produce faulty forms of a particular protein. Nonsense-mediated decay normally destroys all of these mutant proteins, but it may sometimes be better to have faulty versions of a protein than to have none of it. Safely getting rid of nonsense-mediated decay by also eliminating Gadd45 from cells may therefore be a treatment strategy worth exploring.

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suppression when that region was removed by a deficiency (***Figure 1—figure supplement 1B***). Rather, deficiencies that suppressed NMD-mutant lethality clustered in three genomic regions (***Figure 1—figure supplement 1A***). These findings suggest that NMD mutant lethality is not the result of a global excess of nonspecific mRNAs, but rather is mediated by specific genes residing within the few identified regions.

We expected that any specific genes mediating NMD-mutant lethality would have increased expression levels in an NMD mutant and be a direct NMD target. The only gene located within the suppressing regions to fit these criteria is *Gadd45* (***Figure 1C***, ***Figure 1—figure supplement 2A–C***) (***Chapin et al., 2014***). To determine if NMD targeting of *Gadd45* mRNA is critical for viability, we generated a *Gadd45* null allele, *F17*, which completely removes the *Gadd45* coding region (***Figure 1—figure supplement 3A***) and eliminates *Gadd45* mRNA expression (***Figure 1—figure supplement 2A***). As a heterozygote, $Gadd45^{F17}$ suppressed $Upf2^{25G}$lethality as strongly as the corresponding deficiency identified by our screen (***Figure 1D***). We found that $Gadd45^{F17}$ homozygous mutants are fully viable (***Figure 1—figure supplement 3B***), allowing us to test complete loss of *Gadd45* for the suppression of NMD-mutant lethality. Homozygous $Gadd45^{F17}$ restored full viability to $Upf2^{25G}$mutants, and remarkably even partially suppressed the complete lethality observed in null *Upf1* and *Upf2* mutants (***Frizzell et al., 2012***; ***Metzstein and Krasnow, 2006***) (***Figure 1D***). Importantly, neither reducing nor eliminating *Gadd45* restored NMD function to $Upf2^{25G}$ mutants, as measured by the expression of both an endogenous NMD target (***Figure 1—figure supplement 4A***) and PTC-containing mRNAs (***Figure 1—figure supplement 4B***).

In mammals, GADD45 activates the MTK1/MEKK4 kinase in a well-defined stress response pathway (***Takekawa and Saito, 1998***). Strikingly, the *Drosophila* MTK1 orthologue, *Mekk1*, resides within another $Upf2^{25G}$ suppressing region (***Figure 1E***). Similar to *Gadd45*, we found that *Mekk1* null mutants (***Inoue et al., 2001***) suppressed *Upf1* and *Upf2* mutant lethality (***Figure 1F***). This suppression was not as strong as that caused by a loss of *Gadd45*, revealing that although MEKK1 mediates

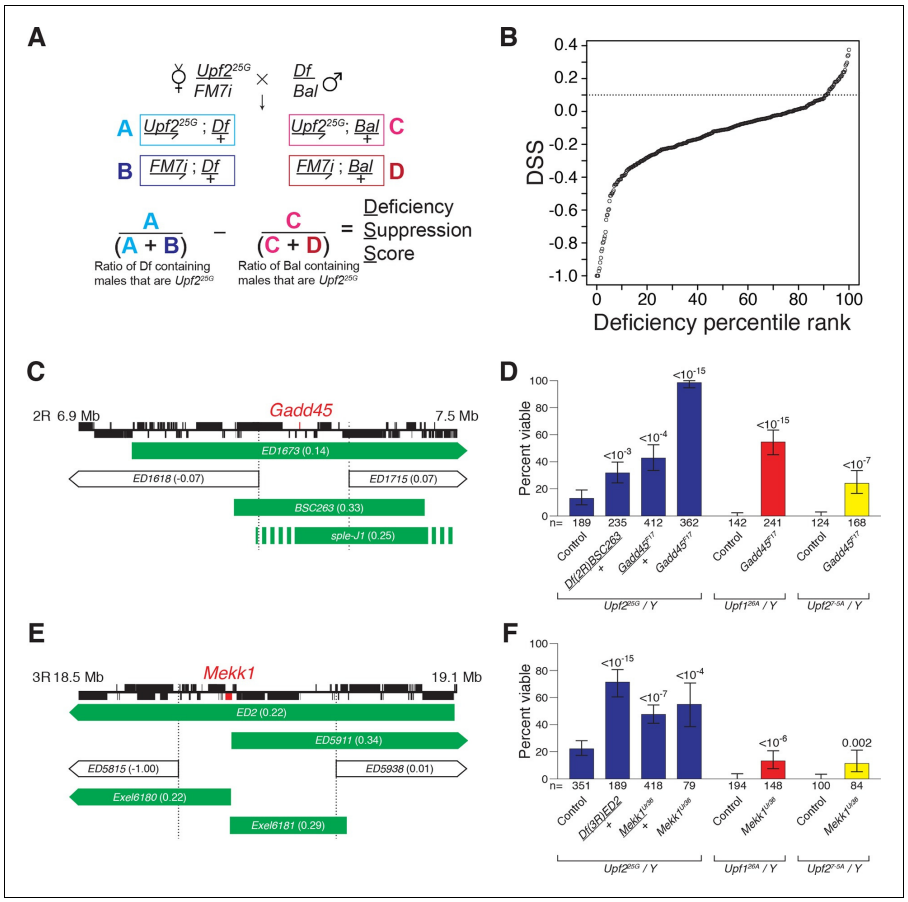

Figure 1. *Drosophila* suppressor screen identifies the *Gadd45* pathway as the inducer of NMD-mutant lethality. (**A**) Scheme to screen deficiencies for the suppression of $Upf2^{25G}$ partial lethality. The Deficiency Suppression Score (DSS) represents the relative difference in $Upf2^{25G}$ viability when crossed to a heterozygous deficiency (*Df*) compared to when crossed to a balancer (*Bal*) (See Methods). (**B**) DSS from 376 screened deficiencies ranked by score. A DSS greater than 0.1 (dotted line) indicates that deficiency suppresses $Upf2^{25G}$ lethality. (**C** and **E**) Candidate suppressing regions uncovering *Gadd45* (**C**) and *Mekk1* (**E**). DSSs are shown in parenthesis. Dotted lines denote extent of regions deleted by suppressing deficiencies but not non-suppressing deficiencies. Filled blocks on chromosomes indicate predicted gene spans, *Gadd45* pathway genes are indicated in red; suppressing deficiencies indicated in green, *sple-J1* has undefined breakpoints located within hashed regions. (**D** and **F**) NMD mutant adult viability in combination with $Gadd45^{F17}$ (**D**) or $Mekk1^{Ur36}$ (**F**) mutants. $Upf1^{26A}$ and $Upf2^{7-5A}$ are null alleles (***Frizzell et al., 2012***; ***Metzstein and Krasnow, 2006***). p-value compared to controls determined by the test of equal or given proportions indicated. Error bars represent 95% confidence interval of the binomial distribution. n equals total number of animals scored in each cross.

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The following figure supplements are available for figure 1:

Figure supplement 1. Reduced expression of specific loci, not overall mRNA abundance, produces NMD mutant suppression by deficiencies.

DOI: 10.7554/eLife.12876.004

Figure supplement 2. Drosophila Gadd45 is an endogenous direct NMD target.

Figure 1 continued on next page

Figure 1 continued

DOI: 10.7554/eLife.12876.005

Figure supplement 3. F17 is a null allele of Gadd45.

DOI: 10.7554/eLife.12876.006

Figure supplement 4. Loss of Gadd45 does not restore NMD activity in NMD mutants.

DOI: 10.7554/eLife.12876.007

NMD mutant lethality, it is likely that GADD45 has additional downstream effectors that influence viability. Overall, our findings reveal that increased *Gadd45* mRNA stability is the major factor inducing NMD mutant lethality, primarily via increased MEKK1 activity.

Activation of MTK1 in mammals triggers a MAPK signaling cascade that promotes apoptosis (***Takekawa and Saito, 1998***). Over-expression of *Gadd45* in *Drosophila* also induces apoptosis (***Peretz et al., 2007***). Interestingly, *Drosophila* cells lacking NMD function show excess cell death in a variety of tissues (***Avery et al., 2011***; ***Frizzell et al., 2012***; ***Metzstein and Krasnow, 2006***). To test if increased *Gadd45* contributes to this excess death, we used TUNEL staining to examine cell death in wing imaginal discs from $Upf2^{25G}$ mutant third instar larvae. This analysis revealed elevated levels of cell death compared to controls (***Figure 2A, B, E***), and this defect was completely suppressed by $Gadd45^{F17}$ (***Figure 2C–E***). To confirm that, this effect was not specific to the *Upf2* gene or *25G* allele, we examined the wing discs in mutants of another essential NMD gene, *Smg5*. We found that *Smg5* discs also showed elevated TUNEL signal, which was eliminated by loss of *Gadd45* (***Figure 2—figure supplement 1A–E***). These results demonstrate that excess *Gadd45* accounts for ectopic cell death in NMD mutant tissues.

To test if *Gadd45*-induced cell death is the only cellular defect in NMD mutants, we examined NMD function in the developing eye. NMD is required for proper development of eye cells, as clonal patches of NMD mutant cells in eyes are reduced in size (***Frizzell et al., 2012***; ***Metzstein and Krasnow, 2006***). We found that *Gadd45* is partially responsible for this defect, as the size of eye-cell clones lacking NMD activity in a $Gadd45^{F17}$ background was increased, although not fully restored (***Figure 2F–J***). These results indicate that some, but not all, defects associated with loss of NMD are dependent on *Gadd45*.

Gadd45 is one of the few genes that is directly regulated by NMD in both flies and mammals (***Huang et al., 2011***; ***Tani et al., 2012***; ***Viegas et al., 2007***), raising the possibility that excess *Gadd45* abundance may also contribute to the NMD-mutant lethality observed in mammalian cells (***Azzalin and Lingner, 2006***; ***Li et al., 2015***; ***Medghalchi et al., 2001***; ***Weischenfeldt et al., 2008***). To test this hypothesis, we analyzed the effects of *Gadd45* and *Upf1* depletion in mouse NIH-3T3 cells. *Gadd45b* mRNA (also known as *MyD118*), which is expressed at least 10-fold higher than any other *Gadd45* paralogue in these cells (***Yue et al., 2014***), was degraded rapidly in a partially *Upf1*-dependent manner after transcription was blocked with actinomycin D (***Figure 3A***), and had increased expression during *Upf1* knockdown (***Figure 3D***), confirming it is sensitive to NMD. We found that transfection of 3T3 cells with siRNAs targeting *Upf1* resulted in significant reduction in cell counts after 48 hr (***Figure 3B***), but co-transfection with siRNAs targeting both *Upf1* and *Gadd45b* largely reversed this effect (***Figure 3B***). The reduction in cell counts was primarily due to increased cell death, as we found that ~25% of cells transfected with *Upf1* siRNA were undergoing apoptosis (***Figure 3C***). Co-transfection of siRNA targeting *Gadd45b* almost entirely eliminated this increase (***Figure 3C***), indicating the excess apoptosis observed in *Upf1*-knockdown cells was mostly due to increased *Gadd45* activity. However, while *Gadd45b* knockdown very greatly suppresses this excess death, it does not as fully rescue cell numbers, suggesting loss of NMD may lead to both Gadd45b-dependent cell death as well as a Gadd45b-independent effect on proliferation. This mirrors the conclusions we made about the partial suppression of cell number defects in the *Drosophila* eye. Importantly, *Upf1* mRNA expression was equivalently reduced and the expression of the mammalian endogenous NMD targets *Rassf1* and *CRCP* (***Tani et al., 2012***) was equivalently increased in both the single and double knockdown experiments (***Figure 3D***), indicating that the restoration of viability was not due to a recovery of NMD pathway activity.

To extend our analysis to other mammalian cells, we analyzed the role of *Gadd45* mediating the effects of loss of NMD in HEK293 cells. We found, similarly to 3T3 cells, that siRNA knockdown of *UPF1* in HEK293 cells led to increased *GADD45A* expression and reduced cell numbers compared

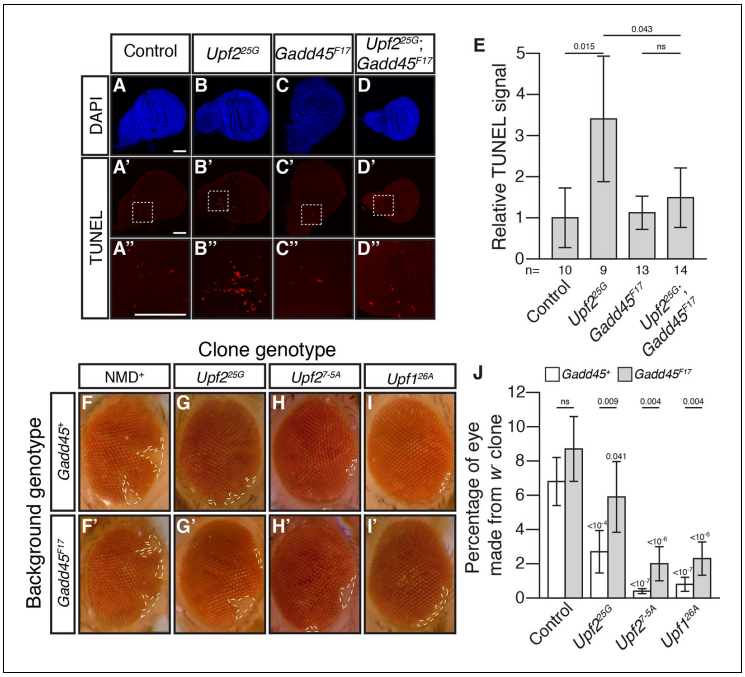

Figure 2. Loss of Gadd45 suppresses NMD-mutant cell death. (**A** to **D**) DAPI (blue) and (**A'**to **D'**) TUNEL (red) staining in late 3rd instar larval wing discs from control (**A**); *Upf2*^{25G} (**B**); *Gadd45*^{F17} (**C**); and *Upf2*^{25G}; *Gadd45*^{F17} (**D**) animals. (**A''** to **D''**) are 4x view of outlined section at the base of the blade of the wing disc from **A'-D'**, respectively. Scale bar represents 100 μm. (**E**) Relative TUNEL signal in control and mutant wing discs, normalized to control. p-value between indicated samples using a two-sided Student's t-test are displayed. ns indicates a p-value greater than 0.05. Error bars represent 2 SEM. n equals total number of discs scored. (**F** to **I**) w^- eye clones in *Gadd45*$^+$ and *Gadd45*^{F17} backgrounds. Dashed lines indicate clone boundaries. (**J**) Quantification of the fraction of the eye composed of w^- cells in control and mutant eyes. p-values indicate differences between *Gadd45* mutant and control in the same NMD background (indicated by horizontal bars) or NMD mutant and control in the same *Gadd45* background (indicated by value above each individual bar), using a two-sided Student's t-test. ns indicates a p-value greater than 0.05. Error bars represent 2 SEM. n = 20 eyes for all conditions.

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The following figure supplement is available for figure 2:

Figure supplement 1. Loss of *Gadd45* suppresses ectopic cell death in *Smg5* mutant wing discs.

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to control siRNA (***Figure 3—figure supplement 1A,B***). Although transfection of siRNA targeting *GADD45A* alone slightly reduced HEK293 cell numbers, co-transfection with *UPF1* siRNA did not further reduce cell count (***Figure 3—figure supplement 1B***), and *UPF1* expression was equivalently reduced in the single and double knockdown conditions (***Figure 3—figure supplement 1C***). These results suggest that *UPF1* knockdown is no longer detrimental to HEK293 cell viability in the absence of *GADD45A* expression. We conclude that increased expression of mammalian *Gadd45* genes contributes to lethality in NMD-deficient mouse and human cells, as *Gadd45* does in *Drosophila*.

Deconvoluting the contributions to organismal viability of the PTC-surveillance versus gene-regulatory functions of NMD has been historically difficult (***Hwang and Maquat, 2011***). Here, we show that viability can be restored to *Drosophila* lacking core NMD factors when a single endogenous

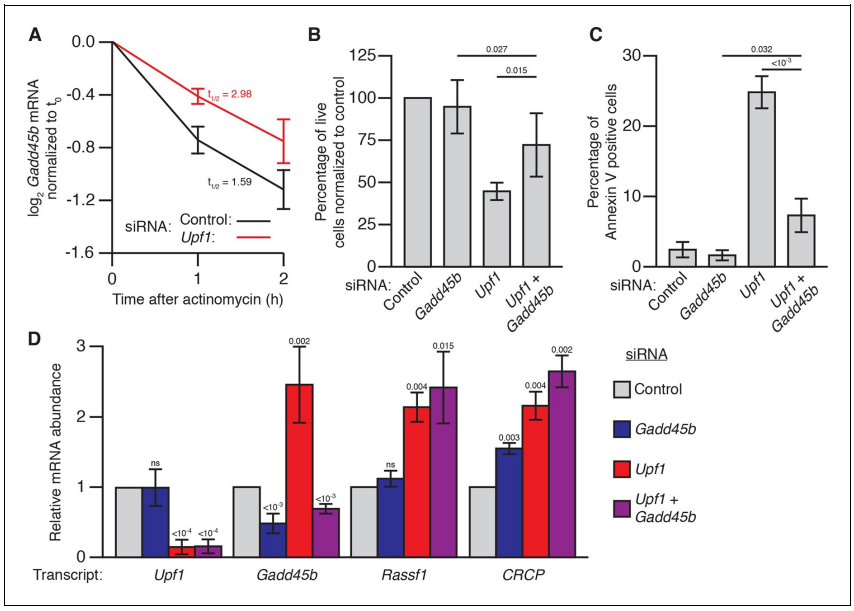

Figure 3. Gadd45b mediates cell lethality in Upf1 siRNA knockdown 3T3 mouse embryonic fibroblasts. (**A**) Relative *Gadd45b* mRNA expression measured by qRT-PCR in NIH-3T3 cells after 48 hr of control (black) or *Upf1* (red) siRNA treatment and 0 to 2 hr of actinomycin D treatment, normalized to expression prior to actinomycin treatment. The half-life calculated for each decay curve is indicated. (**B**) Relative viable cell count of *Upf1* and *Gadd45b* single and double siRNA treatment normalized to control siRNA. p-values display two-sided Student's t-test between indicated conditions. (**C**) Quantification of apoptosis as measured by annexin V staining. p-values display two-sided Student's t-test between indicated conditions. (**D**) Relative mRNA expression of *Upf1*, *Gadd45b*, and two mammalian endogenous NMD targets, *Rassf1* and *CRCP* (***Tani et al., 2012***) measured by qRT-PCR in *Gadd45b* and *Upf1* single and double siRNA knockdown cells, normalized to expression in the control siRNA condition. p-values display one-sided Student's t-test for each condition compared to control. Error bars represent 2 SEM.

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The following figure supplement is available for figure 3:

Figure supplement 1. *GADD45A* mediates cell lethality in *Upf1* knockdown HEK293 cells.

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NMD target, *Gadd45*, is eliminated, and that the requirement for the regulation of *Gadd45* by NMD is evolutionarily conserved from flies to mammals. Although our data suggest that up-regulation of *Gadd45* is a major factor contributing to lethality when NMD activity is lost, it is likely that other NMD targets also contribute to the observed lethality. In particular, viability is not restored to 100% in null *Upf1;* or *Upf2; Gadd45* double mutants. In addition, loss of *Gadd45* suppresses programmed cell death caused by defects in NMD, but not additional cell cycle defects, as implied by the incomplete suppression in the *Drosophila* eye and mammalian cell culture. Such defects in the cell cycle may be particularly pronounced during the development of certain tissue, or specific developmental stages. Indeed, NMD has been reported to have differing stage and tissue- specific activities (***Bao et al., 2015***; ***Bruno et al., 2011***; ***Colak et al., 2013***; ***Li et al., 2015***). Whether this is due to a role in surveillance or another specific target remains unclear, but examination of the effects of loss of NMD in *Gadd45* mutants should allow exploration of these possibilities.

The benefit for such a mechanism regulating *Gadd45* expression may lie in a function of NMD in restricting viral growth (***Balistreri et al., 2014***). Because viruses encode *trans*-acting factors to inhibit NMD (***Mocquet et al., 2012***), the resulting accumulation of GADD45 in infected cells may act as a

"molecular tripwire" that rapidly elicits a stress response and cell death. This outcome suggests that regulating responses to infection may underlie a conserved essential function of NMD. Intriguingly, restriction of pathogens via NMD extends to plants (**Garcia et al., 2014**), where NMD mutant lethality in *A. thaliana*, which do not encode *Gadd45* orthologues, may be caused by the overexpression of a subset of immune-related intracellular nucleotide-binding leucine-rich repeat receptors, some of which are endogenous NMD targets (**Gloggnitzer et al., 2014**). In contrast, eukaryotes that do not rely on the activation of programmed cell death to protect against viruses, such as *S. cerevisiae*, *S. pombe*, and *C. elegans*, do not require NMD for viability (**Hodgkin et al., 1989**; **Leeds et al., 1991**; **Mendell et al., 2000**). Together these observations suggest a potential novel role for NMD and *Gadd45* in immune responses, triggering the death of infected cells during pathogenic challenges.

Restoring the expression of PTC-containing alleles via NMD inhibition has been proposed as a promising therapy for a wide range of recessive genetic diseases (**Keeling et al., 2014**). Translation of stable PTC-containing mRNAs would produce truncated proteins that may be partially functional and alleviate disease symptoms normally caused by complete loss of the protein. However, the essential function for NMD in viability has raised the concern that these therapies may have prohibitive side effects. Our findings reveal a molecular basis for dealing with this obstacle by suggesting that inhibiting both the NMD and *Gadd45* pathways (**Tornatore et al., 2014**) in combination could provide an effective and safe treatment for patients with debilitating genetic disorders.

Materials and methods

Fly genetics

Drosophila melanogaster stocks were raised on standard cornmeal/dextrose food at 25°. The NMD mutant alleles $Upf2^{25G}$, $Upf2^{7\text{-}5A}$, and $Upf1^{26A}$ (**Frizzell et al., 2012**; **Metzstein and Krasnow, 2006**) are on *y w* FRT^{19A} chromosomes. These alleles were balanced over *FM7i, P{ActGFP}JMR3* (**Reichhart and Ferrandon, 1998**). $Smg5^{G115}$ and $Smg5^{C391}$ are null alleles of *Smg5* (J.O.N., D. Förster, S. Luschnig, and M.M.M., unpublished) and will be described in detail later. The *Smg5* alleles are balanced over *CyO, P{Dfd:eYFP* w^{+}*}* (**Le et al., 2006**). Other alleles used were *P{w[+mC]=EPg} HP20647* (**Staudt et al., 2005**), $Mekk1^{Ur36}$ (**Inoue et al., 2001**) recombined on FRT^{82B} by D. Ryoo, *ey-FLP* (**Newsome et al., 2000**), pcm^{14} (**Waldron et al., 2015**), Adh^{n4} (**Chia et al., 1987**) and $DHR78^{3}$ (**Fisk and Thummel, 1998**). Control chromosomes were *y w* FRT^{19A} (for *Upf1* and *Upf2*) and FRT^{82B} (for *Mekk1*) (**Xu and Rubin, 1993**). For all experiments using $Gadd45^{F17}$ we used the $Gadd45^{E8}$ precise excision as a control.

For viability assays, we mated flies for 3 days and collected all progeny each day for 10 days, starting 10 days after the cross was initiated. The total numbers of F1 mutant and balancer males were scored, and the ratio of mutant males to balancer males was used to determine mutant animal viability. To control for balancer viability within each experiment, we normalized the ratio of mutant to balancer animals to a ratio of the appropriate control chromosome to balancer animals produced from a parallel cross.

Deficiency suppressor screen

We screened autosomal deficiencies from the DrosDel collection (**Ryder et al., 2007**). All deficiencies scored can be found in **Supplementary file 1**. Deficiencies on chromosome 2 were balanced over *CyO*, and deficiencies on chromosome 3 were balanced over *TM6C*. We mated males from each deficiency stock to *y w* $Upf2^{25G}$ FRT^{19A}*/FM7i, P{ActGFP}JMR3* females and scored all F1 males for the presence or absence of each balancer. For any given deficiency tested, the percentage of *Deficiency / +* males that are $Upf2^{25G}$ mutants, less the percentage of *Balancer / +* males that are $Upf2^{25G}$ mutants was calculated, producing a Deficiency Suppression Score (DSS), which represents the effect of an individual deficiency on the increase or decrease in $Upf2^{25G}$ viability, while controlling for each deficiency's general influence on viability. A DSS greater than 0.1 indicates suppression of lethality. Supplemental deficiencies used were from the Exelixis collection (**Parks et al., 2004**) and *Df(2R)sple-J1* (**Heitzler et al., 1993**). Deficiency mapping to the *Drosophila* genome was performed using the 5.1 genome release.

RNA-seq data sets were acquired from **Chapin et al. (2014)** (archives SRR896609, SRR896616, SRR503415, and SRR503416) and aligned using Bowtie and TopHat alignment with standard

remapping parameters to the 5.1 *Drosophila* genome release. SAMtools accessory scripts were used to retrieve read counts for deficiency and control regions. All read counts were normalized to reads per million within each data set. Average normalized reads in $Upf2^{25G}$ samples were normalized to the relative reads of 74 ribosomal proteins in $Upf2^{25G}$ samples compared to control samples. Total normalized reads within the regions removed by each deficiency were averaged between biological replicates, and the difference between the $Upf2^{25G}$and control samples was divided by one million to determine percent increase in genomic load across each deficiency region.

Generation of *Gadd45* mutants

We produced P-element excision lines from the *P{w[+mC]=EPg}HP20647* P-element insertion line crossed to a *Δ2–3* transposase stock. We mated F1 males containing the P-element and transposase on a *CyO* balancer to *w; Tft / CyO* females. Cy^{+} *Tft* white-eyed F2 males were then individually mated to *w; Tft /* CyO females. We then collected Tft^{+}, *Cy* males and females to create an isogenic stock from each individually mated F2 male. To identify precise excisions we used the primers Gadd45_F1 / Gadd45_R1 flanking the P-element insert site to amplify a region across the excised P-element. Lines that failed to amplify with these primers were candidate imprecise excisions, which we then tested with Gadd45_F1 / Gadd45_R3 primers for deletions. Any detected deletions were subsequently sequenced using these same primers. Primer sequences are found in ***Supplementary file 2***.

Induction and analysis of eye clones

We generated eye clones with the FLP/FRT system using the *ey-FLP* driver (***Newsome et al., 2000***) to induce recombination. We imaged eyes on a Leica MZ125 stereo microscope with a Retiga-2000R camera (QImaging, Canada) with QCapture 3.1.2 software (QImaging). We focused images using the ImageJ stack focuser plugin and quantified relative eye clone size using the ImageJ analyzer tools. A total of 20 eyes from 20 individual animals were scored for each condition.

Cell death assays

For TUNEL assays, third instar larval wing discs were dissected as described in Sullivan *et al.* (***Sullivan et al., 2000***). TUNEL staining was performed using the Apoptag Red in situ Apoptosis Detection Kit (Chimicon International Inc., Billerica, MA) according to Chakraborty *et al.* (***Chakraborty et al., 2015***). We DAPI stained wing discs (1:5000) for 5 min prior to mounting. Confocal images were acquired using a Zeiss LSM710 laser scanning confocal microscope (Carl Zeiss AG, Germany). 3-dimensional datasets were acquired with a Plan-Apochromat 20X/0.8 lens, 1.34 µm z-step, using the Zeiss ZEN software. To measure TUNEL signal intensity z-projections images were summed with ImageJ. Background signal was removed by using the ImageJ MaxEntropy auto-threshold. Relative total TUNEL signal intensity was calculated using the ImageJ analyzer tools to measure the total pixel intensity within the wing discs of TUNEL images and normalized to the average intensity in control conditions.

For annexin V staining, we collected media (including floating cells) from siRNA treated cells. We spun down media at 950g for 4 min to pellet cells, and then aspirated remaining media. Concurrently, we trypsinized siRNA-treated cells still on plates and added them to the same respective tube as previously spun-down media. Following the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Abcam, UK) protocol, we stained for apoptotic cells. We visualized cells on an Olympus IX51 microscope (Olympus, Japan) with 20X objective. We collected bright field as well as fluorescent images using a FITC filter with a QImaging QICam Fast1394 camera and QCaptureP software (QImaging). We analyzed cells by counting all cells within a bright field image as well as the annexin V positive cells from the same image. The number of annexin V positive cells was divided by total cell number to generate the fraction of apoptotic cells for each treatment. >3000 total cells were counted across three biological replicates for each treatment.

Cell culture experiments

We cultured mouse NIH-3T3 cells (ATCC) or HEK293 cells (ATCC) in DMEM (ThermoFisher, Waltham, MA) supplemented with 10% fetal bovine serum and glutamine. For siRNA experiments, we transfected cells using RNAiMax and 24 pmol of negative control siRNA

(Qiagen, Netherlands), *Upf1* siRNA (Qiagen), or *Gadd45b* siRNA (Sigma-Aldrich) for 3T3 cell experiments, or negative control siRNA (Qiagen), *UPF1* siRNA (Qiagen), or *GADD45A* siRNA (Sigma-Aldrich, St. Louis, MO) for HEK293 experiments. For double siRNA-treated cells, we used 24 pmol of each *Upf1* and *Gadd45b* siRNA for 3T3 experiments or *UPF1* and *GADD45A* siRNA for HEK293 experiments.

For actinomycin experiments, we incubated cells with siRNA for 48 hr before changing the media and then incubated with 2 µg/mL actinomycin (Sigma-Aldrich) for 1 or 2 hr. mRNA half-life was determined by fitting an exponential decay curve to the relative expression at each time point (***Tani et al., 2012***). $t_{1/2}$ was calculated based on the average expression at each time point, and the mean $t_{1/2}$ for each condition is represented.

For cell counting experiments, we trypsinized cells, incubated a small aliquot with Trypan Blue at a final concentration of 0.04% in complete media, and counted Trypan Blue negative cells. RNA was collected from the remaining cells, and relative mRNA levels were measured as described below.

RNA isolation and quantitative RT-PCR

For *Drosophila* qRT-PCR analyses, we collected 5–10 adult animals frozen in liquid nitrogen. We isolated total RNA using TRIzol reagent (Invitrogen) and phase-lock tubes (5-Prime), and the RNeasy mini kit (Qiagen). We used on-column RNase-free DNase treatment (Qiagen) to reduce genomic contamination. We determined RNA concentration by spectrophotometer and normalized concentration for reverse transcription. For reverse transcription, we used random decamers and MMLV8 reverse transcriptase (Retroscript Kit, Thermo-FIsher). We performed qRT-PCR analysis using the SYBR Green qPCR Supermix (Bio-Rad, Hercules, CA) and the Bio-Rad iCycler thermocycler. All experimental reactions were performed using three technical replicates and a minimum of three biological replicates per condition, and the expression level of all experimental assays was normalized to *RpL32* mRNA expression.

For cell culture qRT-PCR analyses, we collected RNA following the Zymo Research Quick RNA MiniPrep kits protocol, and synthesized cDNA using MMLV reverse transcriptase (NEB, Ipswich, MA) with a template of 1 µg of total RNA and priming with a T18 oligo. We measured relative mRNA levels by qRT-PCR using the Masterplex ep realplex (Eppendorf, Germany) with SYBR green fluorescent dye. Each sample was measured with technical triplicates and three biological replicates, and target mRNA levels were normalized to those of ribosomal protein 19 (*Rpl19*) mRNA.

For all qRT-PCR analyses we also measured samples that had been made without reverse transcriptase to ensure that signal was not due to genomic DNA. Primer sequences can be found in ***Supplementary file 2***.

3′ UTR cloning and sensitivity assay

We cloned the *UAS-GFP::Gadd45 3′ UTR* and control *UAS-GFP::Act5C 3′ UTR* constructs using the primers G45_3U_X1_F / G45_3U_S1_R or Act5C_X1_F / Act5C_S1_R (***Supplementary file 2***) to amplify the *Gadd45* and *Act5C* 3′ UTRs, respectively, from genomic DNA. PCR fragments were inserted into the Zero Blunt TOPO vector (Thermo-Fisher), sequenced to assure fidelity, and digested and cloned into a pUAST-attB GFP vector using standard cloning procedures to replace the SV40 3′ UTR. Plasmids were injected by BestGene (Chino Hills, CA) into a stock containing the VK00027 attP site (***Venken et al., 2006***) for *phiC31* directed integration. We used previously described *UAS-GFP::SV40 3′ UTR* animals (***Metzstein and Krasnow, 2006***). For imaging, wandering late L3 larvae were collected and examined using a Leica MZ 16F microscope and the Leica DFC340 FX camera with the Leica Application Suite v3.3.0 software.

Analysis of *dHR78^{3}* and *Adh^{n4}* PTC allele stability

We collected adult F1 *Upf2^{+}; Gadd45$^{E8/+}$*, *Upf2^{25G}; Gadd45$^{E8/+}$*, and *Upf2^{25G}; Gadd45$^{F17/+}$*males that were also heterozygous for either the *dHR78^{3}* or *Adh^{n4}*. The *Adh^{n4}*allele is a PTC-containing allele and has been demonstrated to be a direct NMD target based on cleavage by Smg6 (***Gatfield and Izaurralde, 2004***). The *dHR78^{3}* allele is also a PTC-containing allele and thus is presumably degraded by NMD (***Fisk and Thummel, 1998***). At least three biological replicates were collected for each condition. We isolated RNA and generated cDNA as described in methods above and used this cDNA as a template for PCR amplification of the *dHR78* transcript with the DRH78_F3

/ DHR78_R3 primers and the *Adh* transcript with the Adh_F and Adh_R primers (***Supplementary file 2***), which flank the nonsense mutation in the respective transcripts. To compare the relative abundance of the $dHR78^{3}$ allele to the wild-type allele, PCR products were Sanger sequenced, and the relative peak intensity for a T ($dHR78^{3}$ allele) compared to a C (wild-type allele) at nucleotide 1063 was compared. To compare the relative abundance of the Adh^{n4} allele to the wild-type allele, PCR products were digested with *Pvu*II (a site disrupted by the *n4* mutation), separated on a 1% agarose gel and stained with ethidium bromide. The relative intensity of the cut and uncut bands was determined using ImageJ and normalized for fragment length. All samples were ran on the same gel and compared under identical conditions. All ratios were normalized to the ratio in the $Upf2^{25G}$; $Gadd45^{E8/+}$condition.

Statistical analysis

All figures displaying viability assays represent a proportion of animals of the indicated genotypes that survive to adulthood; error bars for these figures represent the 95% confidence interval of the binomial distribution, and the Test of qual or Given Proportions was used to determine significance difference in these proportions between genotypes. All other figures represent the mean value of multiple replicates have error bars depicting ± 2 SEM, which is a close approximation of the 95% confidence interval (***Krzywinski and Altman, 2013***). For tests between two variable measures, a two-sided paired Student's t-test was used to determine significance difference between mean value data. For most qPCR experiments, data was compared to a normalized control, set to a constant of 1, so these tests were performed with a one-sided Student's t-test.

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Author contributions

JON, MMM, Conception and design, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; KAM, JH, Acquisition of data, Analysis and interpretation of data, Drafting or revising the article; AC, Conception and design, Analysis and interpretation of data, Drafting or revising the article, Contributed unpublished essential data or reagents

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Additional files

Supplementary files

• Supplementary file 1. Deficiencies used in deficiency suppressor screen.
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• Supplementary file 2. PCR primers used in this study.
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Major datasets

The following previously published dataset was used:

Author(s)	Year	Dataset title	Dataset URL	Database, license, and accessibility information
	2014	D.melanogaster Nonsense-Mediated mRNA Decay study	http://trace.ncbi.nlm.nih.gov/Traces/sra/?study=SRP025939	http://trace.ncbi.nlm.nih.gov/Traces/sra/

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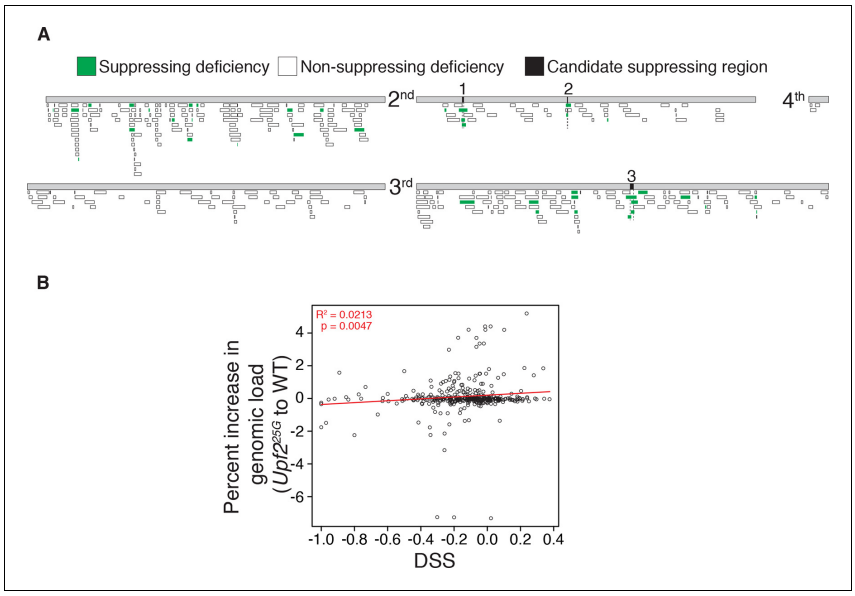

Figure 1—figure supplement 1. Reduced expression of specific loci, not overall mRNA abundance, produces NMD mutant suppression by deficiencies. (**A**) Map of 376 autosomal DrosDel deficiencies with an isogenic background and molecularly defined breakpoints (***Ryder et al., 2007***) and eight other deficiencies used to further test candidate suppressing regions without overlapping DrosDel deficiencies. 39 total deficiencies suppress *Upf2*^{25G} lethality, shown in green. Regions deleted by any non-suppressing deficiencies were eliminated as candidate suppressing regions, removing false positives and reducing the size of the candidate intervals. The three candidate suppressing regions that are deleted only by suppressing deficiencies are indicated in black and labeled 1–3. (**B**) Each deficiency's Deficiency Suppression Score (DSS) compared to percent increase in RNA abundance in *Upf2*^{25G} compared to wild-type from loci removed by that deficiency according to RNA-seq from ***Chapin et al. (2014)***. Trend line in red; statistics calculated using Pearson correlation test.

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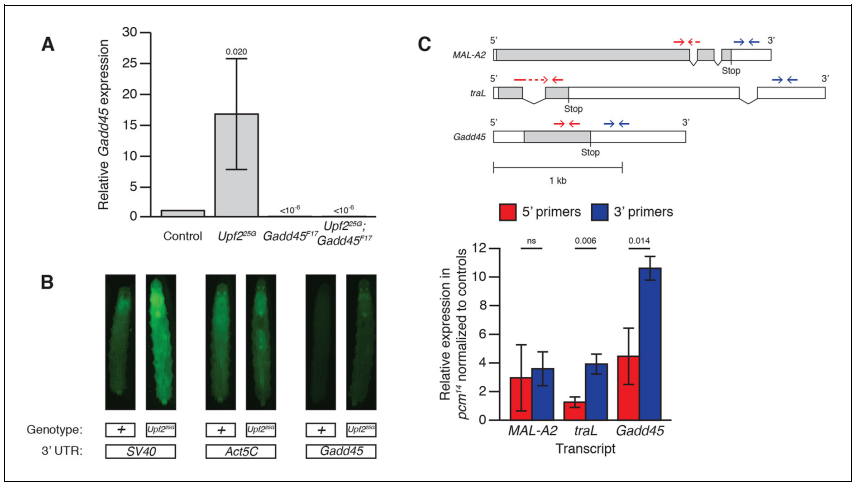

Figure 1—figure supplement 2. Drosophila Gadd45 is an endogenous direct NMD target. (**A**) *Gadd45* mRNA expression in adults of the given genotypes measured by qRT-PCR. *Gadd45* mRNA expression is increased 16.7-fold in $Upf2^{25G}$ mutants, and is eliminated by $Gadd45^{F17}$ mutants. p-values display one-sided Student's t-test of indicated condition compared to control. Error bars represent 2 SEM. (**B**) Fluorescence of GFP transgenes with *SV40*, *Act5C*, or *Gadd45* 3′ UTRs expressed by UAS driven by *Actin:GAL4* in $Upf2^{+}$ or $Upf2^{25G}$ third instar larvae. *SV40* and *Gadd45* 3′ UTR constructs show significantly increased fluorescence in $Upf2^{25G}$ animals compared to $Upf2^{+}$, indicating NMD-dependent post-transcriptional degradation of mRNAs containing these UTRs. The *Act5C* 3′ UTR construct has similar fluorescence in both backgrounds, indicating NMD does not regulate the post-transcriptional stability of this UTR. Micrographs show dorsal views with anterior at top. (**C**) *MAL-A2*, *traL*, and *Gadd45* 5′ and 3′ fragment mRNA expression measured by qRT-PCR in pcm^{14} null mutants (***Waldron et al., 2015***) normalized to controls. Transcript structures of a non-NMD-target, *maltase A2 (MAL-A2)*; a known NMD-targeted transcript, the non-sex specific isoform of *transformer (traL)* (***Rehwinkel, 2005***; ***Metzstein and Krasnow, 2006***); and the *Gadd45* transcript (note *Gadd45* has no introns). Open boxes indicate UTRs; grey boxes indicate coding regions. NMD targeting initiates endonucleolytic cleavage near the stop codon (***Gatfield and Izaurralde, 2004***), producing 5′ and 3′ fragments with unprotected ends, which are then subjected to degradation by cytoplasmic 3′-to-5′ and 5′-to-3′ exonucleases, respectively. qRT-PCR primer pairs 5′ (red) and 3′ (blue) to the cleavage site can be used to differentially measure the quantity of these fragments. The *Drosophila* 5′-to-3′ exonuclease is encoded by the *XRN1* homologue *pacman (pcm)*, and fragments 3′ to an endonucleolytic NMD cleavage accumulate in *Drosophila* cells with reduced XRN1 activity (***Gatfield and Izaurralde, 2004***). The *MAL-A2* 3′ primers show no difference in relative expression in pcm^{14} mutants compared to the 5′ primers, while *tra* and *Gadd45* have relatively increased levels of a 3′ fragment in pcm^{14} mutants, revealing endonucleolytic cleavage has occurred between the primer pairs, probably near the stop codon, indicative of NMD-initiated degradation. p-value between indicated samples using a two-sided Student's t-test are displayed. ns indicates a p-value greater than 0.05. Error bars represent 2 SEM.

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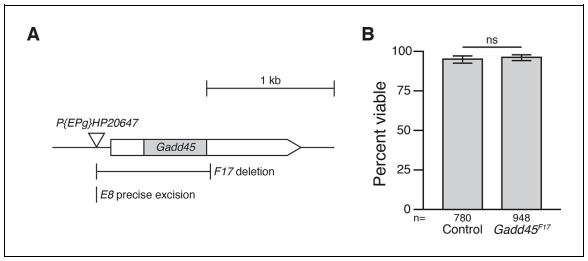

Figure 1—figure supplement 3. F17 is a null allele of Gadd45. (**A**) *Gadd45*^{F17} is an imprecise excision of the P-element *P{EPg}HP20647*, deleting a 894 bp region that includes the entire *Gadd45* coding region. *Gadd45*^{E8} is a precise excision of the same P-element, leaving *Gadd45* intact. Coding region in grey; untranslated regions in white. Arrowhead indicates direction of transcription. (**B**) Adult viability of control and *Gadd45*^{F17} mutants. p = 0.4463 between *Gadd45*^{F17} and controls, using the test of equal or given proportions. Error bars represent 95% confidence interval of the binomial distribution. n equals total number of animals scored in each cross.
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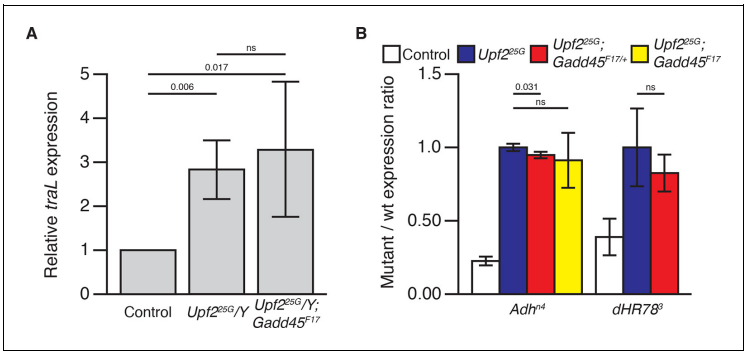

Figure 1—figure supplement 4. Loss of Gadd45 does not restore NMD activity in NMD mutants. (**A**) Expression of the endogenous NMD target transcript *traL* (***Rehwinkel, 2005***; ***Metzstein and Krasnow, 2006***) in control male, $Upf2^{25G}/Y$, and $Upf2^{25G}/Y$; $Gadd45^{F17}$animals, measured by qRT-PCR. There is no significant difference in *traL* expression between $Upf2^{25G}/Y$ and $Upf2^{25G}/Y$; $Gadd45^{F17}$animals. p-values determined by one-sided Student's t-test between indicated conditions are displayed. ns indicates a p-value greater than 0.05. (**B**) Relative abundance of PTC-containing Adh^{n4} (***Chia et al., 1987***) and $dHR78^{3}$ (***Fisk and Thummel, 1998***) allele mRNAs compared to wild-type allele mRNA abundance in animals heterozygous for Adh^{n4}or $dHR78^{3}$in each indicated genotype (stabilization of $dHR78^{3}$ was not determined in $Upf2^{25G}/Y$; $Gadd45^{F17}$ animals). Neither reduction nor elimination of *Gadd45* restored destabilization of these alleles. p-values determined by two-sided Student's t-test between indicated conditions are displayed. ns indicates a p-value greater than 0.05. Error represents 2 SEM.
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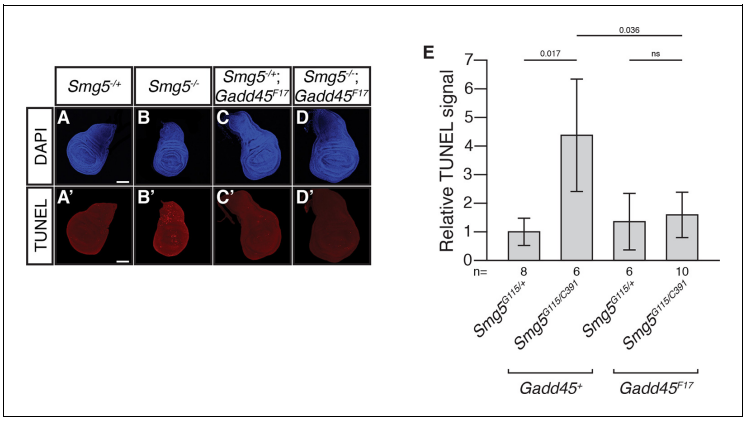

Figure 2—figure supplement 1. Loss of *Gadd45* suppresses ectopic cell death in *Smg5* mutant wing discs. (**A** to **D**) DAPI (blue) and (**A'** to **D'**) TUNEL (red) staining in late 3rd instar larval wing discs from $Smg5^{G115/+}$ (**A**); $Smg5^{G115/C391}$ (**B**); $Smg5^{G115/+}$ $Gadd45^{F17}$ (**C**); and $Smg5^{G115/C391}$ $Gadd45^{F17}$ (**D**) animals. $Smg5^{G115}$ and $Smg5^{C391}$ are null *Smg5* alleles (J.O.N. and M.M.M., unpublished). Scale bar represents 100 μm. (**E**) Relative TUNEL signal in wing discs, normalized to $Smg5^{G115/+}$. p-values determined by two-sided Student's t-test between indicated conditions are displayed. ns indicates a p-value greater than 0.05. Error bars represent 2 SEM.
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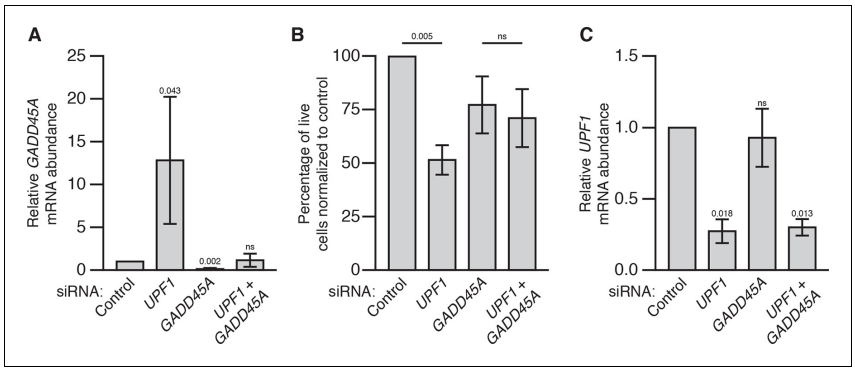

Figure 3—figure supplement 1. *GADD45A* mediates cell lethality in *Upf1* knockdown HEK293 cells. (**A**) Relative *GADD45A* mRNA expression measured by qRT-PCR in *UPF1* and *GADD45A* single and double siRNA knockdown 72 hr after siRNA transfection in HEK293 cells, normalized to expression in the control siRNA condition. p-values display one-sided Student's t-test for each condition compared to control. (**B**) Viable cell count of *UPF1* and *GADD45A* single and double siRNA-treated cells normalized to control siRNA -treated cells. p-values display two-sided Student's t-test between indicated conditions. (**C**) Relative *UPF1* mRNA expression measured by qRT-PCR in *UPF1* and *GADD45A* single and double siRNA knockdown, normalized to expression in control siRNA condition. p-values display one-sided Student's t-test for each condition compared to control. Error bars represent 2 SEM.

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CHAPTER 3

SMG5 IS REQUIRED FOR MULTIPLE NONSENSE-MEDIATED DECAY PATHWAYS IN *DROSOPHILA*

Introduction

Maintaining error-free translation is important to preserve the fidelity of protein function. Eukaryotic cells utilize a number of mRNA surveillance mechanisms to prevent the translation of erroneous proteins (Adjibade and Mazroui, 2014). One of these mechanisms, the nonsense-mediated mRNA decay (NMD) pathway, selectively destroys mRNAs that contain premature termination codons (PTCs) in the open reading frame, preventing their translation (Celik et al., 2015). NMD also degrades many endogenous wild-type mRNAs, acting as a mechanism of post-transcriptional gene regulation (Peccarelli and Kebaara, 2014). NMD is critical for viability in most complex organisms (Arciga-Reyes et al., 2006; Avery et al., 2011; Frizzell et al., 2012; Medghalchi et al., 2001; Metzstein and Krasnow, 2006; Thoren et al., 2010; Weischenfeldt et al., 2008; Wittkopp et al., 2009; Yoine et al., 2006), highlighting the importance of this pathway for cellular function. While the phenomenon of NMD has been well characterized for several decades, the factors required for the activity of this pathway and the mechanisms of target recognition and degradation are still not well understood.

The factors that function in the NMD pathway were first identified in screens in

yeast and *C. elegans* (Cali et al., 1999; Hodgkin et al., 1989; Leeds et al., 1991; 1992). These screens discovered seven proteins that are required for NMD function in most eukaryotes: Upf1, Upf2, and Upf3, which were found in every eukaryote examined, and Smg1, Smg5, Smg6, and Smg7, which are variable in their presence in different species (Siwaszek et al., 2014). After these genes were first identified, a series of genetic analyses in yeast and *C. elegans* mutants lacking NMD genes provided the foundation to build a model of the NMD mechanism. Double and triple mutants between the NMD genes *UPF1*, *UPF2*, and *UPF3* in *S. cerevisiae* were found to all have similar defects to each individual mutant, indicating that all three of these genes are equally required for NMD and probably function together in this process (Atkin et al., 1997). Additionally, double mutant combinations of *smg-1* through *smg-6* in *C. elegans* also revealed that there is no synergistic effect on PTC-containing mRNA stabilization (Hodgkin et al., 1989). These findings indicated that all known NMD factors are equivalently required for all NMD activity, providing context to interpret the characterization of the molecular interactions between these factors, suggesting that they function together in a complex (Weng et al., 1996; Yamashita et al., 2001). *C. elegans* double mutants also revealed that *smg-1*, *smg-3* (*Upf2*), and *smg-4* (*Upf3*) are required for phosphorylation of SMG-2 (UPF1), but *smg-5*, *smg-6*, and *smg-7* are required to prevent SMG-2 hyper-phosphorylation (Page et al., 1999), suggesting that regulation of Upf1 phosphorylation may be a critical step in the NMD process. This discovery linking Upf1 phosphorylation to NMD function informed how the biochemical interactions of Smg5, Smg6, and Smg7 binding to phosphorylated residues on Upf1 may contribute to NMD activity (Chiu et al., 2003; Ohnishi et al., 2003; Okada-Katsuhata et al., 2012). Combining an understanding

of the molecular interactions of the NMD factors with genetic analysis of the requirements of these genes for NMD function has shaped the model of the NMD mechanism.

Even though genetic analysis has provided critical context for interpreting the molecular interactions of NMD factors, there has been very little characterization of the individual contribution of NMD genes *in vivo* in organisms other than yeast and *C. elegans*. The lack of genetic analysis in these organisms may be due to the fact that reduced expression of any NMD gene causes lethality in plants, zebrafish, mice, and human cells (Arciga-Reyes et al., 2006; Kerényi et al., 2008; Li et al., 2015; Medghalchi et al., 2001; Thoren et al., 2010; Weischenfeldt et al., 2008; Wittkopp et al., 2009; Yoine et al., 2006), indicating NMD is an essential feature of biology in these more complex organisms. Additionally, inhibition of any individual NMD factor in *Drosophila* cells produces a similar genomic profile of gene expression changes due to stabilization of endogenous and PTC-containing NMD targets (Rehwinkel et al., 2005). The similar phenotypes between the reduced activity of individual NMD factors in these systems is consistent with the idea that all NMD genes are equivalently required for any NMD function, as observed in yeast and *C. elegans*. However, most of these studies were done using RNA interference to reduce expression of genes required for NMD, and not with genetic ablation of gene activity. It is possible that the similar phenotypes are the result of incomplete and variable expression knockdown, masking the true phenotype that may occur upon complete loss of each individual NMD factor. Mutations in *A. thaliana upf1, upf3*, and *smg7* cause early developmental lethality (Hori and Watanabe, 2005; Riehs et al., 2008; Yoine et al., 2006), and mouse mutants lacking *Upf1*, *Upf2*, *Smg1*, or *Smg6* all

die during early embryonic development (Li et al., 2015; McIlwain et al., 2010; Medghalchi et al., 2001; Weischenfeldt et al., 2008), consistent with each gene being similarly required for NMD function. However, these mutants may have subtle differences in the efficiency of NMD activity, which has been difficult to determine due to this very early lethality, and the potential combinatorial effects of these mutations are yet to be tested in either organism. The lack of genetic tools to test the requirement of each NMD factor has limited the ability to characterize the individual contribution of each gene in a native setting.

Drosophila melanogaster mutants are the only other genetic models of loss of NMD in an organism where it is required for viability; however, *Drosophila* is the exception to the trend that loss of any NMD factor has similar effects on viability. *Drosophila* with null mutations in *Upf1* or *Upf2* die during early larval stages, and these animals never survive to adulthood (Chapin et al., 2014; Metzstein and Krasnow, 2006). However, *Drosophila* with mutations in *Upf3, Smg1*, and *Smg6* can survive to adulthood (Avery et al., 2011; Chen et al., 2005; Frizzell et al., 2012; Metzstein and Krasnow, 2006). It has been suggested that this difference in requirement for these NMD factors is due to non-NMD functions of Upf1 and Upf2 being required for viability, and not differences in NMD activity (Avery et al., 2011). However, we have recently shown that *Upf1* and *Upf2* mutants can survive to adulthood by eliminating the expression of a single endogenous NMD target (Nelson et al., 2016), indicating that the lethality of *Upf1* and *Upf2* mutants is in fact likely due to the specific loss of NMD activity. This finding further suggests that the differences in viability between *Upf1* and *Upf2* mutants and *Upf3, Smg1,* and *Smg6* mutants is likely due to differences in their requirement for the

NMD process. In particular, the viability of *Upf3, Smg1,* and *Smg6* mutants suggests that these animals have sufficient NMD activity to survive to adulthood. This possibility would imply that in the absence of one of these factors a redundant mechanism can be used to recognize and degrade NMD targets in *Drosophila*. It is possible that these three genes function in three independent NMD mechanisms, allowing for residual NMD activity to occur when any one of these genes are lost.

Smg5 is the only known NMD gene for which loss-of-function mutations are yet to be characterized in *Drosophila* or any other species that require NMD for viability. Smg5 is perhaps the least well understood NMD factor, and it is possible that it may account for some of the residual NMD activity in viable *Drosophila* NMD mutants. Here we describe the first analysis of *Drosophila Smg5* mutants and discover that *Smg5* is a critical NMD gene required for viability. To understand how *Smg5* is required for NMD, we performed the first double-mutant analysis of NMD genes in *Drosophila*. Our findings suggest *Drosophila* utilize multiple independent mechanisms to initiate NMD target decay, providing the first *in vivo* context for potential alternative NMD mechanisms.

Methods

Fly genetics

Drosophila melanogaster stocks were raised on standard cornmeal/dextrose food at 25°. The NMD mutant allele $Smg1^{32AP}$ (Metzstein and Krasnow, 2006) is on a *y w* FRT^{19A} chromosome and $Smg6^{292}$ (Frizzell et al., 2012) is on an FRT^{82B} chromosome and balanced over *TM6B, P{Dfd-EYFP} Sb*^{1} *ca*^{1} (Le et al., 2006). All *Smg5* alleles are on FRT^{40A} chromosomes. Other alleles used were $Gadd45^{F17}$ (Nelson et al., 2016), pcm^{14}

(Waldron et al., 2015) and *DHR78*^{3} (Fisk and Thummel, 1998). *y w FRT*^{19A} was used as a control chromosome for all experiments.

For the genetic screen, *FRT*^{40A} males were starved for 8 hours, and then fed on sucrose with 1% ethyl methanesulfonate over night to induce mutagenesis. Mutagenized males were then mated with *FRT*^{40A}; *P{Da-GAL4 w*$^{+}$*} P{UAS-FLP} P{UAS-eGFP::SV40 3'UTR}* females, and F1 late wandering L3 larvae were collected in glycerol and scored for mosaic enhanced GFP fluorescence using a Leica MZ 16F microscope. Mosaic animals were cleaned in PBS and placed in vials with food to continue development. After eclosion, candidate mutant lines were established and retested to confirm an NMD defect. Candidate alleles were tested for complementation with *Df(2L)BSC345*, which deletes the *Smg5* locus, and lines that failed to complement this deficiency were sequenced at the *Smg5* locus. Isolated *Smg5* alleles are balanced over *CyO, P{Dfd:eYFP w*$^{+}$*}* (Le et al., 2006).

For viability tests, animals containing mutant alleles over a balancer were mated for three days, and offspring were collected for 10 days, beginning 10 days after mating began. All balancer^{-} and balancer^{+} animals were scored, and percent expected viable was determined by the ratio of balancer^{-} animals to balancer^{+} animals.

RNA isolation and quantification

For qRT-PCR analyses, we collected five to ten 0-4 h L3 larvae and froze them in liquid nitrogen. We isolated total RNA using TRIzol reagent (Invitrogen) and phase-lock tubes (5-Prime), and the RNeasy mini kit (QIAGEN). We used on-column RNase-free DNase treatment (QIAGEN) to reduce genomic contamination. We determined RNA concentration by spectrophotometer and normalized concentration for reverse

transcription. For reverse transcription, we used random decamers and MMLV8 reverse transcriptase (Retroscript Kit, Ambion). We performed qRT-PCR analysis using the SYBR Green qPCR Supermix (Bio-Rad) and the Bio-Rad iCycler thermocycler. All experimental reactions were performed using three technical replicates and a minimum of three biological replicates per condition, and the expression level of all experimental assays was normalized to *RpL32* mRNA expression. For all qRT-PCR analyses, we also measured samples that had been made without reverse transcriptase to ensure that signal was not due to genomic DNA.

Primer sequences used were RpL32_1 (ATGCTAAGCTGTCGCACAAA), RpL32_2 (CGATGTTGGGGCATCAGATAC), Gadd45_5'_1 (CATCAACGTGCTCTCCAAGTC), Gadd45_5'_2 (CGTAGATGTCGTTCTCGTAGC), Gadd45_3'_1 (ACAGCCAGATGTCACAGAATT), and Gadd45_3'_2 (CCAGCAACTGGTTTCCATTAG). All *Gadd45* qPCR analysis was done using the Gadd45_5' primer pair unless otherwise noted.

Analysis of *dHR78*^{3} PTC allele stability

We collected adult *Smg5*$^{+/G115}$ or *Smg5*$^{C391/G115}$ males that were also heterozygous for the *dHR78*^{3} allele. The *dHR78*^{3} allele is a PTC-containing allele that has lower expression than the wild-type allele, and thus is presumably degraded by NMD (Fisk and Thummel, 1998). At least three biological replicates were collected for each condition. We isolated RNA and generated cDNA, as described above, and used this cDNA as a template for PCR amplification of the *dHR78* transcript with the DRH78_F3 / DHR78_R3 primers (TGGGGCTTATTCAGAGTTCG /

ATTAATGCTGGCCACACTCC), which flank the nonsense mutation. To compare the relative abundance of the *dHR78*^{3} allele to the wild-type allele, PCR products were Sanger sequenced, and the relative peak intensity for a thymine (*dHR78*^{3} allele) compared to a cytosine (wild-type allele) at nucleotide 1063 was compared.

Lethal phase and larval development analysis

For lethal phase and larval development analysis, first-instar larvae were collected 20-24 hours after egg lay. Animals were examined every 24 hours, their developmental stage was recorded, and they were transferred to fresh food. Larval stage was determined based on physical characteristics of the mouth hooks. Once animals entered pupariation, pupae were transferred to vials and scored for eclosion to adulthood five days later.

Results

***Drosophila Smg5* is an essential NMD factor**

To identify novel *Drosophila Smg5* mutant alleles, we performed an EMS mutagenesis screen in animals expressing an NMD-sensitive GFP reporter. This reporter expresses the GFP coding sequence from a *UAS* promoter and has the *SV40* 3'UTR (Metzstein and Krasnow, 2006). The *SV40* 3' UTR is targeted by NMD, so cells with defective NMD activity have increased reporter expression and GFP fluorescence (Metzstein and Krasnow, 2006). Since mutations in *Smg5* are potentially lethal, like *Upf1* and *Upf2* mutations, we screened genetic mosaic animals for loss of NMD activity in individual cells. To do so, we used the *da-GAL4* driver to ubiquitously express the NMD sensitive *GFP::SV40 3' UTR* reporter and *FLP* recombinase from *UAS* promoters

in *Drosophila* larvae. FLP-mediated recombination of *FRT* sites located near a centromere produces daughter cells homozygous for the chromosome arm with the *FRT* sequence. If the recombined chromosome arms are heterozygous for a mutation in a gene required for NMD, then cells that become homozygous for that mutation after *FRT* recombination will show increased reporter GFP fluorescence compared to other cells **(Figure 3.1A)**. We observed that mosaic loss of NMD activity causes late L3 larvae to show multiple patches of brighter GFP fluorescence, but non-mosaic animals express a uniform fluorescent signal across the entire animal **(Figure 3.1B)**. The mosaic enhanced fluorescence phenotype is easy to distinguish, and mosaic animals remain viable and fertile, so lines can be established from individual identified mutants. An added benefit of this approach is that mutagenizing animals that have an *FRT* site located near the centromere on the left arm of the second chromosome (FRT^{40A}) will isolate mutations only on this chromosome arm. Since *Smg5* is the only known NMD gene located on the left arm of the second chromosome, mutations identified from the screen will be enriched for alleles of *Smg5*.

Using this approach, we screened 12,554 larvae and identified three mutants with mosaic GFP fluorescence enhancement. These three mutants, *A1*, *EI1*, and *MI1*, were all homozygous lethal. To test if these alleles had mutations in *Smg5*, we crossed each allele to a deficiency that deletes *Smg5*. We found that all three of these alleles were lethal over this deficiency **(Figure 3.2A)**, indicating that they had mutations in *Smg5*. Sequencing of the *Smg5* locus identified that each allele had a nonsense mutation in the *Smg5* coding sequence **(Figure 3.2B)**, suggesting they are loss-of-function alleles. In a parallel screen that expressed the *GFP::SV40 3' UTR* reporter in the larval tracheal system (Förster et

al., 2010), we also identified four mutants that had increased GFP fluorescence **(Figure S3.1A-E)**. These mutations were mapped to the second chromosome, indicating they may be alleles of either *Smg5* or *Upf3*, the only known NMD genes located on that chromosome. These alleles were also homozygous lethal, so they were crossed to the deficiency that deletes *Smg5* and tested for viability. Two of these alleles, *G115* and *C391*, were lethal over this deficiency **(Figure 3.2A)**, and sequencing of the *Smg5* locus revealed they respectively had a nonsense mutation and an altered splice acceptor site in *Smg5* **(Figure 3.2B)**. The nature of these mutations suggested that they were also loss-of-function alleles, and when the viability of these alleles was tested as trans-heterozygotes, they were also found to be lethal **(Figure 3.1B)**, confirming that they are null mutations. The other two alleles, *Q454* and *Q376*, were viable over the deficiency that deletes *Smg5* **(Figure 3.2A)**, but they also had increased fluorescence of the *GFP::SV40 3' UTR* reporter when over this deficiency (Data not shown). These results suggested they may have been viable hypomorphic alleles of *Smg5* with linked lethal mutations. We sequenced *Smg5* in these mutants, and discovered they both had missense mutations in highly conserved alpha-helices of the *Smg5* 14-3-3-like domain **(Figure 3.2B)** (Fukuhara et al., 2005). All together we isolated seven total *Smg5* mutants from these two screen – five likely null alleles and two hypomorphic alleles. Importantly, the near complete lethality of these *Smg5* loss-of-function mutants indicates that *Smg5* is required for viability.

Lethal *Drosophila* NMD mutations generally have more severe defects in NMD function than viable mutations, as measured by increased expression of endogenous NMD targets (Frizzell et al., 2012; Metzstein and Krasnow, 2006). To test if lethal *Smg5*

mutant alleles also have strong defects in NMD activity, we used qRT-PCR to measure the expression of the endogenous NMD target *Gadd45* (Chapin et al., 2014; Nelson et al., 2016). Since *Gadd45* is directly targeted by NMD, the amount of increased *Gadd45* expression in *Smg5* mutants compared to controls serves as an indirect measure of the decrease in NMD activity. We measured *Gadd45* expression in early third instar larvae and found that $Smg5^{C391/G115}$ mutants had a strong increase in *Gadd45* mRNA, while $Smg5^{Q454/G115}$ hypomorphs had a weaker expression increase **(Figure 3.2C)**. These findings are consistent with the previously observed trend that lethal mutations in NMD genes have more severe defects in NMD activity than viable mutations (Frizzell et al., 2012). $Smg5^{C391/G115}$ mutants also fully stabilized the expression of PTC containing mRNAs **(Figure 3.2D)**, indicating that this defect in NMD function is not restricted to the degradation of endogenous NMD targets, but that *Smg5* mutants essentially have a complete loss of NMD activity.

Upf1 and *Upf2* null mutants have delayed development and die during larval stages (Chapin et al., 2014). We found that $Smg5^{C391/G115}$ mutants also have developmental delays, with *Smg5* mutants spending almost twice as long in larval stages as control animals **(Figure S3.2B)**, and most $Smg5^{C391/G115}$ mutants die during pupariation **(Figure S3.2A)**. This developmental delay and lethal phase is similar to, but somewhat weaker than, the defects in *Upf1* and *Upf2* mutants (Chapin et al., 2014). This difference in the timing of lethality in *Upf1* and *Upf2* mutants compared to *Smg5* mutants suggests that these animals may be dying due to different causes, even if all three conditions lack NMD function. Increased *Gadd45* expression is a major factor contributing to the death of *Upf1* and *Upf2* mutants, and loss of *Gadd45* suppresses *Upf1*

and *Upf2* mutant lethality (Nelson et al., 2016). Given the strong increase of *Gadd45* expression in $Smg5^{C391/G115}$ mutants, it is likely that these animals are also dying due to excessive *Gadd45*. We found that loss of *Gadd45* also suppresses the lethality of $Smg5^{C391/G115}$ mutants **(Figure 3.2E)**, indicating that these animals are dying due to a similar loss of NMD function as *Upf1* or *Upf2* mutants. These results combined strongly suggest that *Smg5* mutant lethality is specifically due to a loss of NMD activity, and not due to loss of any potential NMD-independent Smg5 function.

Smg5 is required for Smg6 endonuclease activity

Smg6 is an endonuclease that cleaves NMD-targeted mRNAs near the stop codon, exposing free 5' and 3' ends, which can then be degraded by the cells general exonucleases (Gatfield and Izaurralde, 2004; Huntzinger et al., 2008). Smg6-mediated cleavage is the only known mechanism for degradation of NMD targets in *Drosophila*; however, Smg6-independent decay must occur since *Smg6* mutants are viable and have weak NMD defects (Frizzell et al., 2012). To test if *Smg5* mutants lose Smg6 endonuclease activity, we tested NMD target cleavage in *Drosophila* lacking the lone cytoplasmic 5'-to-3' exonuclease Xrn1, which is encoded by the gene *pacman* (*pcm*). pcm^{14} mutants are null and no longer have functioning 5'-to-3' exonuclease activity (Waldron et al., 2015), and thus mRNAs cleaved by Smg6 in this mutant show increased expression of the fragment 3' to the cleavage site compared to the 5' fragment (Nelson et al., 2016). This difference in the expression of the 3' and 5' region of NMD targets likely occurs in pcm^{14} mutants because 3' fragments produced after Smg6 cleavage are no longer degraded by Xrn1, but the 5' fragments are still degraded by the functioning 3'-to-5' exonuclease. We found that in pcm^{14}; $Smg6^{292/Df}$ double mutants, the preferential

stabilization of the 3' fragment of *Gadd45* is lost **(Figure 3.3A, B)**, indicating that the preferential increase in expression of the 3' region is due to Smg6 endonuclease activity. Interestingly, we found that the preferential stabilization of the 3' fragment is also lost in pcm^{14}; $Smg5^{C391/G115}$ double mutants **(Figure 3.3A, B)**. These results indicate that *Smg5* is required for any Smg6 endonuclease activity to occur.

Smg5 is not required for decapping and Xrn1-mediated decay

Although we found that *Smg5* is required for NMD targets to be degraded by the Smg6 endonuclease, *Smg5* mutants have much stronger defects than *Smg6* mutants, indicating that *Smg5* is also required for an additional Smg6-indpendent decay mechanism. Human Smg5 can indirectly interact with both decapping and deadenylation complexes (Cho et al., 2013; Loh et al., 2013); however, whether either of these interactions occur during native NMD conditions is unclear. Any decay mechanism that occurs via decapping would require 5'-to-3' exonucleases to complete degradation of the mRNA, and a deadenylation mechanism would require 3'-to-5' exonucleases. To test if decapping-mediated decay contributes at all to the degradation of NMD targets in *Drosophila*, we measured *Gadd45* expression in pcm^{14} mutants and controls using the q-PCR primer pair 5' to the *Gadd45* termination codon **(Figure 3.3A)**. Xrn1 would only degrade *Gadd45* mRNA in this region after decapping occurs, but not after Smg6 endonuclease activity, as with the 3' primer pair, so this assay allows us to determine the contribution of Xrn1 decay specifically after decapping of this NMD target. We found a small increase in *Gadd45* expression in pcm^{14} mutants compared to controls **(Figure 3.3C)**, suggesting that this mRNA is subjected to a low amount of decapping and 5'-to-3' degradation, but we cannot yet determine if this decay occurs due to NMD activity.

Decapping followed by Xrn1 endonuclease activity can occur due to many different mechanisms that are independent from NMD function (Siwaszek et al., 2014). The relative expression in *pcm*^{14} mutants compared to control animals represents the increased stability that occurs due to loss of both Smg5-independent and any potential Smg5-dependent Xrn1 decay activity. To separate the two potential Xrn1 decay activities, we measured *Gadd45* expression in *pcm*^{14}; *Smg5*$^{C391/G115}$ double mutants compared to *Smg5*$^{C391/G115}$ mutants alone using the 5' q-PCR primers. Any decay activity that requires both Smg5 and Xrn1 will already be lost in a *Smg5* mutant, so the relative expression in the double mutant compared to the *Smg5* mutant will represent only the increased stability due to loss of specifically Smg5-independent Xrn1 decay activity. If any Smg5-dependent Xrn1 decay activity exists, then we expect the relative *Gadd45* expression in *pcm*^{14} mutants compared to control animals to be greater than the relative expression in *pcm*^{14}*; Smg5*$^{C391/G115}$ double mutants compared to *Smg5*$^{C391/G115}$ mutants. If no Smg5-dependent Xrn1 decay activity exists, then there should be no difference in relative *Gadd45* expression in these two comparisons, because in both experiments, only Smg5-independent Xrn1 decay is being lost. We found that there was no difference in the relative expression between *pcm*^{14} mutants compared to control animals and *pcm*^{14}*; Smg5*$^{C391/G115}$ animals compared to *Smg5*$^{C391/G115}$ mutants **(Figure 3.3C, D)**. These results indicate that Xrn1 does not require Smg5 to degrade the 5' region of *Gadd45* mRNA, suggesting that Smg5 does not promote decapping of NMD targets. However, these results do not mean that NMD does not induce target decapping at all in *Drosophila*, as decapping enzymes may be directly recruited by Upf1 independent from Smg5 function.

Smg1 and Smg6 function together to degrade NMD targets

Smg6 binds Upf1 at phosphorylated residues (Fukuhara et al., 2005; Okada-Katsuhata et al., 2012), suggesting that Smg1 kinase activity is required to first phosphorylate Upf1 for Smg6 to cleave NMD targets. However, *Drosophila Smg6* mutants have much stronger defects in viability and NMD activity than *Smg1* mutants (Chen et al., 2005; Frizzell et al., 2012; Metzstein and Krasnow, 2006), and Smg6 has been shown to be capable of binding Upf1 in the absence of functional Smg1 in human cells (Chakrabarti et al., 2014; Nicholson et al., 2014). These reports suggest an alternative model in which Smg6 can cleave NMD targets without Smg1 kinase activity. The contribution of Smg1 to NMD may then be independent of Smg6 function, or it may act to enhance Smg6 binding to Upf1, even if Smg6 can degrade NMD targets in the absence of Smg1. These possibilities can be distinguished by examining the potential additive NMD defects of *Smg1; Smg6* double mutants. If *Smg1* and *Smg6* function in independent decay mechanisms, we expect a *Smg1; Smg6* double mutant would have enhanced defects compared to loss of *Smg6* alone. Alternatively, if Smg1 only functions to recruit Smg6, and thus is dependent on Smg6 for all NMD activity, we expect *Smg1; Smg6* double mutants to have no more severe defects than a *Smg6* mutant. We found that $Smg1^{32AP}$; $Smg6^{292/Df}$ double mutants showed no difference to $Smg6^{292/Df}$ single mutants in viability, *Gadd45* expression, or stage of lethality **(Figure 3.4A-C)**. Together, these data indicate that Smg1 function is completely dependent on Smg6, and suggest that Smg1 contributes to, but is not required for, Smg6 binding to Upf1, and that Smg1 is not required for any other NMD activity.

Smg1 functions in a subset of Smg5 decay activity

Since we found that *Smg5* is required for Smg6 endonuclease activity and that *Smg6* is required for *Smg1* NMD function, it is likely that *Smg1* and *Smg5* function in the same pathway to promote Smg6 decay activity; however, it is possible that they independently contribute to Smg6 function. To determine if *Smg1* and *Smg5* are both required for the same decay mechanism, we tested if *Smg1*; *Smg5* null double mutants have a larger increase in *Gadd45* expression than *Smg5* null mutants alone. We found that loss of *Smg1* did not increase the expression of *Gadd45* in *Smg5* mutants **(Figure 3.5A)**, suggesting that *Smg1* and *Smg5* do function in the same pathway. We could not directly test if loss of *Smg1* enhances *Smg5* null mutant lethality because *Smg5* null mutants are completely lethal **(Figure 3.5B)**; however, we could test if *Smg1*; *Smg5* null double mutants die earlier than *Smg5* mutants. We found that loss of *Smg1* neither enhances the developmental delay observed in *Smg5* mutants, nor shifts the lethality from pupariation to the larval stages, when *Upf1* and *Upf2* mutant die **(Figure S3.2A, B)** (Chapin et al., 2014). These findings indicate that *Smg1* is dispensable for NMD activity in *Smg5* mutants, and suggests that Smg5, like Smg6, is required for all *Smg1*-dependent decay activity.

The more severe defects of *Smg5* mutants than *Smg1* mutants suggests that Smg5 may be required for the residual NMD activity that occurs in *Smg1* mutants. To test if *Smg5* has a Smg1-independent NMD activity, we tested if *Smg5* hypomorphs, which are normally completely viable with weak NMD defects, would enhance the defects of *Smg1* mutants. We found that when *Smg1* is lost in *Smg5* hypomorphs, most of these animals now fail to survive to adulthood **(Figure 3.5C)**, and $Smg1^{32AP}$; $Smg5^{Q454/G115}$ double

mutants have a much larger increase in *Gadd45* expression than either mutation alone **(Figure 3.5D)**. These results indicate that Smg5 is required for NMD activity that is independent of Smg1, and suggest that Smg5 is required for multiple NMD functions, one that also requires Smg1 and one or more that does not.

Discussion

Smg5 is essential for NMD function in *Drosophila*

The degradation of mRNAs containing premature termination codons by the nonsense-mediated mRNA decay (NMD) pathway is a crucial cellular quality-control function. While many factors required for NMD have been characterized, the individual contribution of each factor to the recognition and degradation of NMD targets is not fully understood. Most of our understanding of how these factors contribute to NMD has come from discerning the molecular interactions that occur between these proteins during the NMD process, but there is relatively little description of the defects that occur when individual NMD factors are genetically ablated. Through the first analysis of *Drosophila Smg5* mutants and double mutants of NMD genes, we have found that NMD utilizes multiple mechanisms to promote target degradation, potentially via several different molecular interactions. Our data are consistent with the idea that the decay of NMD target mRNAs is preceded by Smg6 endonuclease activity, and this mechanism requires Smg5. Additionally, we show that Smg5 is required for a separate decay process **(Figure 3.6)**. This discovery was surprising given that *Smg5* is the least well characterized NMD factor, and was previously thought only to promote NMD complex recycling and not to be required to stimulate decay activity (Ohnishi et al., 2003). The finding that *Smg5* mutants have as severe defects as *Upf1* and *Upf2* mutants indicates

that *Smg5* is actually a critical factor for promoting decay mechanisms.

Smg1 kinase activity does not contribute to the defects in *Smg5* or *Smg6* mutants

One explanation for the strong defect in *Smg5* mutants may be the loss of dephosphorylation of Upf1. Smg1 is a kinase that phosphorylates Upf1 at multiple residues (Grimson et al., 2004; Yamashita et al., 2001). This phosphorylation in turn can promote Smg5 and Smg6 binding (Anders et al., 2003; Ohnishi et al., 2003). Smg5 also binds the PP2A phosphatase, recruiting it to the NMD complex, and this recruitment is thought to lead to dephosphorylation after target degradation has been initiated (Anders et al., 2003; Ohnishi et al., 2003). In *C. elegans Smg5* mutants, Upf1 (SMG-2) is found to be hyper-phosphorylated in a Smg1-dependent manner (Page et al., 1999). Failure to dephosphorylate Upf1 may be the cause of the strong NMD defect in our *Drosophila Smg5* mutants; however, it is unclear how failure to dephosphorylate Upf1 leads to loss of NMD activity. Though *Smg1* is thought to be necessary for Upf1 phosphorylation, loss of *Smg1* has little effect on NMD activity in *Drosophila* (Chen et al., 2005; Metzstein and Krasnow, 2006), indicating that Upf1 phosphorylation is not a necessary step in the NMD process in this organism. As a corollary to these findings, *Smg1* mutants should suppress *Smg5* mutant defects, since *Smg5* would not longer be required for Upf1 dephosphorylation. However, we found just the opposite: *Smg1* mutants do not suppress *Smg5* mutants at all, and *Smg1* mutants actually enhance the defect of *Smg5* hypomorphic alleles **(Figure 3.5C, D)**. These data suggest *Smg5* has functions independent of *Smg1* in *Drosophila* NMD. It is possible that another PIK-related kinase could phosphorylate Upf1 in the absence of Smg1 in *Drosophila*, allowing for *Smg1*

mutants to have minor defects and also explaining why *Smg1* mutants fail to suppress *Smg5*. However, given that *Smg5* hypomorphic alleles maintain sufficient function for viability, we would expect these alleles to maintain viability in a *Smg1* mutant if another kinase was phosphorylating Upf1. Since *Smg1* has such strong synthetic defects with *Smg5* hypomorphs, it suggests instead that these two genes have redundant functions. Based on these analyses of *Smg1; Smg5* double mutants, we propose that these two NMD factors contribute to parallel decay pathways **(Figure 3.7)**.

Smg5 and Smg6 contain 14-3-3-like domains, which can bind phosphorylated Upf1 (Fukuhara et al., 2005; Okada-Katsuhata et al., 2012), so phosphorylation of Upf1 by Smg1 was thought to be required for Smg6 to bind Upf1 prior to cleaving target mRNAs. However, more recent evidence suggests that Smg6 binds Upf1 in the absence of Smg1 activity, through a domain separate from the 14-3-3-like domain, although it is unclear if this binding occurs during NMD *in vivo* (Chakrabarti et al., 2014; Nicholson et al., 2014). Interestingly, *Drosophila Smg6* mutants have much stronger NMD defects than *Smg1* mutants (Frizzell et al., 2012), consistent with the idea that Smg6 has Smg1-independent NMD activity. Our finding that loss of *Smg1* does not enhance *Smg6* mutant defects is consistent with the idea that Smg1 functions through Smg6 activity **(Figure 3.7)**. This finding is the first *in vivo* context that reveals that Smg1 does require Smg6 activity, but that Smg6 can function in the absence of Smg1.

Smg5 is required for Smg6 endonuclease activity

We found that *Smg5* mutants have much stronger defects in NMD activity than either *Smg1* or *Smg6* mutants, and that *Smg5* mutants have completely lost endonuclease activity on endogenous NMD targets, indicating that Smg6 requires Smg5 function. One

possibility is that Smg5 is required for targeting or activation of Smg6 endonuclease activity, perhaps through features of the inactive PIN domain of Smg5, which has similar structure to the active PIN domain of Smg6 that is required for NMD activity (Glavan et al., 2006; Huntzinger et al., 2008). These PIN domains may be required to interact with one another for Smg6 endonuclease activity to occur. The contribution of individual Smg5 domains to NMD activity must be further investigated to dissect how Smg5 is required for Smg6 endonuclease activity.

Smg5 is required for a Smg6-independent decay activity

The enhanced defects in $Smg1^{32AP}$; $Smg5^{Q454/G115}$ double mutants and that *Smg5* mutants have more severe defects than *Smg6* mutants suggests that *Smg5* is required for Smg6-independent decay activity; however, the mechanism of this decay remains unclear. It is likely that *Smg5* promotes decay activity through recruiting either decapping or deadenylation complexes to induce exonucleolytic decay. There is evidence that human Smg5 interacts with the Dcp decapping complex and Smg7 may interact with the CCR4-NOT deadenylation complex (Loh et al., 2013). *Drosophila* does not encode a direct Smg7 orthologue, but *Drosophila* Smg5 has some conserved regions with human Smg7, and it may have some of these same interactions (Chiu et al., 2003; Fukuhara et al., 2005). Whether either of these interactions are required during normally occurring NMD is not known, and so far, no genetic evidence has indicated that *Smg5* is required for either of these alternative decay mechanisms. We found that *Smg5* decay activity on NMD targets does not overlap with the decay activity of *pcm*, the lone *Drosophila* 5'-to-3' exonuclease, which would degrade mRNAs in the event of decapping (Till et al., 1998). This lack of overlap between *pcm* and *Smg5* function does

not mean that *pcm* does not contribute to NMD, as it is likely that Xrn1 (the protein encoded by *pcm*) is directly recruited by Upf1, independent of Smg5 activity, as previously suggested in human cells (Lejeune et al., 2003). *Drosophila* Smg5 may instead recruit deadenylation complexes, a potential function of Smg7 in human cells (Loh et al., 2013). The possible overlap in decay activity between the exosome, the predominant 3'-to-5' exonuclease, and Smg5 will need to be tested to determine if this is the Smg6-independent Smg5 decay activity.

An alternative model for the requirement of *Smg5* in NMD may be that it is necessary for NMD complex recycling. The *Smg5* mutant defects are unlikely to be due to failure to dephosphorylate Upf1, because *Smg1* mutants do not suppress *Smg5* defects, and instead enhance the defects of the hypomorphic *Smg5* alleles. However, it is possible that *Smg5* is required for complex disassembly independent of Smg1 activity. Lack of complex recycling would explain why the *Smg5* mutant defect is as severe as Upf1 and Upf2 mutants, and why all endonuclease and other decay activity is lost in *Smg5* mutants. While the potential role for Smg5 in complex disassembly has been postulated for a long time, it is yet to be directly tested (Ohnishi et al., 2003). The process of complex recycling itself needs to be closely investigated in *Smg5* mutants to determine if Smg5 is required for this process, and if a defect in recycling accounts for *Smg5* mutant NMD defects.

The differences in the strength of the NMD defects that occur when *Smg1*, *Smg5*, or *Smg6* are lost suggests that there may be a biased preference for which NMD mechanism is used to degrade specific targets. For instance, the very weak defects of *Smg1* mutants indicates that Smg1 actually contributes little to the decay of NMD

targets, suggesting that most degradation does not occur via Smg1 activity. However, loss of *Smg1* actually causes a strong stabilization of the *GFP::SV40 3' UTR* reporter (Metzstein and Krasnow, 2006), which has a viral 3' UTR that contains an intron, suggesting Smg1-dependent NMD may contribute more to the degradation of targets with these features. The incomplete loss of NMD activity in *Smg6* mutants also indicates that Smg6-indepdendent decay is sufficient to maintain most NMD activity. However, the weak defect of *Smg6* mutants does not necessarily mean that most NMD activity occurs through the Smg6-independent pathway. It is possible that under wild-type conditions, most NMD occurs via Smg6 endonuclease activity, and that Smg6-independent decay contributes very little to NMD, but only during loss of *Smg6* does this alternative decay pathway perform the bulk of NMD activity. It is also possible that the preference for which decay mechanism degrades NMD targets may be different between individual NMD targets, or in tissue- or developmental-specific contexts. It will be important to identify the Smg6-independent decay mechanism to parse the relative contribution of each decay pathway to the degradation of NMD targets to understand the potential mechanism of the bias in decay.

Summary

Here we performed the first double mutant analysis of multiple NMD factors, providing genetic analysis of the relative contribution of individual NMD genes. We also characterized the first *Drosophila Smg5* mutants, identifying that *Smg5* is critical for NMD function and viability, similar to *Upf1* and *Upf2*. This is the first genetic analysis of *Smg5* function in a model system where *Smg5* is required for viability. Our findings suggest that NMD utilizes multiple branched decay mechanisms to destroy its targets.

All of these pathways depend on *Smg5*. Our study indicates the importance of *Smg5* has been underappreciated. More closely characterizing the molecular mechanisms of Smg5 in NMD may reveal novel key features of NMD activity that have been thus far undetected.

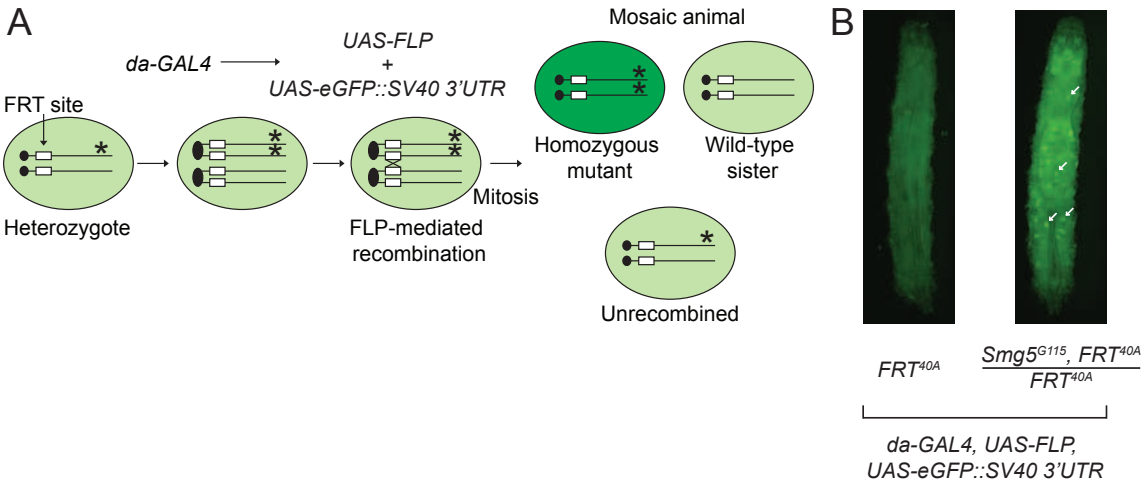


Figure 3.1. Screens for novel NMD defective mutations identify *Smg5* mutants. (**A**) Scheme to detect heterozygous novel mutations in genes required for NMD. The GAL4 transcription factor ubiquitously expressed by the *da* promoter activates transcription at the *UAS* promoter, expressing *FLP* recombinase and the NMD sensitive *eGFP::sv40 3' UTR* fluorescent reporter. This reporter mRNA is degraded by NMD, and thus has increased fluorescence in cells lacking NMD activity. FLP recombinase recombines chromosomes at *FRT* sites (white box). When FLP-mediated recombination occurs during G2 phase, the arms of sister chromatids can be exchanged, and homozygous chromosome arms can be inherited by daughter cells. If recombined chromosome arms contain a mutation in an NMD factor, then the daughter cell and all subsequent lineage will have increased GFP fluorescence compared to homozygous wild-type sisters and unrecombined cells. (**B**) Example of mosaic GFP reporter fluorescence phenotype. Late L3 larvae expressing the NMD sensitive *eGFP::sv40 3' UTR* fluorescent reporter in animals with the FRT^{40A} site that are either homozygous wild-type (left) or heterozygous for a mutation in *Smg5* (right). Clones with increased GFP fluorescence are indicated by white arrows. Dorsal view; Anterior at top.

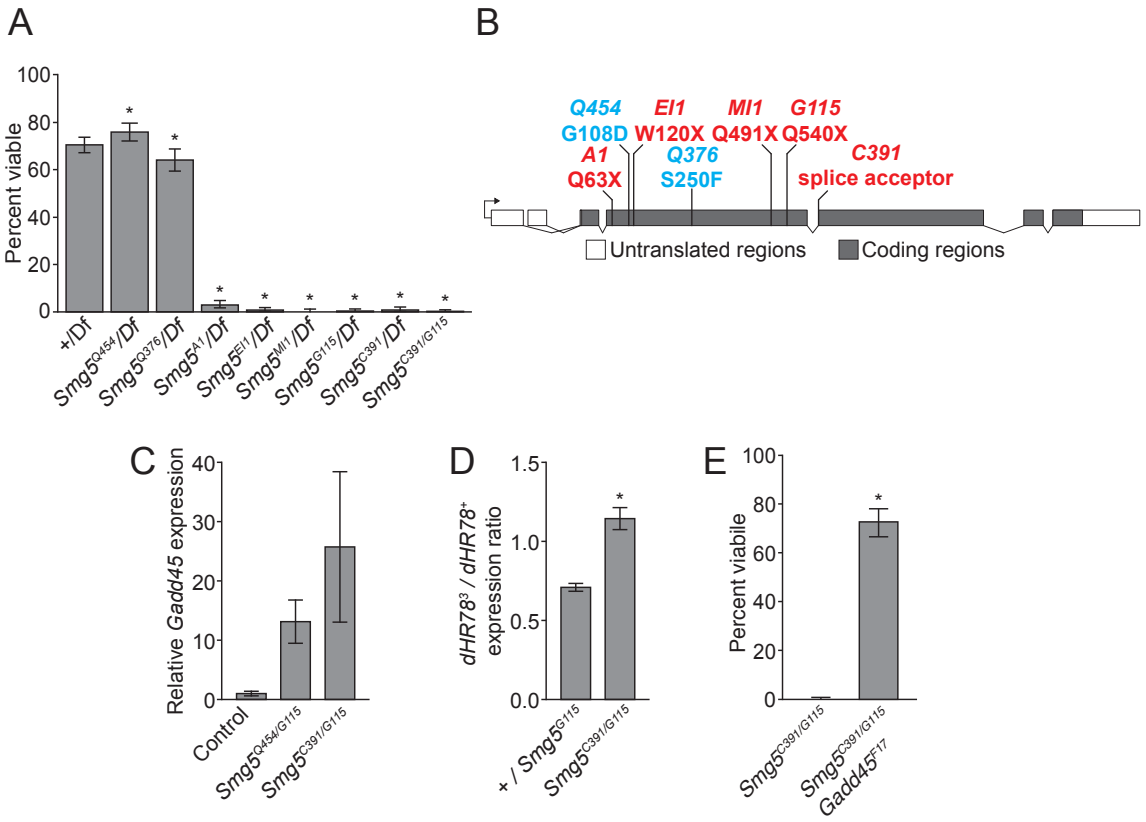
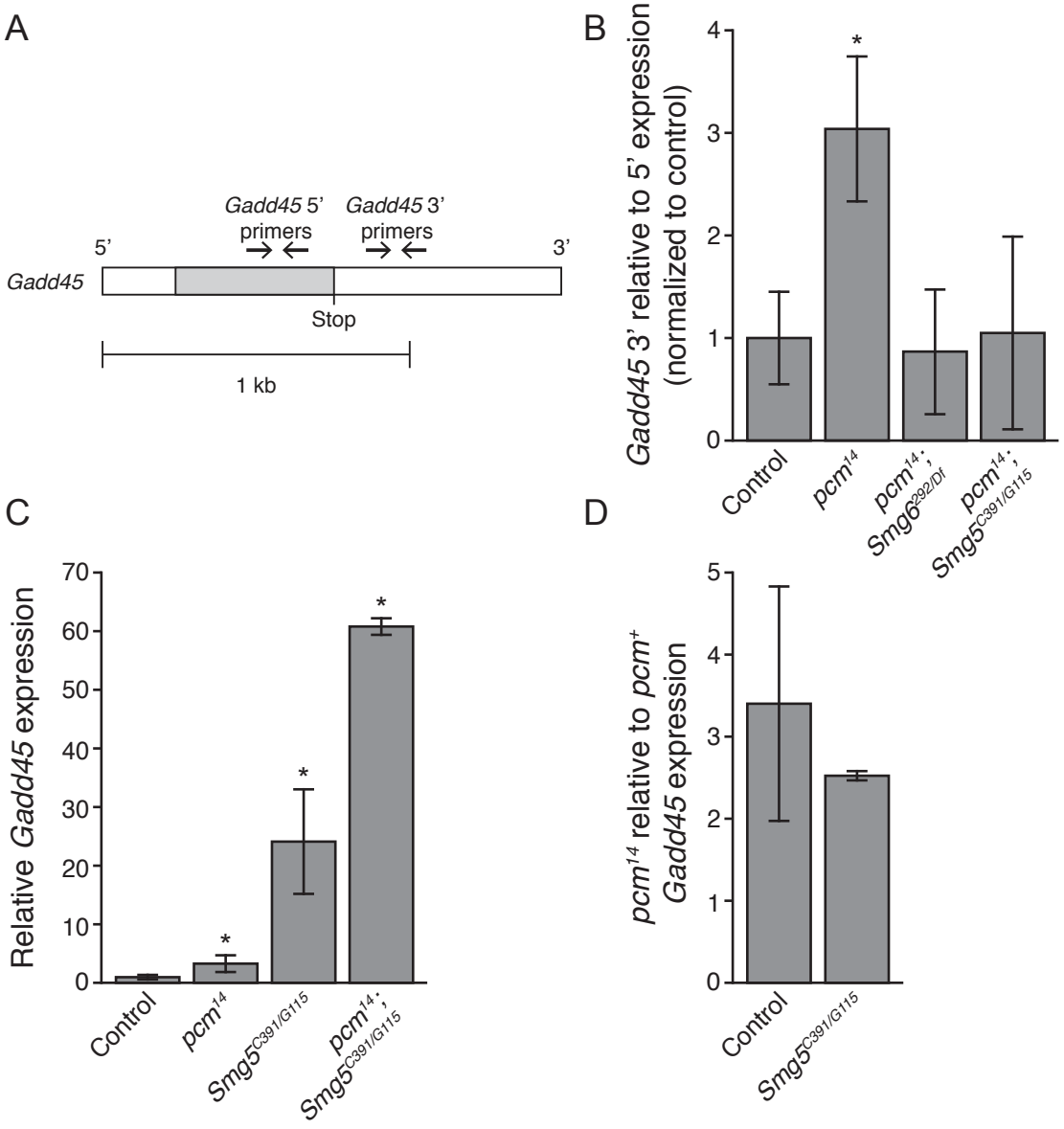


Figure 3.2. *Smg5* is required for viability and all NMD activity in *Drosophila*. (**A**) Adult viability of *Smg5* mutant alleles trans-heterozygous with either a deficiency removing the *Smg5* locus (*Df*) or with two *Smg5* mutant alleles. Error represents 95% confidence interval of the binomial distribution. * indicates $p < 0.05$ compared to + / *Df* condition determined by the test of equal or given proportions. (**B**) Diagram of isolated *Smg5* mutations. Four alleles (*A1, EI1, MI1,* and *G115*) are nonsense mutations. *C391* is a mutation in a splice acceptor site. *Q454* and *Q376* are missense mutations. Red represents loss-of-function alleles; blue represents hypomorphic alleles. Amino acid change is listed under allele names. (**C**) Expression of the endogenous NMD target *Gadd45*, as measure by qRT-PCR. Error bars represent 2 SEM. (**D**) Relative abundance of PTC-containing $dHR78^{3}$ (Fisk and Thummel, 1998) allele mRNA compared to wild-type *dHR78* allele mRNA abundance in animals heterozygous for $dHR78^{3}$ in each indicated genotype. Error represents 2 SEM. * indicates $p < 0.05$ compared to + / $Smg5^{G115}$ condition determined by two-sided Student's t-test. (**E**) Adult viability of $Smg5^{C391/G115}$ and $Smg5^{C391/G115}$ $Gadd45^{F17}$ mutants. Loss of *Gadd45* suppresses *Smg5* mutant lethality. Error represents 95% confidence interval of the binomial distribution. * indicates $p < 10^{-16}$ compared to $Smg5^{C391/G115}$ condition determined by the test of equal or given proportions.

Figure 3.3. *Smg5* is required for endonucleolytic cleavage, but not decapping, of the endogenous NMD target *Gadd45*. (**A**) Diagram of *Gadd45* transcript and 5' and 3' q-PCR primer pairs. Open boxes indicate UTRs; grey boxes indicate coding regions. 5' primer pair is located 5' to the translation stop site and 3' primer pair is 3' to the stop site. (**B**) *Gadd45* 3' primer pair expression relative to 5' primer pair expression in indicated genotypes. The 3' region is preferentially stabilized in pcm^{14} mutants, but this preferential stabilization is lost when either *Smg6* or *Smg5* are lost. Error bars represent 2 SEM. * indicates $p < 0.05$ compared to control condition determined by two-sided Student's t-test. (**C**) Expression of the endogenous NMD target *Gadd45* measured by q-PCR using the 5' primer pair in indicated genotypes, normalized to the expression in the control condition. Error bars represent 2 SEM. * indicates $p < 0.05$ compared to control condition determined by two-sided Student's t-test. (**D**) Relative expression of *Gadd45* in pcm^{14} mutants compared to pcm^{+} animals in either a control or $Smg5^{C391/G115}$ background, measured by q-PCR using the 5' primer pair. Error represents 2 SEM.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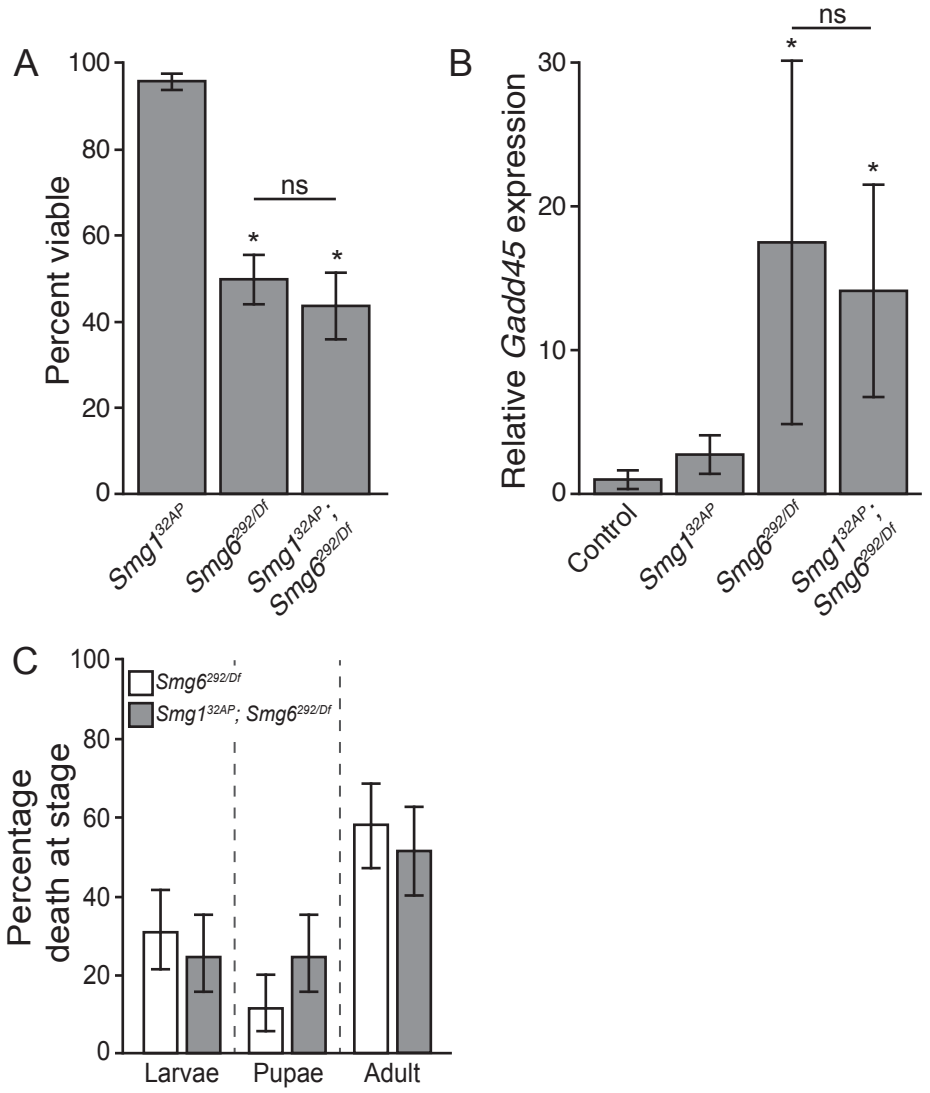
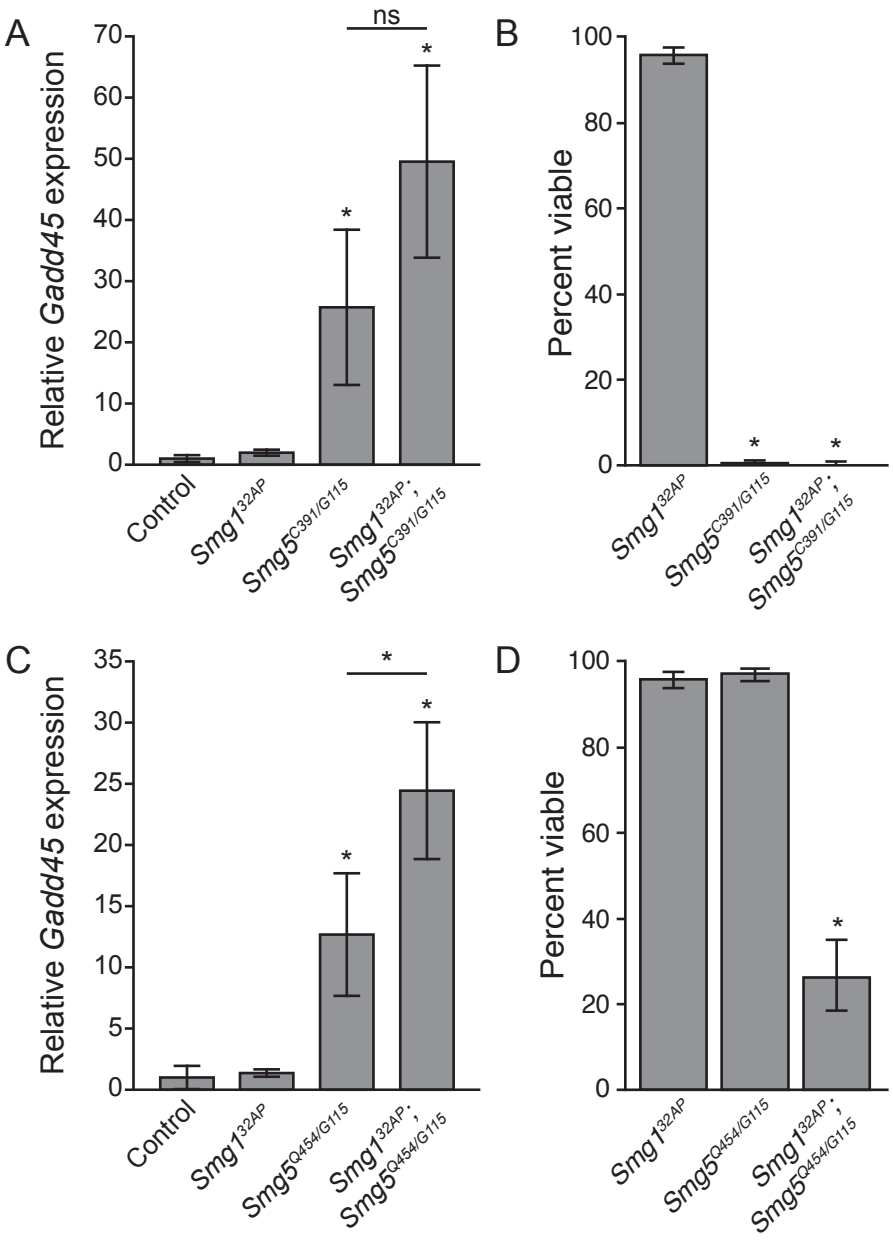


Figure 3.4. *Smg1* NMD function is completely dependent on Smg6 activity. (**A**) Adult viability of *Smg1*^{32AP} and *Smg6*$^{292/Df}$ null mutants, and *Smg1*^{32AP}*; Smg6*$^{292/Df}$ double mutants. Error represents 95% confidence interval of the binomial distribution. * indicates $p < 0.05$ compared to *Smg1*^{32AP} condition determined by the test of equal or given proportions. (**B**) Expression of the endogenous NMD target *Gadd45* in *Smg1*^{32AP} and *Smg6*$^{292/Df}$ null mutants, and *Smg1*^{32AP}*; Smg6*$^{292/Df}$ double mutants. Error represents 2 SEM. * indicates $p < 0.05$ compared to control condition determined by two-sided Student's t-test. (**C**) Percentage of animals that die during larval development, pupal development, or adulthood in *Smg6*$^{292/Df}$ and *Smg1*^{32AP}*; Smg6*$^{292/Df}$ mutants. Error represents 95% confidence interval of the binomial distribution.

Figure 3.5. *Smg1* mutants enhance *Smg5* hypomorph, but not null, mutant defects. (**A**) Expression of the endogenous NMD target *Gadd45* in $Smg1^{32AP}$ and $Smg5^{C391/G115}$ null mutants and $Smg1^{32AP}$; $Smg5^{C391/G115}$ double mutants, as measure by qRT-PCR. $Smg1^{32AP}$ increases the expression of *Gadd45* in $Smg5^{C391/G115}$ mutants. Error bars represent 2 SEM. * indicates $p < 0.05$ compared to control condition determined by two-sided Student's t-test. (**B**) Adult viability of $Smg1^{32AP}$ and $Smg5^{C391/G115}$ null mutants and $Smg1^{32AP}$; $Smg5^{C391/G115}$ double mutants. Error represents 95% confidence interval of the binomial distribution. * indicates $p < 0.05$ compared to $Smg1^{32AP}$ condition determined by the test of equal or given proportions. (**C**) Expression of the endogenous NMD target *Gadd45* in $Smg1^{32AP}$ and $Smg5^{Q454/G115}$ null mutants and $Smg1^{32AP}$; $Smg5^{Q454/G115}$ double mutants, as measure by qRT-PCR. Error bars represent 2 SEM. * indicates $p < 0.05$ compared to control condition determined by two-sided Student's t-test. (**D**) Adult viability of $Smg1^{32AP}$ null mutants, $Smg5^{Q454/G115}$ hypomorphs, and $Smg1^{32AP}$; $Smg5^{Q454/G115}$ double mutants. Error represents 95% confidence interval of the binomial distribution. * indicates $p < 0.05$ compared to $Smg1^{32AP}$ condition determined by the test of equal or given proportions.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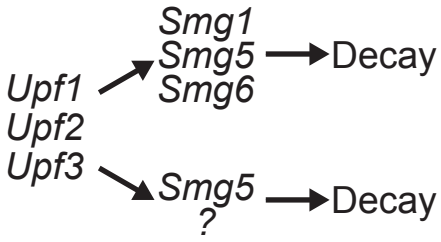

Figure 3.6. Model of branched NMD pathways. Our findings suggest that there are multiple NMD decay mechanisms. We propose that one decay mechanism is Smg6 endonuclease activity, which requires Smg5 for Smg6 to be activated, and Smg1 can enhance, but is not required for, Smg6 function. We propose that Smg5 is also required for a Smg6-independent decay activity. The mechanism for this second decay activity is unknown, but is unlikely to be decapping and 5'-to-3' exonuclease.

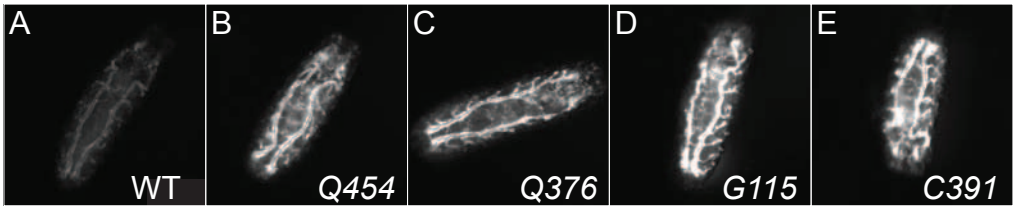

Figure S3.1. *Smg5* mutant alleles have enhanced fluorescence of NMD sensitive GFP reporter in first instar larval trachea. (**A-E)** NMD sensitive *eGFP::sv40 3'UTR* fluorescent reporter is expressed in larval trachea by the *btl-GAL4* driver in control (**A**) and *Smg5* mutant animals isolated in the screen based on Forster et al. (2010) (**B-E**).

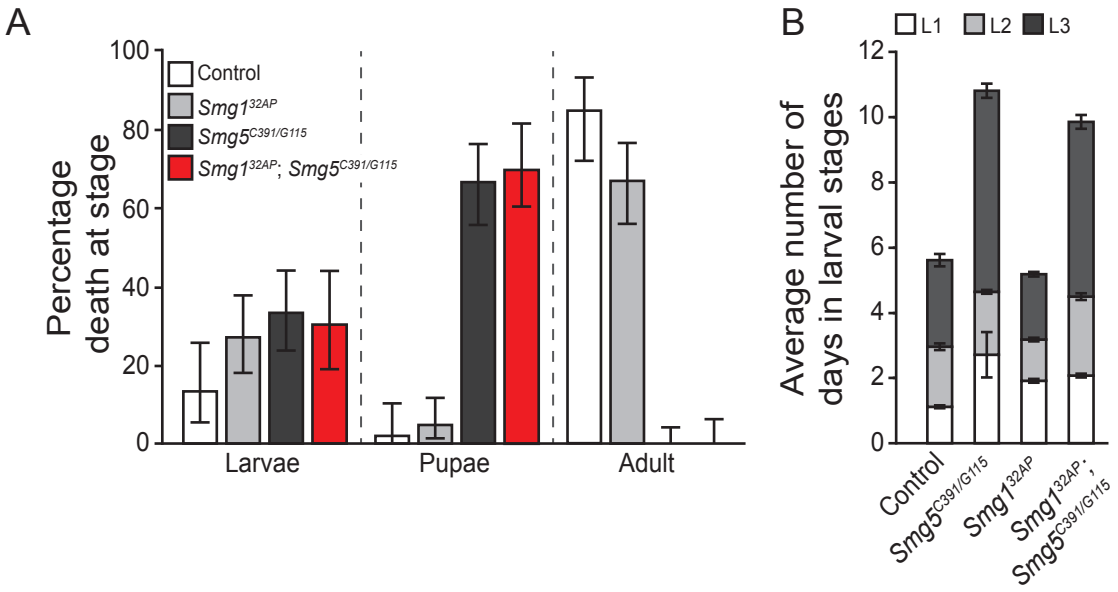

Figure S3.2. *Smg1* mutants do not enhance the developmental delays or stage of lethality of *Smg5* mutants. (**A**) Percentage of animals that die during larval development, pupal development, or adulthood in indicated genotypes. Error represents 95% confidence interval of the binomial distribution. (**B**) Average number of days spent during larval stages of animals that entered pupariation in each indicated genotype. Error bars represent 2 SEM.

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CHAPTER 4

ARC1 AND *ARC2* MRNA POTENTIALLY CONTRIBUTE TO NMD MUTANT LETHALITY

Introduction

The eukaryotic nonsense-mediated mRNA decay (NMD) pathway is an important mechanism to destroy erroneous mRNAs that contain premature termination codons (PTCs). The destruction of PTC-containing mRNAs is a cellular quality-control mechanism that prevents the accumulation of truncated proteins that would otherwise be produced by these mRNAs, which can have detrimental effects in the cell (Celik et al., 2015). In addition to this quality-control function, NMD also degrades many wild-type endogenous mRNAs as a feature of post-transcriptional gene regulation (Peccarelli and Kebaara, 2014). These aspects of NMD are conserved throughout eukaryotes (Conti and Izaurralde, 2005), and the importance of NMD pathway function is highlighted by the requirement of NMD for viability in most complex organisms (Hwang and Maquat, 2011). Until recently, it has been unclear whether the source of the lethality of cells lacking NMD activity is due to loss of the quality-control function or the gene regulatory feature of NMD (Hwang and Maquat, 2011). We have recently identified that a major factor contributing to the lethality of *Drosophila* with mutations in core NMD genes is the stabilization and ensuing increased expression of a single endogenous NMD target, *Gadd45* (Nelson et al., 2016). We also found that *Gadd45* orthologues contribute to

lethality in mammalian cells (Nelson et al., 2016). However, eliminating *Gadd45* expression failed to fully restore viability in either whole *Drosophila* or mammalian cells (Nelson et al., 2016), indicating that there are other factors contributing to NMD mutant lethality.

Upf1 and *Upf2* are key NMD genes and are required for NMD activity. These genes also act in processes other than NMD, such as staufen-mediated decay and translation initiation (Park and Maquat, 2013; Wilkinson, 2005). Defects in these functions may contribute to the residual lethality that occurs when *Gadd45* expression is lost in *Upf1* or *Upf2* mutants. *Smg5* is also an essential NMD factor, and loss of *Gadd45* does not fully restore viability to *Smg5* mutants either (described in Chapter 3). *Smg5* is not known to function in any NMD-independent pathways, but it is possible that an undiscovered NMD-independent function contributes to *Smg5* mutant lethality. However, given that loss of *Gadd45* fails to fully restore viability to multiple different NMD mutants, it is likely that the residual lethality is due to loss of NMD activity itself, and not the individual NMD-independent activity of each of these genes.

The unsuppressed lethality in NMD mutants lacking *Gadd45* is likely due to excess expression of either PTC-containing mRNAs or other endogenous NMD targets. It is very likely additional endogenous NMD targets that influence lethality were missed by the suppression screen used to identify *Gadd45*, since this screen only covered two thirds of the autosomes and none of the X-chromosome (Nelson et al., 2016). Additionally, only two of the three candidate regions identified from that screen contained loci related to increased expression of *Gadd45*, and the reason for suppression by the third region, located on the right arm of the second chromosome, was unidentified

(Nelson et al., 2016). Twenty-four genes have been characterized as endogenous NMD targets that have strongly increased expression in *Drosophila* NMD mutants, one of which is *Gadd45* and two are NMD genes themselves (Chapin et al., 2014). It is possible that one of these 21 remaining genes is the factor accounting for the residual lethality in NMD mutants lacking *Gadd45* expression. Five of these genes are located in regions that did not suppress the lethality of *Upf2* mutants when removed by a deficiency (Chapin et al., 2014; Nelson et al., 2016), and the other 16 reside in untested portions of the genome. Interestingly, the third suppressing region identified in this suppressor screen does not contain any previously characterized endogenous NMD targets, so it is possible the genes responsible for the residual lethality may be unidentified endogenous NMD targets. Additionally, since *Gadd45* is responsible for the majority of the death of NMD mutants, elimination of those factors contributing to the residual lethality may have little or no effect on the viability of NMD mutant on their own. This possibility means that genes that have little or no effect to suppress the lethality of NMD mutants when lost might have a stronger effect when eliminated from an NMD mutant lacking *Gadd45*, so such genes must also be considered.

Here we describe the characterization of candidate loci that may contribute to the lethality of *Drosophila* NMD mutants located within the third region identified from the deficiency suppressor screen we performed previously. We identify two genes, *Arc1* and *Arc2*, that are deleted by the deficiencies that suppress lethality in the uncharacterized candidate region and have increased expression in NMD mutants. Interestingly, these genes are orthologues of a well-characterized mammalian endogenous NMD target, *activity-regulated cytoskeleton-associated protein* (*Arc*) (Giorgi et al., 2007). While

these genes have little effect on NMD mutant viability themselves, they may be responsible for the residual lethality in NMD mutants lacking *Gadd45*. We also found these genes have promoter variation that may influence their expression in response to NMD activity. These findings will help identify the contribution of other NMD regulated genes to the viability of *Drosophila* lacking NMD function, and will help to determine if only endogenous NMD targets contribute to NMD mutant lethality, or if stabilized PTC-containing mRNAs are also responsible for this death.

Methods

Fly genetics

Drosophila melanogaster stocks were raised on standard cornmeal/dextrose food at 25°. The NMD mutant alleles $Upf2^{25G}$ and $Smg1^{32AP}$ (Frizzell et al., 2012; Metzstein and Krasnow, 2006) are on *y w* FRT^{19A} chromosomes. $Upf2^{25G}$ is balanced over *FM7i*, *P{ActGFP}JMR3* (Reichhart and Ferrandon, 1998). $Arc1^{esm18}$ and $Arc1^{esm113}$ mutant alleles and the $Arc1^{esm115}$ precise excision control line (Mattaliano et al., 2007) were provided by Leslie Griffith. The $Arc2^{JON80}$ mutant and $Arc2^{JON5}$ precise excision control were isolated as described below. *y w* FRT^{19A} was used as a control chromosome for all experiments.

For viability tests, we mated flies for three days and collected all progeny each day for 10 days, starting 10 days after the cross was initiated. The total numbers of F1 mutant and balancer males were scored, and the ratio of mutant males to balancer males was used to determine mutant animal viability.

Deficiency suppressor screen

We screened autosomal deficiencies from the DrosDel collection (Ryder et al., 2007). Deficiencies on chromosome 2 were balanced over *CyO*. We mated males from each deficiency stock to *y w* $Upf2^{25G}$ FRT^{19A}*/FM7i, P{ActGFP}JMR3* females and scored all F1 males for the presence or absence of each balancer. For any given deficiency tested, the percentage of *Deficiency* / + males that are $Upf2^{25G}$ mutants, less the percentage of *CyO* / + males that are $Upf2^{25G}$ mutants was calculated, producing a Deficiency Suppression Score (DSS), which represents the effect of an individual deficiency on the increase or decrease in $Upf2^{25G}$ viability, while controlling for each deficiency's general influence on viability. A DSS greater than 0.1 indicates suppression of lethality. Supplemental deficiencies used were from the Exelixis collection (Parks et al., 2004). Deficiency mapping to the *Drosophila* genome was performed using the 5.1 genome release.

RNA isolation and quantification

For qRT-PCR analyses, we collected five to ten adult *Drosophila* frozen in liquid nitrogen. We isolated total RNA using TRIzol reagent (Invitrogen) and phase-lock tubes (5-Prime), and the RNeasy mini kit (QIAGEN). We used on-column RNase-free DNase treatment (QIAGEN) to reduce genomic contamination. We determined RNA concentration by spectrophotometer and normalized concentration for reverse transcription. For reverse transcription, we used random decamers and MMLV8 reverse transcriptase (Retroscript Kit, Ambion). We performed qRT-PCR analysis using the SYBR Green qPCR Supermix (Bio-Rad) and the Bio-Rad iCycler thermocycler. All experimental reactions were performed using three technical replicates and a minimum

of three biological replicates per condition, and the expression level of all experimental assays was normalized to *RpL32* mRNA expression.

For all qRT-PCR analyses, we also measured samples that had been made without reverse transcriptase to ensure that signal was not due to genomic DNA. Primer sequences used were RpL32_1 (ATGCTAAGCTGTCGCACAAA), RpL32_2 (CGATGTTGGGGCATCAGATAC), Arc1_F (CTGGCCATACCGTAGAACAGC), Arc1_R (TGTAGAAGGTATCAGCGACGAGA), Arc2_F(CAACTGCACGGTGAGATTCAGT), Arc2_R (GATGCCCTCCACCTCTTTATACG), eGFP_F(AGGACGACGGCAACTACAAGAC), and eGFP_R (AAGTCGATGCCCTTCAGCTC).

***Arc1* 3' UTR and promoter cloning**

We cloned the *UAS-eGFP::Arc1 3' UTR* construct using the primers Arc1_3'UTR_XbaI_F (GAAGTCTAGACACGAGGAGTAGGCGAC) and Arc1_3'UTR_StuI_R (GAAGAGGCCTACGCAGTTTTCCGTTTCTG) to amplify the entire *Arc1* 3' UTR from genomic DNA. We cloned the *Arc1 promoter::eGFP::Act5C 3'UTR* construct using the primers Arc1_prom_SbfI_F (GAAGCCTGCAGGTTGGAGAGCACAGTTTGTGG) and Arc1_prom_XhoI_R (GAAGCTCGAGTTTCGCTGCTGTGTGAG) to amplify the *Arc1* promoter from genomic DNA. PCR fragments were inserted into the Zero Blunt® TOPO® vector (Invitrogen), sequenced to assure fidelity, and digested and cloned into a pUAST-attB eGFP::Actin5c 3'UTR vector using standard cloning procedures with SbfI and XhoI restriction sites to replace the UAS sequence or XbaI and StuI restriction sites to replace

the Actin5c 3'UTR. Plasmids were injected by Genetic Services Inc. (Cambridge, MA) into a stock containing the VK00027 attP site (Venken et al., 2006) for *phiC31* directed integration. We used previously described *UAS::eGFP::Actin5C 3'UTR* animals (Metzstein and Krasnow, 2006; Nelson et al., 2016). Constructs were expressed by the *e22c-GAL4* or *da-GAL4* drivers.

Generation of *Arc2* mutant alleles

We produced P-element excision lines from the $P\{EPgy2\}Arc2^{EY21260}$ P-element insertion line crossed to a *Δ2-3* transposase stock. We mated F1 males containing the P-element and transposase on a *CyO* balancer to *w*; *Tft* / *CyO* females. Cy^{+} *Tft* white-eyed F2 males were then individually mated to *w*; *Tft* / CyO females. We then collected Tft^{+}, *Cy* males and females to create an isogenic stock from each individually mated F2 male. To identify precise excisions, we used the primers Arc2_Test_F (TTATTTAAAATCTGCCCAATAA) and Arc2_Test_R (CCCATCCACCCCATAAAATA) flanking the P-element insert site to amplify a region across the excised P-element. Any detected deletions were subsequently sequenced using these same primers.

***Arc1* promoter variant determination**

We tested for *Arc1* promoter variants by using two PCR tests. The first tests used the primer pair Arc1_prom_SbfI_F and Arc1_prom_XhoI_R, which flank the variant region. In the *Arc3* variant, these primers amplify a 1594 bp fragment and in the *Narc* variant, these primers amplify a 989 bp fragment. The second test used the Narc_F (AGCAGTTTGAAAGCCGGTAA) and Narc_R (CGCATGCAAGCCTCTACA) primer

pair, which are located within the unique *Narc* sequence. This primer pair will not amplify a fragment in the *Arc3* variant, but it will amplify a 161 bp fragment if the *Narc* sequence is present.

To determine which variant is present in the reference sequence for *Drosophila* species related to *D. melanogaster*, we used the unique *Arc3* or *Narc* sequences in a BLAST search to each species genome using the NCBI blastn tool. Whichever search had a higher match to the region between *Arc1* and *Arc2* for each species was determined to be the variant in that genome.

Results

We previously used a series of deficiencies to identify regions of the genome that when deleted suppress the incomplete lethality of the $Upf2^{25G}$ hypomorphic mutant (Nelson et al., 2016). Two of the three regions identified suppressed $Upf2^{25G}$ lethality due to reduced expression of genes involved in the Gadd45/Mekk1 stress response pathway (Nelson et al., 2016). However, the third region, located from 14,360,425 to 14,363,886 on the right arm of the second chromosome **(Figure 4.1A)**, does not contain any loci known to interact with this pathway. Only one gene, *Arc2*, entirely resides within this region, which is deleted by deficiencies that suppress $Upf2^{25G}$ lethality, but is not deleted by any deficiencies that fail to suppress lethality **(Figure 4.1B)**. A second gene, *Tfb1*, is partially located within this region, but it is also partially removed by a deficiency that does not suppress $Upf2^{25G}$ lethality **(Figure 4.1B)**, so it is not considered a candidate.

Arc2 has not been previously characterized as an endogenous NMD target; however, it does have a very strong increase in expression in $Upf2^{25G}$ mutants **(Figure**

4.1C), indicating it may be a direct or secondary NMD target. Interestingly, the gene *Arc1,* which is paralogous to *Arc2* and resides directly next to it **(Figure 4.1B)**, also has increased expression in $Upf2^{25G}$ mutants **(Figure 4.1C)**. All deficiencies that delete *Arc2* and suppress $Upf2^{25G}$ lethality also delete *Arc1*; however, a deficiency that deletes *Arc1* but not *Arc2* does not suppress lethality **(Figure 4.1B)**. Additionally, these deficiencies that suppress $Upf2^{25G}$ mutant lethality and delete *Arc1* and *Arc2* strongly reduce both *Arc1* and *Arc2* expression, in either a wild-type or $Upf2^{25G}$ mutant background **(Figure 4.1C)**. It may be possible that suppression occurs due to the combined reduced expression of both of these genes, and not necessarily the reduction of one or the other. Given that Arc1 and Arc2 are closely related and may have similar function, it is also possible that the suppression occurs due to an overall reduced expression of *Arc* genes, independent of whether *Arc1*, *Arc2,* or a combination of the two has reduced expression. This possibility means that while loss of a single copy of *Arc1* through a heterozygous deficiency may not be sufficient to suppress $Upf2^{25G}$ mutant lethality, complete elimination of *Arc1* expression may be just as effective at suppressing lethality as the reduced expression of *Arc1* and *Arc2* produced by these deficiencies.

The mammalian orthologue of *Arc1* and *Arc2*, *Arc,* is a well-characterized NMD target (Giorgi et al., 2007), suggesting these genes may be targeted by NMD in *Drosophila*. Interestingly, the *Arc1* 3' UTR is longer than the average *Drosophila* 3' UTR, a feature common to many *Drosophila* endogenous NMD targets (Chapin et al., 2014). To directly test if *Arc1* mRNA is targeted by NMD, we attached the *Arc1* 3' UTR to an eGFP reporter expressed in epidermal cells under GAL4-UAS control using the *e22c*-GAL4 driver. If the *Arc1* 3' UTR is targeted by NMD, this construct will have

enhanced *GFP* expression in NMD mutants compared to control animals (Metzstein and Krasnow, 2006; Nelson et al., 2016). We found that *eGFP::Arc1 3'UTR* mRNA expression has a two-fold increase in $Upf2^{25G}$ mutants compared to control animals **(Figure 4.1D)**. Expression of the same eGFP reporter with a 3' UTR that is not targeted by NMD, the *Act5c* 3' UTR (Metzstein and Krasnow, 2006), does not have increased expression in $Upf2^{25G}$ mutations **(Figure 4.1D)**. These results suggest that the *Arc1* 3'UTR is sufficient to induce sensitivity to NMD, indicating *Arc1* mRNA may be directly targeted by NMD. We also wanted to test if NMD influences transcription at the *Arc1* promoter site as an alternative explanation for the large increase in *Arc1* expression in NMD mutants. We cloned the region 991 base pairs upstream of the *Arc1* open reading frame start site (including both promoter and 5' UTR) as the *Arc1* promoter. This region was attached to an *eGFP* reporter with the *Act5c* 3' UTR, so that mRNA stability would not be affected by NMD. If *Arc1* transcription is repressed by NMD activity, then this reporter should have increased *eGFP* expression in an NMD mutant compared to a control animal. We found that this reporter may have very slightly increased *eGFP* mRNA expression in mutants lacking the NMD factor *Smg1* compared to controls **(Figure 4.1E)**, suggesting that *Arc1* transcription may also be influenced by NMD activity. The weak increase in reporter expression may partially be due to *Smg1* being the NMD factor eliminated in this test, since *Smg1* mutants have only a weak increase in the expression of endogenous NMD targets (Chen et al., 2005; Metzstein and Krasnow, 2006). However, the increase in *eGFP* expression was much smaller than the increase of endogenous *Arc1* in these mutants, indicating that this promoter region contributes a small amount to the *Arc1* mRNA increase in NMD mutants. Importantly,

these experiments have only been performed with a single biological replicate, and thus are extremely preliminary and should be repeated to substantiate these conclusions.

To determine if *Arc1* or *Arc2* contribute to the death of NMD mutants, we tested if *Arc1* and *Arc2* mutants suppress the lethality of $Upf2^{25G}$ mutants. We tested heterozygous *Arc1* mutants (Mattaliano et al., 2007) for suppression of $Upf2^{25G}$ mutant lethality, and found that they, like the deficiency that only removed one copy of *Arc1* without reducing *Arc2* expression, did not increase viability compared to controls **(Figure 4.2A)**. We did find that trans-heterozygous $Arc1^{esm18/ems113}$ mutants partially suppressed $Upf2^{25G}$ mutant lethality, increasing viability by ~10% **(Figure 4.2A)**. *Arc2* mutants have not been previously described in the literature, so we isolated a novel mutation of *Arc2* by imprecise excision of the $P\{EPgy2\}Arc2^{EY21260}$ P-element, which is inserted at the 3' end of the *Arc2* coding sequence **(Figure 4.2B)**. This allele, called *JON80,* is a deletion of 1049 base pairs, removing most of the *Arc2* coding region and some of the *Arc2* promoter without affecting any nearby genes **(Figure 4.2B)**. $Arc2^{JON80}$ is lethal as a homozygote, so we could only test heterozygous *Arc2* mutants; however, *Arc2* is not an essential gene, as $Arc2^{JON80}$ over a deficiency that deletes *Arc2* is viable (data not shown). We did not test the suppressing effect of $Arc2^{JON80}$ mutants over a deficiency due to the deficiencies removing *Arc2* already suppressing $Upf2^{25G}$ mutant lethality for reasons that may be independent of *Arc2*. Interestingly, we found that heterozygous $Arc2^{JON80}$ mutants very weakly increase the viability of $Upf2^{25G}$ mutants by ~5% compared to the precise excision control, $Arc2^{JON5}$ **(Figure 4.2C),** suggesting that *Arc2* may also partially contribute to the death of these animals. Together these results indicate overall *Arc* expression, independent of whether it is *Arc1* or *Arc2*, has a minor

impact on the viability of *Upf2*^{25G} mutants. Additionally, it appears that increased *Arc2* expression may have a more significant impact than *Arc1*, because heterozygous *Arc2* mutants can increase *Upf2*^{25G} mutant viability, while only loss of both copies of *Arc1* could increase viability.

Since the deficiencies that uncover *Arc1* and *Arc2* are the only deficiencies in a candidate region identified from our suppressor screen that do not remove a known Gadd45 pathway gene (Nelson et al., 2016), we wanted to test if *Arc1* or *Arc2* potentially act downstream of *Gadd45*. We measured *Arc1* and *Arc2* mRNA expression in *Upf2*^{25G} mutants and *Upf2*^{25G}; *Gadd45*^{F17} double mutants. We found that loss of *Gadd45* partially reduces the expression of *Arc1*, but not *Arc2*, in a *Upf2*^{25G} mutant **(Figure 4.2D)**. This finding suggests that *Arc1* expression is regulated by activity of the *Gadd45* stress response pathway; however, *Arc2* expression is not influenced by *Gadd45*. This difference in the response of *Arc1* and *Arc2* expression to *Gadd45* activity is surprising given that *Arc1* and *Arc2* share the same region of the genome as a promoter, although in different orientations.

The *Arc1*/*Arc2* promoter region itself is a particularly interesting feature. In the reference genome, *Arc1* and *Arc2* are oriented in opposite directions, with a region of 1627 base pairs in between them. This region serves as the promoter for both of these genes. The pseudogene *Arc3*, a duplication of most of the *Arc1* 3' UTR that is not expressed, also resides in this region (Mattaliano et al., 2007), and presumably contributes to the regulation of the transcription of these two genes. Interestingly, when cloning the *Arc1* promoter, we discovered that many *Drosophila* lines have a completely different region between *Arc1* and *Arc2*. This previously uncharacterized region is

shorter than the reference sequence, and completely lacks the *Arc3* pseudogene, instead replacing it with an entirely different sequence that is shorter than *Arc3* and unrelated to any other region of the genome **(Figure 4.3A)**. We termed this novel region "not *Arc*," or "*Narc*." At first we thought perhaps the inclusion of *Arc3* in this region was a potential error in the assembly of the *Drosophila* reference genome; however, when we tested this region from the *Drosophila* line used to assemble the reference genome, we found that it contained *Arc3* and not *Narc* **(Figure 4.3B)**. This difference between the presence of *Arc3* or *Narc* in the region in between *Arc1* and *Arc2* suggests that there may be variation in the *Drosophila melanogaster* population of which promoter sequence is used. We found that among multiple laboratory and wild *Drosophila melanogaster* strains tested, the *Narc* sequence was the only variant identified **(Figure 4.3C)**. The $Upf2^{25G}$ mutant also has the *Narc* variant, so when cloning the *Arc1* promoter, we used the *Narc* sequence instead of *Arc3*. To test if *Arc3* is unique to *Drosophila melanogaster*, we looked at presence of *Narc* or *Arc3* in the promoter of *Arc1* in related *Drosophila* species. We found *Narc* in the reference genome for those species most closely related to *Drosophila melanogaster*, while *Arc3* is found in those species more divergent from *Drosophila melanogaster* **(Figure 4.3D)**. This finding suggests that *Arc3* may be an ancestral variant of the *Arc1* promoter, but that there is variation in which sequence is used in *Drosophila melanogaster* and other closely related species.

Discussion

The regulation of gene expression is an important feature to maintain cellular homeostasis. The nonsense-mediated mRNA decay (NMD) pathway is one of many post-transcriptional gene regulation mechanisms that destroy mRNAs in order to reduce

or eliminate their expression (Adjibade and Mazroui, 2014). NMD degrades erroneous mRNAs that contain premature termination codons (PTCs), as well as many endogenous mRNAs (Kurosaki and Maquat, 2016). Since NMD is required for viability in most complex organisms, it has long been unclear if the requirement for NMD activity is due to a necessity to degrade PTC containing mRNAs or to regulate the expression of those endogenous NMD targets (Hwang and Maquat, 2011). Recently, we identified that *Drosophila* and mammalian cells lacking NMD activity can have their viability partially restored when eliminating a single endogenous NMD target, *Gadd45* (Nelson et al., 2016). However, it has remained unclear if the expression of other endogenous NMD targets accounts for the remaining lethality in these conditions, or if PTC-containing mRNAs are responsible for this death. Here we described how the genes *Arc1* and *Arc2*, which were identified in the same screen that identified *Gadd45* as a suppressor of NMD mutant lethality, are potential factors that may contribute to NMD mutant lethality in *Drosophila*.

The deficiency suppressor screen that identified *Gadd45* pathway genes as suppressors of NMD mutant lethality had one candidate suppressing region that did not contain any known *Gadd45*-related genes (Nelson et al., 2016). Interestingly, this region only contained one gene, *Arc2*. *Arc2*, and the nearby paralogue *Arc1*, which is also deleted by the same deficiencies that suppress NMD mutant lethality, had increased expression in NMD mutants. Although loss of a single copy of *Arc1* alone, either by a deficiency or mutation, is not sufficient to suppress NMD mutant lethality, loss of both copies of *Arc1*, or heterozygous *Arc2* mutants, slightly increased viability. The slight suppression in each of these conditions could be due to individual activity of *Arc1* or

Arc2, or it is possible that combined *Arc* gene expression contributes to the death of NMD mutants. The combined loss of *Arc1* and *Arc2* may have a stronger effect on viability than loss of each individual gene alone, and *Arc1 Arc2* double mutants should be tested for more complete suppression of NMD mutant lethality.

While *Arc1* and *Arc2* have not been characterized as endogenous NMD targets in *Drosophila*, their mammalian orthologue, *Arc*, is a well-described endogenous NMD target (Giorgi et al., 2007). *Arc* is located in the dendritic segments of neurons and functions in activity-dependent synaptic receptor trafficking (Rodríguez et al., 2005; Steward et al., 1998). The regulation of *Arc* mRNA by NMD is thought to be critical for restricting Arc to location and temporally specific activity (Bramham et al., 2008). Interestingly, restoration of NMD activity specifically in the nervous system of *Drosophila Upf2* mutants is able to partially suppress the lethality of these mutants (personal communication, A. Chapin), suggesting NMD may have a key function in the nervous system during *Drosophila* development. Given that mammalian *Arc* functions in the nervous system, its possible that misregulation of *Arc1* and *Arc2* mRNA in the nervous system contributes to the lethality of *Drosophila* NMD mutants. However, *Arc1* has been found to not be necessary for activity-dependent vesicle trafficking in the *Drosophila* neural-muscular junction (Mattaliano et al., 2007), suggesting that the function for the *Arc* genes may not be conserved from *Drosophila* to mammals. On the other hand, *Drosophila* NMD mutants have reduced synaptic vesicle trafficking (Long et al., 2010), which could be the result of excessive *Arc1* or *Arc2* expression. Given that *Drosophila* express two *Arc* paralogues while only one is expressed in mammals, it is possible that these genes have redundant functions, and both would need to be

eliminated to identify a defect in vesicle trafficking in *Drosophila*. The link between *Arc* neuronal function and the potential key role for NMD in the nervous system during development suggests the enticing possibility that over-expression of *Arc1* and *Arc2* specifically in the nervous system contributes to NMD mutant lethality. Interestingly, over-expression of *Arc1* in the nervous system causes a depletion in body fat (Mosher et al., 2015), so NMD mutants may also have depleted body fat, which may contribute to their failure to develop to adulthood. It should be tested if over-expression of *Arc1* and *Arc2* together in the nervous system can induce lethality, and if RNAi knockdown of *Arc1* and *Arc2* expression in this tissue can suppress NMD mutant lethality to determine if *Arc* gene expression specifically in the nervous system contributes to NMD mutant lethality.

Arc1 and *Arc2* have not been previously identified as endogenous NMD targets in *Drosophila*. Our analysis suggests that both *Arc1* transcription and stability may be regulated by NMD. The increase in *Arc1* transcription in NMD mutants may partially be a secondary effect due to increased *Gadd45* mRNA stability, because the increased expression of *Arc1* in $Upf2^{25G}$ mutants is reduced when *Gadd45* is eliminated. The potential for *Gadd45* to increase *Arc1* transcription in NMD mutants should be further examined by testing the expression of the *Arc1 promoter*::*eGFP* reporter in NMD mutants lacking *Gadd45*. *Arc2* should also be tested for being an endogenous NMD target and if transcription of *Arc2* is restricted by NMD activity. We found that *Gadd45* expression does not increase *Arc2* expression, so if *Arc2* is not an endogenous NMD target, it will be important to understand how *Arc2* transcription increases in NMD mutants.

The region between *Arc1* and *Arc2* is likely the promoter for these two genes. In the *Drosophila* reference strain, this region is approximately 1.5 kb and includes a pseudogene, *Arc3*, which is a repeat of most of the *Arc1* sequence and is not expressed. We found that almost all *Drosophila melanogaster* strains we examined do not have the *Arc3* pseudogene, and this region is instead replaced with an entirely novel sequence that is ~600 bp shorter than *Arc3*. This novel region, which we called *Narc*, is also found in the reference sequence of the four *Drosophila* species most closely related to *D. melanogaster*, while less related *Drosophila* species have *Arc3* at this location in their genome. The variation at the location of the likely *Arc1* and *Arc2* promoter suggests that there could be variation in the expression pattern of these genes between these strains. Additionally, the potential influence NMD may have on the transcription of these genes could be different depending on which variant promoter is present. It will be important to determine the impact on the expression pattern that these variant promoter regions have on *Arc1* and *Arc2* in both an NMD^+ and NMD^- context. More strains of the other related *Drosophila* species should be tested for this variant to determine if these strains also have variation at this region like *D. melanogaster*, or if these species only have one version of the *Arc1* and *Arc2* promoter. The potential of transcriptional differences between these promoters and standing variation of these promoters across multiple *Drosophila* species may reveal an interesting evolutionary phenomenon. It is possible that the degradation of *Arc1* and *Arc2* mRNA by NMD is a more important regulator of their expression than transcription, allowing for variant promoters to exist.

Here we uncovered two potential endogenous NMD targets, *Arc1* and *Arc2*, that may be contributing to lethality in NMD mutants. The increased expression of these

genes is partially dependent on *Gadd45* expression, but most of their expression appears to be independent of this pathway. It is possible then that *Arc1* and *Arc2* may be responsible for the residual lethality that occurs in NMD mutants lacking *Gadd45*, as opposed to being entirely dependent on *Gadd45* activity. These mutants should be tested for their capacity to increase the viability of NMD mutants lacking *Gadd45* expression. The potential for *Arc1* and *Arc2* to be critical NMD targets needs to be further investigated and validated, but they currently are promising candidates.

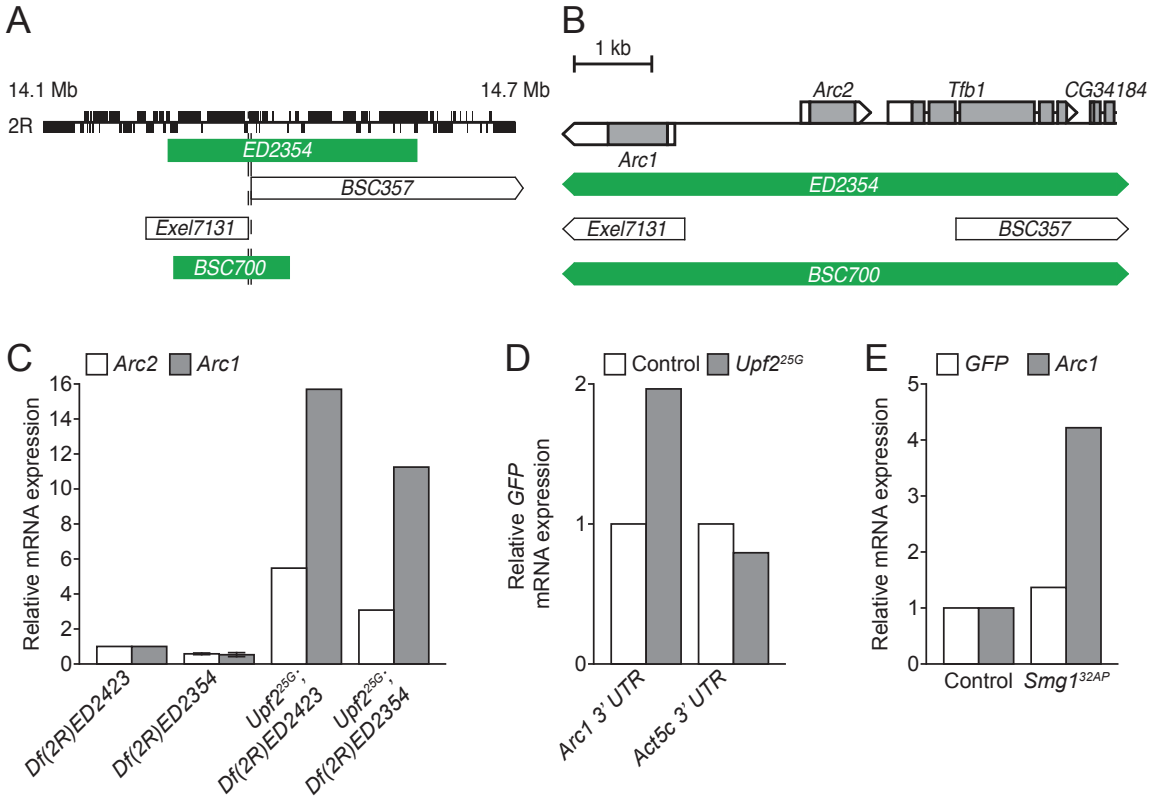
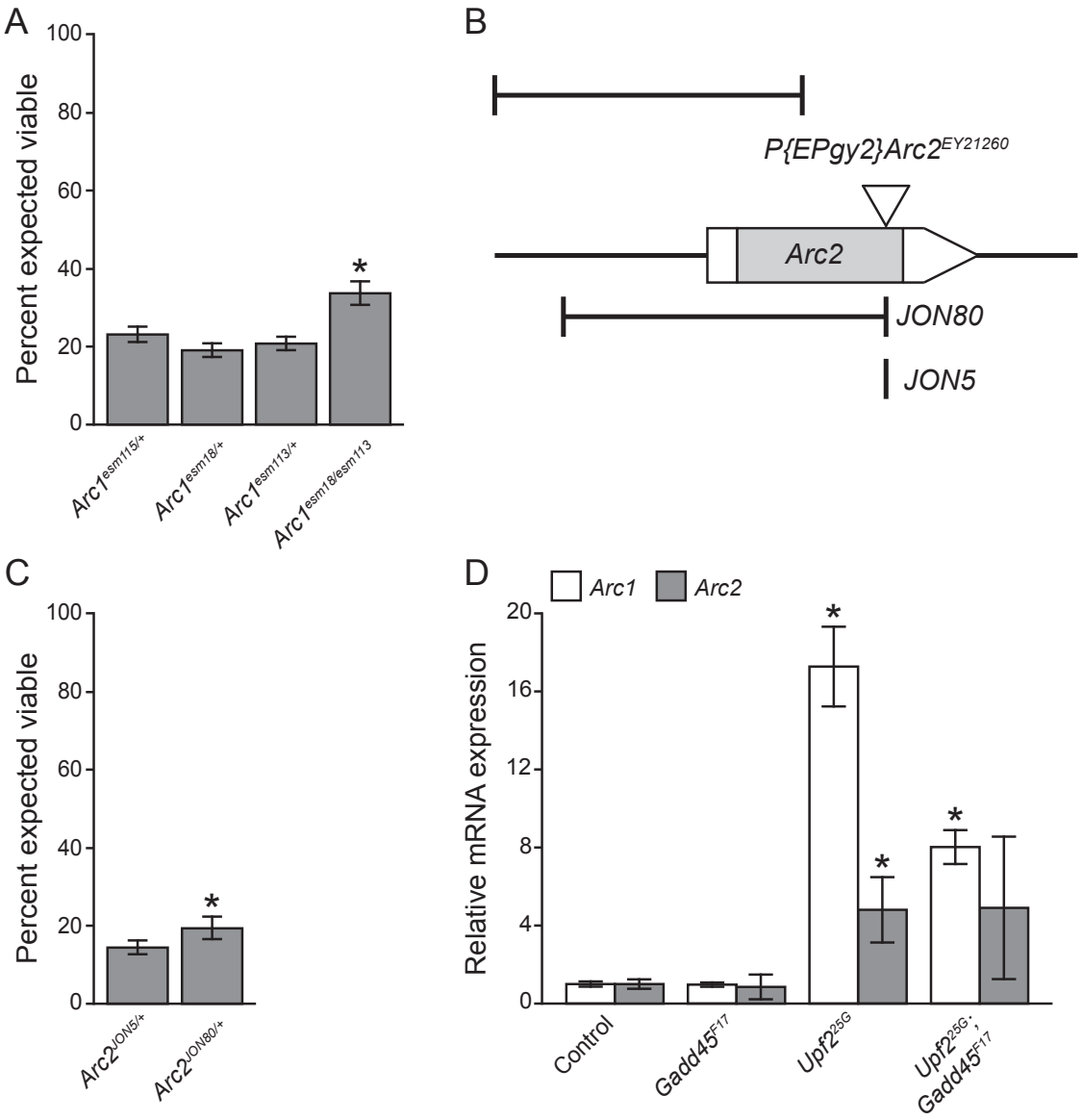


Figure 4.1. *Arc1* and *Arc2* candidate NMD targets that mediate NMD mutant lethality. (**A**) The region from 14,360,425 to 14,363,886 on the right arm of the second chromosome is removed by deficiencies that suppress *Upf2*^{25G} lethality (Green) but not any deficiencies that fail to suppress *Upf2*^{25G} mutant lethality (White) from Nelson et al., 2016. Black boxes on chromosome above deficiencies indicate gene locations. (**B**) Smaller scale representation of the 3.4 kb candidate suppressing region. White boxes indicate untranslated regions; gray boxes represent coding region; line between boxes represents introns; arrow head indicates direction of transcription. (**C**) *Arc1* and *Arc2* mRNA expression, measured by qRT-PCR, in indicated genotypes. *Df(2R)ED2354* is a deficiency that deletes *Arc1* and *Arc2* and suppresses *Upf2*^{25G} mutant lethality. *Df(2R)2423* is a control deficiency that does not delete *Arc1* or *Arc2* and does not suppress *Upf2*^{25G} mutant lethality, but has an identical background to *Df(2R)ED2354*. All mRNA expression is normalized to expression in the *Df(2R)ED2423* condition. Error bars represent 1 SD. *Upf2*^{25G}; *Df(2R)ED2423* and *Upf2*^{25G}; *Df(2R)ED2354* conditions do not have error bars because they represent a single replicate. (**D**) Relative *GFP* mRNA expression in control and *Upf2*^{25G} mutant animals expressing the *GFP* coding sequence with either the *Arc1 3'UTR* or the *Act5c 3'UTR* via *e22c-GAL4*. Expression is normalized to the control condition. There are no error bars because each condition represents a single replicate. (**E**) Relative *GFP* and *Arc1* mRNA expression in animals expressing *GFP::Act4c 3'UTR* from the *Arc1* promoter in control or *Smg1*^{32AP} mutant background. Expression for each mRNA is normalized to the control condition. There are no error bars because each condition represents a single replicate.

Figure 4.2. *Arc1* and *Arc2* potentially contribute to NMD mutant lethality. (**A**) Percentage of expected animals that survive to adulthood in $Upf2^{25G}$ mutant animals in the indicated backgrounds. *esm115* is a control allele; *esm18* and *esm113* are *Arc1* mutant alleles (Mattaliano et al., 2007). Error bars represent the 95% confidence interval of the binomial distribution. * indicates $p < 0.05$ between condition and control determined by the test of equal or given proportions. (**B**) Schematic of the $Arc2^{JON80}$ imprecise P-element excision mutant allele. *JON80* is an imprecise excision of the $P\{EPgy2\}Arc2^{EY21260}$ P-element that deletes 1049 bp, removing most of the *Arc2* coding sequence. *JON5* is a precise excision of this P-element. Coding region in gray; untranslated regions in white. (**C**) Percentage of expected animals that survive to adulthood in $Upf2^{25G}$ mutant animals in $Arc2^{JON5/+}$ or $Arc2^{JON80/+}$ backgrounds. Error bars represent the 95% confidence interval of the binomial distribution. * indicates $p < 0.05$ between condition and control determined by the test of equal or given proportions. (**D**) *Arc1* and *Arc2* mRNA expression, measured by qRT-PCR, in $Upf2^{25G}$ mutants and controls with and without *Gadd45*. All expression is normalized to the control condition. Error bars represent 1 SD. * indicates $p < 0.05$ between condition and control determined by two-sided Student's t-test.


A
Percent expected viable
100
80
60
40
20
0
*
Arc1esm115/+
Arc1esm18/+
Arc1esm113/+
Arc1esm18/esm113
B
P{EPgy2}Arc2EY21260
Arc2
JON80
JON5
C
Percent expected viable
100
80
60
40
20
0
*
Arc2JON5/+
Arc2JON80/+
D
Arc1
Arc2
Relative mRNA expression
20
16
12
8
4
0
*
*
*
Control
Gadd45F17
Upf225G
Upf225G; Gadd45F17

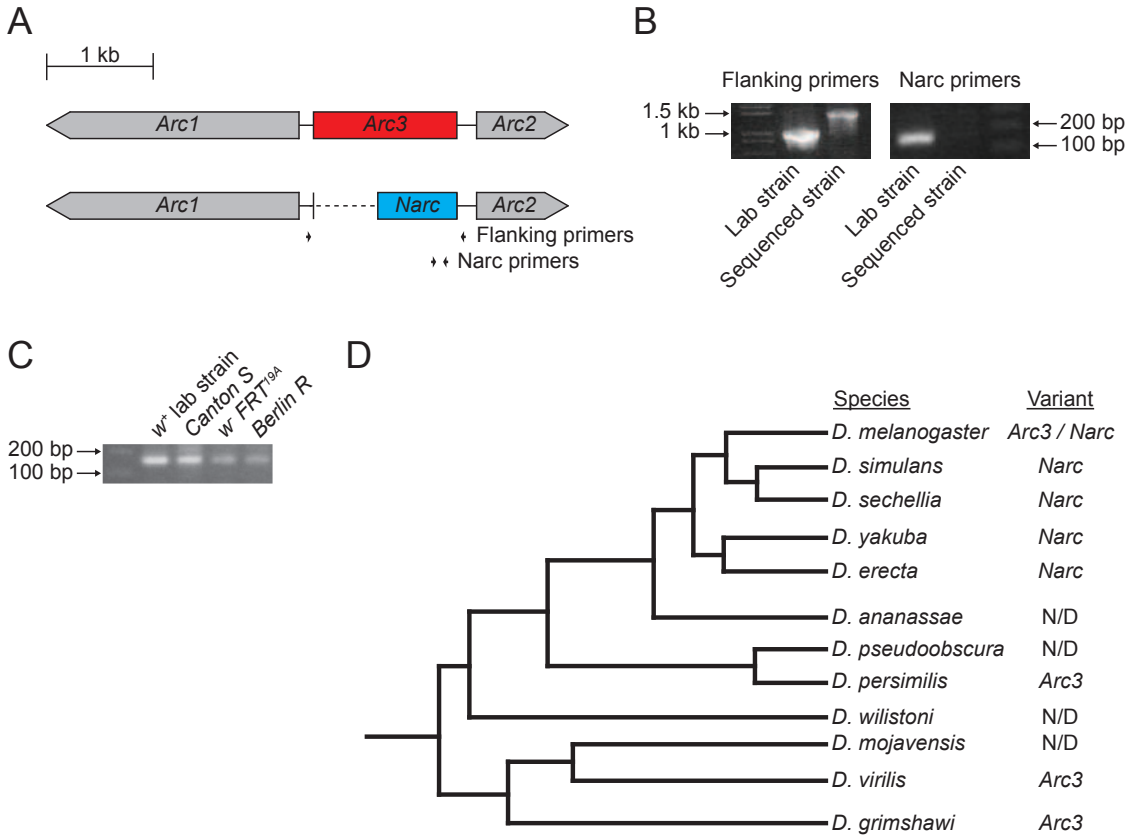


Figure 4.3. *Arc1* and *Arc2* have a variable promoter. (**A**) Schematic of the two variants for the region between *Arc1* and *Arc2*. *Arc3* (red box) is a 1,347 bp region and *Narc* (blue) is 733 bp long. Arrow heads indicate primer pairs used to genotype region for *Arc3* or *Narc*. The Narc primers are specific to the unique *Narc* sequence. (**B**) *Drosophila melanogaster* lab strains contain the *Narc* sequence and the reference sequence strain contains *Arc3* when genotyped with flanking primer pair or *Narc* specific primers. (**C**) *Narc* genotyping in wild and lab *D. melanogaster* strains. All strains tested contain the *Narc* sequence. (**D**) Variant presence in *Drosophila* species related to *D. melanogaster*. Phylogeny according to flybase.org.

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CHAPTER 5

CONCLUSIONS

Nonsense-mediated mRNA decay (NMD) is a critical mRNA degradation pathway that serves dual functions as a cellular quality-control mechanism and a feature of post-transcriptional gene regulation. NMD degrades both erroneous mRNAs that contain premature termination codons -- preventing the expression of potentially harmful truncated proteins (Celik et al., 2015) -- and error-free endogenous mRNAs, thereby silencing their expression (Peccarelli and Kebaara, 2014). While the quality-control feature has generally been focused on as the primary NMD function, NMD influences the expression of approximately 10% of the genome in many species, indicating that it has a significant impact on gene expression (Barberan-Soler et al., 2009; Chapin et al., 2014; Hansen et al., 2009; He et al., 2003; Lewis et al., 2003; Nguyen et al., 2014; Ramani et al., 2009; Rehwinkel, 2005; Tani et al., 2012). Importantly, NMD is required for viability in most complex organisms (Frizzell et al., 2012; Kerényi et al., 2008; Li et al., 2015; McIlwain et al., 2010; Medghalchi et al., 2001; Metzstein and Krasnow, 2006; Thoren et al., 2010; Weischenfeldt et al., 2008; Wittkopp et al., 2009), highlighting the key contributions of this pathway for development and cellular homeostasis. The dual roles of NMD activity and the requirement of this pathway for viability raise two important questions: 1) Why is NMD required for viability? and 2) How does NMD recognize and degrade these two types of targets? The work described in this dissertation

will contribute to understanding these key questions about the NMD pathway. Specifically, this dissertation identifies the relative contribution of the separate NMD functions to the lethality of animals and cells lacking NMD activity, and reveals branched NMD mechanisms for target degradation. These findings uncover new aspects of NMD activity and raise several questions about the role of the NMD pathway in RNA metabolism that prompt future experiments that will be discussed throughout this chapter.

NMD-Mediated Gene Regulation Is Critical for Viability

Chapter 2 of this dissertation addressed understanding the source of lethality in animals and cells lacking NMD activity. We approached this question with the hypothesis that the cause of lethality when NMD is lost is the increased expression, and thus over-activity, of specific endogenous NMD targets that can reduce cell viability. To test our hypothesis, we sought to suppress the incomplete lethality of a *Drosophila* hypomorphic allele of the core NMD factor *Upf2* (Metzstein and Krasnow, 2006) through elimination or reduced expression of individual genes. We identified three regions of the genome that suppressed the lethality of these mutants when deleted, revealing approximately 80 candidate genes that contribute to NMD mutant lethality. One of these genes, *Gadd45*, is an endogenous NMD target that has increased expression in NMD mutants (Chapin et al., 2014). We also identified that *Mekk1*, an orthologue of mammalian MTK1/MEKK4 (Inoue et al., 2001), which is activated by Gadd45 protein in a concentration-dependent manner (Takekawa and Saito, 1998), was located within another suppressing region. We found that elimination of either *Gadd45* or *Mekk1* alone was sufficient to restore viability to animals completely lacking NMD activity, revealing

that increased expression of *Gadd45* is a major factor contributing to NMD mutant lethality. We then established that excessive *Gadd45* is responsible for the ectopic cell death in the developing wing disc of NMD mutant larvae, and that mammalian orthologues of *Gadd45* also contribute to the death of mammalian cells lacking NMD function.

The findings described in Chapter 2 have many important implications for understanding NMD function and potentially developing therapies to treat genetic disease. Since NMD was first discovered to be required for viability, it has been unclear if cells lacking NMD activity die due to loss of the gene regulatory function or the quality control function (Hwang and Maquat, 2011). Our findings indicate that the gene regulatory feature is the key aspect of NMD activity that is essential for viability. NMD mutants lacking the single endogenous target *Gadd45* have restored viability, indicating that the surveillance feature of the NMD pathway is not required for these animals to survive. We also confirmed that PTC-containing mRNAs are fully stabilized in viable NMD mutants lacking *Gadd45* expression, indicating that expression of PTC-containing mRNAs *per se* do not induce cell death. However, some NMD mutants lacking *Gadd45* still die, which may be due to an accumulation of many different or a specific subset of PTC-containing mRNAs in these animals. PTC-containing mRNAs are spontaneously created due to errors that occur in transcription and splicing at a low, but appreciable, rate of around 10^{-4} (Rosenberger and Foskett, 1981; Shaw et al., 2002), meaning random truncated polypeptides are likely being translated in NMD mutant cells. The cumulative expression of multiple truncated proteins, or perhaps the unfortunate production of a single particularly harmful truncated protein, may reduce cellular viability in these

animals. The NMD mutants lacking *Gadd45* may be a useful tool to identify those PTC-containing mRNAs that are tolerable, and the combination or individual PTCs that can induce lethality. *Upf2*; *Gadd45* double mutants could be crossed to a series of *Drosophila* lines containing recessive nonsense mutations, and test which mutations, singly or in combination, induce heterozygous lethality specifically in an NMD mutant background. Characterizing those alleles that are recessive in an animal with functioning NMD but cause dominant lethality in animals lacking NMD will help to understand the detrimental nature of truncated polypeptides. This understanding may potentially be used to develop a method to use sequence alone to predict if a particular nonsense mutation is possibly harmful or benign in the absence of NMD activity.

It is important to identify what PTC-containing mRNAs may be tolerable if stabilized because suppression of NMD is considered a potential therapy for a wide range of recessive genetic diseases (Miller and Pearce, 2014). Recessive loss of function diseases are often the result of both copies of a gene having nonsense mutations, so all mRNA transcribed from those loci are degraded by NMD, causing loss of all gene function. In some cases, such as mutations in *DMD* and *CFTR*, PTC-containing alleles that are not degraded by NMD cause less severe symptoms than those alleles that are degraded by NMD (Kerr et al., 2001; Rowntree and Harris, 2003). Inhibiting NMD in cases where expression of a PTC-containing mRNA may be better than loss of gene function would provide a general therapy for many loss of function diseases, including rare diseases that my not otherwise have therapies established. The lethality of loss of NMD function has blocked development of this potential therapy, but the identification that *Gadd45* as a critical factor causing this lethality suggests that co-inhibition of

Gadd45 with NMD may be a viable treatment. Gadd45 inhibitors already exist (Tornatore et al., 2014), so progress for this potential therapy could be rather quick, and it should be immediately tested. Given that approximately a third of genetic diseases are due to loss-of-function mutations (Khajavi et al., 2006), a general therapy for treating many of these diseases would have a wide impact on medicine.

The discovery that degradation of *Gadd45* mRNA by NMD is required for viability raises an important new question: why would this mechanism to regulate *Gadd45* expression have evolved? There are two likely possibilities that may explain the benefit of this post-transcriptional regulation of a gene that can induce cell death. The first explanation is that this mechanism may limit the amount of protein produced by each *Gadd45* mRNA molecule. NMD is translation-dependent (Popp and Maquat, 2014), so *Gadd45* mRNAs would translate at least one molecule of Gadd45 protein before being degraded by NMD. *Gadd45* is transcribed in response to septic injury in *Drosophila* (Peretz et al., 2007) or a variety of cell stresses in mammalian cells (Papathanasiou et al., 1991; Takekawa and Saito, 1998), initiating a signaling cascade that promotes cell death (Harkin et al., 1999). It is possible that quick degradation of *Gadd45* mRNAs is essential after their translation to ensure an acute response to these stressors, creating a burst of protein synthesis with the burst of transcription. Prolonged translation of Gadd45 may produce a chronic response that would be harmful to the cell. A similar explanation is proposed for the degradation of *Arc* mRNA by NMD in the mammalian nervous system (Bramham et al., 2008). The second possible explanation for why NMD would degrade *Gadd45* mRNA may be that this gene regulatory mechanism would cause a rapid increase in *Gadd45* expression if NMD is inhibited. NMD can

restrict the replication of some viruses (Balistreri et al., 2014), likely because many viral transcripts are polycistronic and thus look like PTC-containing mRNAs, which may be degraded by NMD. To combat the potential anti-viral NMD activity, many viruses have evolved *trans*-acting mechanisms to inhibit NMD function (Mocquet et al., 2012). Regulation of *Gadd45* expression by NMD may serve to act as a "molecular tripwire" that senses this viral activity and causes a quick increase in *Gadd45* expression upon infection to induce cell death and prevent viral replication. Interestingly, NMD degrades the mRNAs encoding a subset of immune-related receptors that can promote cell death in *Arabidopsis*, and this regulation also is thought to function as a similar anti-viral activity (Gloggnitzer et al., 2014). While the specific NMD targets are not conserved in these cases, it is possible that the function of NMD as a molecular sensor to stimulate an immune response upon infection is conserved between plants and animals. These two potential functions for the repression of *Gadd45* expression by NMD are not necessarily mutually exclusive, and both would tie NMD function to innate immune activity. The potential role of NMD and *Gadd45* in the innate immune response of *Drosophila* should be further investigated to test these possibilities and better understand the this regulatory mechanism.

Smg5 Is Required for NMD Targets to Be Degraded by Multiple Independent Decay Mechanisms

Chapter 3 described the first genetic analysis of animals with mutations in multiple NMD factors in a model organism where NMD is required for viability. Most model organisms that require NMD die when any individual NMD gene is eliminated (Frizzell et al., 2012; Kerényi et al., 2008; Li et al., 2015; McIlwain et al., 2010;

Medghalchi et al., 2001; Metzstein and Krasnow, 2006; Thoren et al., 2010; Weischenfeldt et al., 2008; Wittkopp et al., 2009); however, *Drosophila* with mutations in individual NMD genes have variable effects on viability. *Drosophila* can survive when *Upf3*, *Smg1*, or *Smg6* function is eliminated (Avery et al., 2011; Chen et al., 2005; Frizzell et al., 2012; Metzstein and Krasnow, 2006), but loss of *Upf1*, *Upf2*, or, as described in Chapter 3, *Smg5* activity causes the animals to die before reaching adulthood (Frizzell et al., 2012; Metzstein and Krasnow, 2006). This study is the first in-depth genetic analysis of *Smg5* function in any model organism, and identified that *Smg5* is a critical component of the NMD pathway, like *Upf1* and *Upf2*. Importantly, our double mutant analysis revealed that the variability in the requirement of individual NMD genes for *Drosophila* viability is due to there being multiple mechanisms for NMD target degradation. We identified that in addition to the well-characterized Smg6 endonuclease, at least one other mechanism exists to degrade NMD targets, and propose that *Smg5* is required for both of these degradation activities. Additionally, we found that all *Smg1* decay activity requires *Smg6* function, consistent with the models proposed from biochemical analysis of Smg1 (Hug et al., 2016). However, we also identified that *Smg6* likely has *Smg1*-independent activity, providing an *in vivo* context for recent claims that Smg6 can bind Upf1 without Smg1 kinase activity (Chakrabarti et al., 2014; Nicholson et al., 2014). Together, these analyses indicate that, at least in *Drosophila*, the activity of the NMD pathway is much more complex that previously proposed.

This analysis is the first time that multiple NMD mechanisms have been described in *Drosophila*, and the first time that *Smg5* has been implicated in the degradation of NMD targets during native NMD conditions. However, this study is not

the first time that multiple NMD mechanisms have been proposed. Co-depletion of *Smg6* and *Smg7* in HeLa cells has previously been shown to enhance defects in NMD function compared to depletion of *Smg6* alone (Loh et al., 2013; Metze et al., 2013), suggesting independent decay mechanisms exist. However, this additive defect may have been the result of compounding incomplete knockdown of two factors required for the same function. By using true complete loss of function conditions via genetic mutations, we found that additive defects between *Smg1* and *Smg5* hypomorphs confirm that there are multiple NMD mechanisms. The complete set of NMD double mutants should be tested in *Drosophila* for enhanced defects in NMD activity to dissect the distinct activities of each NMD factor and fully understand the multiple decay mechanisms utilized by this pathway.

Smg5 and the paralogous *Smg7* (which is not encoded for in the *Drosophila* genome) have been implicated to potentially recruit both decapping and deadenylation machinery to NMD targets to promote exonucleolytic decay, but it is unclear if these functions occur during native NMD activity (Cho et al., 2013; Loh et al., 2013). In HeLa cells, Smg5 can bind PNRC2, which bridges an interaction with Dcp1a (Cho et al., 2013; 2009), a member of the DCP decapping complex. Artificial tethering of Smg5 to an mRNA can elicit RNA degradation, but depletion of PNRC2 prevents Smg5-tethered mRNAs from being degraded (Cho et al., 2013), suggesting Smg5 interacting with PNRC2, and thus Dcp1a, is required for Smg5-dependent decay. These findings suggest that Smg5 may recruit a decapping complex to NMD targets to initiate 5'-to-3' exonuclease activity in mammalian and potentially *Drosophila* cells. However, we found that the decay activity of the sole cytoplasmic 5'-to-3' exonuclease Xrn1 (encoded by

pcm) is completely independent from *Smg5* function in *Drosophila*, indicating that Smg5 does not recruit any decapping complex in the fly. This finding does not mean that NMD-mediated decapping does not occur in *Drosophila*, but simply that Smg5 is not required for this activity. Upf1 directly recruits a decapping complex in both yeast and mammalian cells (Lykke-Andersen, 2002; Tarassov et al., 2008), so it is likely that Upf1 has similar activity in *Drosophila*, and the potential overlap between *Upf1* and *pcm* function should be tested. As an alternative explanation for *Smg5*-dependent decay activity, it has been shown in HeLa cells that Smg7 can directly interact with POP2, a component of the CCR4-NOT deadenylase complex (Loh et al., 2013), potentially promoting deadenylation and subsequent 3'-to-5' exonucleolytic cleavage of NMD targets. *Smg7* is paralogous to *Smg5*, and while the *Drosophila* genome does not encode a *Smg7* orthologue, *Drosophila* Smg5 has many conserved regions with human Smg7 (Chiu et al., 2003; Fukuhara et al., 2005) and may have some of the same interactions. It is then possible that *Drosophila Smg5* may recruit a deadenylation complex to promote NMD target decay. The findings from Chapter 3 suggest that it is likely that Smg5 recruits a deadenylase complex, given that Smg5 does not contribute to 5'-to-3' exonuclease decay, but is necessary for some endonuclease-independent decay mechanism. Double mutants for *Smg5* and *POP2*, or other factors essential for deadenylation or the cytoplasmic 3'-to-5' exonuclease, should be tested for enhanced defects in NMD activity to determine if this potential mechanism occurs *in vivo* in *Drosophila*.

The potential for multiple NMD mechanisms described in Chapter 3 opens the possibility that there is preference for one mechanism over the other in a target-

dependent context. Depletion of individual NMD factors has varying impacts on NMD targets containing or lacking an exon-junction complex (EJC) depending on which factor is removed (Metze et al., 2013), suggesting EJC presence may be a signal to prefer one decay mechanism over another. Additionally, analysis in human cells identified that Smg6 endonuclease activity is the primary decay mechanism for endogenous NMD targets (Lykke-Andersen et al., 2014), but it is unclear if there is difference in this preference between individual targets or classes of targets. One decay mechanism may preferentially degrade endogenous NMD targets rather than PTC-containing mRNAs, or there may be a difference in which mechanism is used based on the length of the 3' UTR of a target. The null mutant for the lone *Drosophila* cytoplasmic 5'-to-3' exonuclease *pcm* (Till et al., 1998) may be a useful tool to determine if there is variability in the decay mechanism between different endogenous NMD targets. *pcm* mutants have increased stability of the 3' regions of NMD targets cleaved by Smg6 compared to the 5' regions (Nelson et al., 2016). This bias in the abundance of two parts of a single mRNA occurs because Smg6 cleaves the mRNA near the stop codon, and the 5' fragment is degraded by a 3'-to-5' exonuclease, but the 3' fragment is not degraded because *pcm* is removed. The difference in abundance between the 3' region and the 5' region of mRNAs in *pcm* mutants may serve as a readout for Smg6 endonuclease activity. Comparing the stabilization of 3' fragments in *pcm* mutants to the total increase in expression of a given mRNA during complete loss of NMD function would be able to determine the relative contribution of Smg6 endonuclease activity to any given NMD target. For example, if the difference in expression of an mRNA between the 3' region and 5' region in a *pcm* mutant is equal to the difference in expression between complete

loss of NMD activity and a control condition, it suggests that all NMD activity on that mRNA occurs through Smg6. Alternatively, a transcript may have no difference in expression between the 3' region and 5' region in a *pcm* mutant, but has increased expression in NMD mutants, indicating all NMD activity on that mRNA is Smg6-independent. RNA-sequencing analysis in *pcm* mutants, NMD mutants, and controls would be able to make this comparison across all transcripts, and could determine if there is variation in the relative amount of Smg6 activity for any individual NMD targeted gene. Identifying the potential variation in preference for the independent NMD mechanisms would provide a new foundation for understanding the mechanisms of how targets are degraded.

Tissue Specific Regulation of *Arc1* and *Arc2* by NMD May Be Essential for Viability

Chapter 4 described how two related genes, *Arc1* and *Arc2*, may also contribute to the lethality of NMD mutants. These genes both have increased expression in *Upf2* mutants, and heterozygous deficiencies that simultaneously delete both genes partially suppress *Upf2* mutant lethality. *Arc1* and *Arc2* have not been previously characterized as endogenous NMD targets; however, we found that the *Arc1* 3' UTR may be sensitive to NMD. Additionally, we found the increase in expression of *Arc1*, but not *Arc2*, in *Upf2* mutants is partially dependent on *Gadd45* expression, suggesting *Arc1* may function downstream of *Gadd45* activity. These findings may indicate that *Arc1* and *Arc2* contribute to the lethality that occurs in some NMD mutants lacking *Gadd45* expression, and would further clarify the source of lethality in animals lacking NMD activity.

Arc1 and *Arc2* are particularly interesting candidates for being endogenous NMD

targets that partially mediate the lethality of NMD mutants because the mammalian orthologue of these genes, *Arc*, is a well-characterized endogenous NMD target (Giorgi et al., 2007). *Arc* functions in synaptic vesicle trafficking in neuronal cells (Rodríguez et al., 2005; Steward et al., 1998), and restoration of NMD in the *Drosophila* nervous system is sufficient to partially suppress the lethality of NMD mutants (Personal Communication, A. Chapin). An enticing possibility is that increased expression of *Arc1* and *Arc2* specifically in the nervous system may be a source of lethality in *Drosophila* NMD mutants. However, loss of *Arc1* was found to not have an effect on synaptic vesicle trafficking in the *Drosophila* neural-muscular junction (Mattaliano et al., 2007), suggesting *Arc* gene function is not conserved in these species. It is possible that *Arc1* and *Arc2* have redundant functions in vesicle trafficking though, and double mutants should be tested for defects in this process. Additionally, while reduced expression of either *Arc1* or *Arc2* had a minor increase in NMD mutant viability, elimination of both *Arc1* and *Arc2* might have a stronger effect on suppression of NMD mutant lethality than loss of either single gene.

The potential that increased expression of *Arc1* and *Arc2* represent tissue-specific consequences of loss of NMD is exciting because it would begin to uncover the tissue specific functions of this ubiquitous mRNA degradation pathway. It is possible that NMD activity may be differently modulated in specific cell types or during certain developmental stages, so uncovering those tissues where NMD is required for viability will be the first step to understanding the potential modulation of NMD function. Interestingly, the response to reduced expression of individual NMD factors varies between cell types in mice (Huang et al., 2011), and NMD activity is endogenously

reduced specifically in developing mouse neuronal cells via expression of a miRNA that targets *Upf1* (Bruno et al., 2011). These reports support the idea that differential modulation in NMD activity exists, at least between tissues. *Arc1* and *Arc2* are enticing possibilities for being additional contributing factors to NMD mutant lethality in *Drosophila*, and the potential that their over-expression in the nervous system induces lethality should be tested. Additionally, neuronal specific reduction in *Arc1* and *Arc2* expression should be tested for partial suppression of NMD mutant lethality to test if there is a tissue-specific effect on viability in these mutants.

Summary

All together, this dissertation describes several advancements to the understanding of the mechanisms and biological roles of the nonsense-mediated mRNA decay pathway. The discovery that the endogenous NMD target *Gadd45* is a major factor causing the lethality of both *Drosophila* and mammalian cells lacking NMD answers a long-standing question in the field. This finding will push the field to further understand and appreciate the gene regulatory role of the NMD pathway, and opens the possibility for NMD inhibition therapies to treat loss-of-function genetic diseases. Identifying that there are multiple NMD mechanisms in *Drosophila* reshapes the model of how the NMD process occurs, and may reveal a new understanding of how to define different NMD targets. The existing model of NMD function has been formed mostly through the characterization of the biochemical interactions that can occur between the NMD factors, but these interactions will need to be re-interpreted through the *in vivo* genetic analysis that has been presented in this dissertation. Additionally, uncovering that *Smg5* is a critical NMD gene required for these branched pathways highlights *Smg5*

as a previously underappreciated NMD factor, which has been of relatively little research focus compared to other NMD genes. There should be a shift to emphasize understanding the role of Smg5 in the NMD mechanism to better characterize this newly revealed crucial factor. These findings will provide the critical foundation for future research to better understand the NMD pathway.

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