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Alternative Splicing Coupled To Nonsense-Mediated Decay in Regulation of Splicing Factor Expression in *Caenorhabditis elegans*

A thesis submitted by

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March 2012

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Abstract

Alternative splicing of pre-mRNA can produce alternative isoforms that contain premature termination codons (PTCs). These PTC-containing messages, if translated, would produce truncated proteins. However, these mRNAs are rapidly degraded by nonsense-mediated decay (NMD), which preferentially degrades PTC-containing transcripts. NMD is thought to act upon PTC-containing isoforms as a type of quality control mechanism to degrade mRNAs with PTCs that arise from mutation, thus preventing their translation. New evidence suggests that not all NMD substrates are efficiently degraded, and it is unknown as to whether these are ever translated. Using the C. elegans model system, our lab has discovered examples of splicing factor transcripts with PTC- containing alternative isoforms that are inefficiently degraded by NMD. We also identified many alternative splicing events that change in NMD mutant strains, even though the alternative products do not contain PTCs. We propose that translation of PTC-containing splicing factor transcripts may produce truncated splicing factors proteins, and these may affect splicing patterns throughout the animal. In order to detect whether truncated splicing factors are indeed expressed, an experimental system was developed to epitope-tag candidate splicing factor genes. An N-terminal FLAG tag was inserted into splicing factor genes, and these were inserted as transgenes in both wild-type and NMD mutant backgrounds. Reverse transcription and PCR were performed to confirm the expected alternative splicing of the transgenes. Immunoblots are being performed to detect whether the truncated proteins are expressed from the transgenes. If expression of the truncated proteins can be detected, it would suggest that the production of truncated splicing factors may be responsible for the global changes in alternative splicing previously observed in NMD mutants.

Introduction

Alternative splicing is the process of splicing together exons from the same pre-mRNA transcript to create multiple different isoforms of mRNA. In the event that an alternative splicing event or a point mutation results in a message that contains a premature termination codon (PTC), a mechanism called nonsense-mediated mRNA decay (NMD) acts to destabilize these messages (Leeds et al., 1991). 35% of alternatively spliced genes produce a PTC-containing isoform in humans (Lewis et al., 2003).

This coupling of alternative splicing to NMD (AS-NMD) can serve as a way to regulate gene expression post-transcriptionally. There is extensive research into the mechanisms of NMD, but the idea of NMD as a path for gene regulation is relatively new and under-researched aspect of NMD (Pan et al., 2006). The developmental regulation of NMD and the varying degrees of NMD efficiency are a poorly understood area of gene expression regulation. However, there is strong evidence suggesting that NMD does not act to degrade all PTC containing messages all the time (Barberan-Soler et al., 2009). The timed and proper expression of genes is required for development and the life of the organism. Any mutations that may affect the ratios of genes expressed at any given moment can tip the balance and cause disease. Many human diseases have been attributed to mutations that cause mistakes in pre-mRNA splicing and alternative splicing, so an improved understanding about how alternative splicing isoform ratios are regulated could change the way we understand human disease.

Premature Termination Codons (PTCs):

Premature termination codons (PTCs) are stop codons that occur more than 55 nucleotides upstream of the last exon-exon junction in the transcript. PTCs are usually the direct

result of a shift in the reading frame of a protein due to additions or deletions in the DNA, or a nonsense mutation that changes a normal codon into a stop codon in the wrong place. Additionally, alternative splicing can also lead to a PTC in the mRNA in two ways. The inclusion or exclusion of an alternative exon can either shift the reading frame of the mRNA to induce a PTC farther downstream of the included exon, or the exon included may already contain a PTC. In humans, 35% of alternatively spliced genes produce a PTC-containing isoform (Lewis et al., 2003).

Nonsense Mediated Decay (NMD):

Nonsense mediated decay (NMD) is a mechanism used by the cell to degrade messenger RNAs that contain a PTC. The NMD machinery identifies mRNAs during the initial pioneer round of translation and targets them for destruction. In *C. elegans*, seven genes were initially identified as required for the NMD pathway to function. These were named SMG1 through SMG7 (Suppressor with Morphological effects on Genitalia), after the abnormally swollen hermaphrodite vulva and male bursa phenotype that the SMG mutants exhibited (Cali et al., 1999; Hodgkin et al., 1989; Pulak and Anderson, 1993). Up to eleven essential NMD factors have now been identified in mammals that have *C. elegans* homologs. (Yamashita et al., 2009; Longman et al., 2007)

NMD has been shown to be essential for development in *Drosophila*—NMD is highly active during development and is required for the proper expression of dozens of genes and for larval viability (Metzstein and Krasnow, 2006). In mammalian embryogenesis, embryos that have mutations in the NMD pathway stop development at day 8.5 (Mcllwain et al., 2010). *C.*

elegans that are defective in NMD are still viable, making this a great animal model in which to study the global effects of loss of NMD.

NMD is a way to degrade aberrant mRNA transcripts that have been mutated to include a PTC. If translated, these PTC-containing transcripts may produce truncated proteins that could compete with the normal proteins, causing great amounts of damage in the cell. NMD is likely important for stopping translation of a mutant allele of a gene in a heterozygous background.

Mechanism of NMD:

After splicing, the exon junction complex (EJC) is deposited on the mRNA 20-24 nucleotides upstream of the exon-exon junction, and stays with the mRNA as it travels into the cytoplasm (Le Hir and Andersen, 2008). In the pioneer round of mRNA translation, the ribosome displaces these EJCs as it translates the message (Dostie and Dreyfuss, al., 2010). There is also evidence that the nuclear cap binding complex proteins that are still bound to the mRNA during the first round of translation get replaced by the eIF4E cap binding complex during bulk translation. Co-immunoprecipitation experiments have shown that EJC components and SMG components are associated with the CBC-containing mRNPs but not with eIF4E-containing mRNPs (Ishigaki et al., 2001). A protein called UPF1 is recruited to the ribosome when it comes across a stop codon by interacting with eRF (eukaryotic ribosome release factor), and UPF 2 and UPF3 associate with the EJC. A complex called SURF (SMG1, UPF1, eRF) assembles at the ribosome at a stop codon. The phosphorylation of UPF1 by SMG1 is thought to be the signal for activating the NMD pathway for a message. The SURF complex interacting with UPF2/3 at the downstream EJC is important for inducing phosphorylation of UPF1.

To degrade the message, SMG 5/6/7 bind to UPF1 and recruit a phosphatase to dephosphorylate UPF1, which signals the mRNA decapping process, followed by exonuclease degradation of the exposed mRNA.

Alternative Splicing Coupled to Nonsense Mediated Decay (AS-NMD):

Alternative splicing isoforms containing PTCs are degraded by the NMD pathway. It is estimated that, in humans, up to 35% of alternative splicing events generate substrates for NMD. NMD may act as a type of quality control mechanism, to degrade transcripts with PTCs that arose by mutation (Lewis et al, 2003). However, NMD could act post-transcriptionally to regulate gene expression, by changing alternative splicing patterns. If the pre-mRNA becomes spliced to generate more PTC-containing message and less non-PTC-containing message, there would be a higher frequency of transcripts targeted for degradation by the cell and less non-PTC-containing isoform for proteins to be translated from. Essentially, AS-NMD can be used as a regulatory mechanism to turn off gene expression after transcription.

Splicing Factor Expression Regulation and AS-NMD:

Splicing factors are proteins (*trans*-factors) that bind to *cis*-elements in the pre-mRNA to inhibit or promote the inclusion of alternative exons. The pre-mRNA encoding many splicing factors can be alternatively spliced to produce both PTC-containing and non-PTC-containing isoforms, the first of which can be subject to degradation through NMD. Many splicing factors act on their own messages post-transcriptionally by coupling splicing patterns to message stability through NMD as a form of autoregulatory negative feedback (Lareau et al., 2007; Ni et al., 2007; Saltzman et al., 2008). By changing splicing patterns to decrease the production of stable mRNA isoforms and increase the production of mRNAs targeted by NMD for destruction,

splicing factors can cause a decrease in the amount of splicing factor translated. Slight changes in splicing factor concentrations can affect the expression of all genes whose splicing is regulated by the splicing factor (Caceres et al., 1994; Karni et al., 2007). Therefore, the message stability of splicing factors plays an important role in the maintenance and well being of the organism.

If the PTC-containing isoform of the splicing factor mRNA were to be translated, it would result in a truncated protein that would keep some functional domains of the splicing factor and be missing other functional domains. Proteins with SR (Ser/Arg rich) domains select the alternative splice sites to be utilized and compete with other SR proteins for the same sites. RRM (RNA-recognition Motif) domains recognize specific RNA sequences. Truncated splicing factors that retain an RRM domain but lack the SR domain would be able to bind to transcripts in the cell, but would be unable to activate splicing of that pre-mRNA. This could lead to a dominant-negative effect on the cell, since truncated splicing factors with RRM domains intact could compete with their full-length versions for pre-mRNA binding sites, changing splicing patterns throughout the cell. The embryonic lethality observed in other organisms lacking the NMD machinery could be due to dangerous changes in alternative splicing that are a consequence of the increase in the stability and expression of PTC-containing messages of splicing factors.

Varying Degrees of NMD Efficiency and Secondary Targets of NMD

Using a custom DNA microarray assay that can measure the relative steady-state levels of mRNA isoforms in the cytoplasm, the Zahler lab compared the steady-state levels of mRNA in the cytoplasm of wild-type worms and NMD-mutants (each defective in a different SMG gene). The relative amounts of each mRNA isoform measured reflect a combination of the rate at which

alternative splicing produces these isoforms and the relative stabilities of each mRNA isoform in the cytoplasm. Out of 352 alternative splicing events, 21% showed a greater than 2-fold change in steady-state levels of alternative isoforms in the NMD deficient background. 59% of the alternative splicing events that showed a change with NMD absent were not predicted targets of NMD; meaning that these alternative isoforms did not contain PTCs and shared the same stop codon as their partner isoforms (Barberan-Soler et al., 2009). Since splicing factor pre-mRNAs can be spliced to include a PTC, this group of proteins would be considered primary targets of NMD. The loss of the ability to degrade PTC-containing isoforms of splicing factors in NMD mutants could affect the relative ratios of secondary alternative splicing events that are not direct targets for NMD. If anything, the large proportion of secondary splicing events affected by the loss of NMD emphasizes the effect that a change in the stability of splicing factor isoforms due to loss of NMD could have on global alternative splicing. We hypothesize that changes in splicing factor isoform message abundace in NMD mutants, and the subsequent translation of stablized mRNAs containing PTCs leads to production of truncated, partially functional proteins. These truncated splicing factors in turn can have downstream effects on the alternative splicing patterns of many genes.

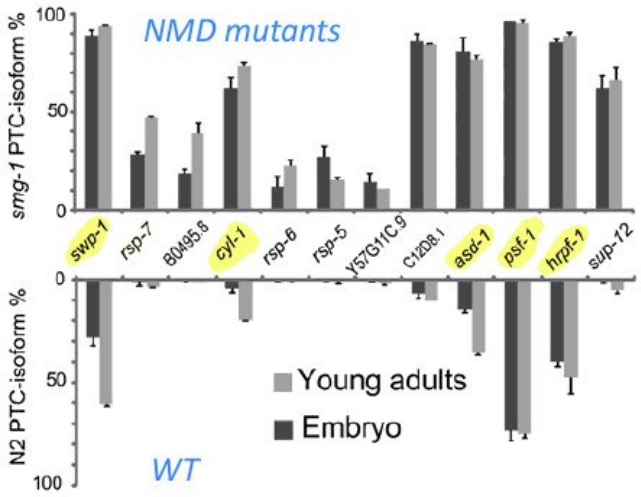

Figure 1. Developmental regulation of AS-NMD. RT-PCR comparison of wild-type (N2) and *smg-1* NMD mutant worms for two developmental time points (embryo and young adults) for 12 splicing factors identified as regulated by AS-NMD. *swp-1*, *cyl-1*, *asd-1*, *psf-1* and *hrpf-1* show inefficient degradation of PTC-containing isoforms. *swp-1*, *cyl-1* and *asd-1* show developmental regulation of NMD degradation efficiency.

Barberan-Soler et al. 2009

For 12 splicing factor genes that can be spliced to generate mRNA substrates for NMD,

RT-PCR was used to compare the relative levels of the PTC containing and non-PTC isoforms in wild-type vs. NMD mutant strains, and in embryos vs. adults (Figure 1) (Barberan-Soler et al., 2009). For 6 of the 12 splicing factor genes, it was found that their PTC-containing isoforms were very efficiently degraded, such that they are barely detectable in the wild-type worms, but are present in up to 60% of the messages in the NMD mutant strains (for example, *rsp-7* and *sup-12*). This reveals that these pre-mRNAs are spliced 60% of the time in a way that produces a PTC-containing isoform, but that isoform is almost completely degraded in wild-type worms by the NMD machinery. For 5 of the 12 splicing factor genes, there was inefficient degradation of PTC-containing isoform in wild-type worms (yellow highlights), showing for the first time that NMD is not an all-or-nothing event—there are varying degrees of NMD efficiency. There are also examples of developmental regulation in at least 3 of the 12 splicing factor genes (for example, *swp-1*, *cyl-1*, *asd-1*). Each of these PTC-containing isoforms is present in NMD mutants in both embryos and young adults in similar amounts, but are present in much lower amounts in wild-type embryos versus wild-type young adults. Since the lack of NMD reveals similar splice site choice in the nucleus between embryos and young adults, the change in steady-state isoform ratios in the wild-type worms from the NMD mutants must be due to the fact that NMD efficiency is much higher in embyros, since not as much PTC-containing isoform was detected, and NMD gets less efficient as the worm develops into a young adult.

The goal of this project is to test the hypothesis that changes in alternative splicing seen in an NMD mutant background are the result of translation of truncated forms of splicing factor proteins whose messages are normally degraded by NMD. To do this, we created 5 different epitope tagged transgenes of splicing factors regulated by alternative splicing coupled to NMD and tested whether the PTC-containing isoforms of these factors are indeed translated in NMD

mutant backgrounds. If the truncated proteins are present in wild-type worms, then it would suggest that inefficient NMD of these factors plays a role in splicing regulation. If truncated proteins are expressed in the NMD mutants at high levels, it suggests that the production of truncated splicing factors may be responsible for the global changes in alternative splicing seen in the microarray performed by Barberan-Soler et al., (2009). Truncated splicing factor proteins that retain an RNA binding domain and lack a splicing activation domain would be able to compete with their full-length counterparts for pre-mRNA binding sites, affecting splicing patterns throughout the cell.

Specific Aim

My project builds on the prior research done by Barbaren-Soler et al. (2009), to understand the relationship between alternative splicing and nonsense-mediated decay. We will determine whether the PTC containing isoforms encoding 6 different splicing factors are expressed as truncated proteins in either wild-type worms or NMD mutants, and look to understand the downstream effects of their potential translation on global alternative splicing.

Materials and Methods

Polymerase Chain Reaction

Long-range PCR of genomic DNA was performed on six different splicing factor genes that can be alternatively spliced to include a PTC—*hrpf-1*, *cyl-1*, *swp-1*, *psf-1*, *rsp-5*, and *rsp-6*. The PCR primers were designed so that the products include the promoter region of DNA upstream of the start codon, the entire coding region and downstream sequences to the neighboring gene (Table 1). It was important for the experimental design to include the native promoter, and extend down to the end of the gene past any polyadenylation signal. The final PCR products had sizes ranging from 3 Kb to 6.1 Kb in length. A Long-Range PCR Kit (Qiagen) was used for the reactions. We found that adding 5µL MgCl to the 50µL reaction mixture, increasing the extension time by 20 seconds every cycle for the last 28 cycles, and finding the optimum annealing temperature by using a gradient of temperatures in the PCR machine to be helpful, although most of the PCR products are so long that each one required several rounds of troubleshooting to find the best PCR variables for amplification (Table 2).

Cloning, Transformation, and Restriction Digest

PCR products were cloned using a TOPO TA Cloning Kit (Invitrogen), into a pCR 2.1-TOPO Vector (Invitrogen), and transformed into E.coli following the protocols provided by Invitrogen. Ampicillin resistant colonies that were negative for beta galactosidase activity were then grown overnight in LB ampicillin and plasmid DNA was purified using the Qiagen PCR Purification Kit according to manufacturer's instructions. Isolated plasmid DNA was digested with Hind-III, EcoRI, or another appropriate restriction enzyme to confirm that the correct DNA

sequence had been cloned. If the restriction digest looked correct, the plasmids were sent off for sequencing at the Berkeley DNA Sequencing Facility to confirm that the correct PCR product was cloned.

Table 1. Forward and reverse primers used in each PCR reaction are listed together with the expected size of the resulting PCR product.

	Forward Primer	**Reverse Primer**	**PCR Product Size (bp)**
Cyl-1	ATTTTTACATCCGTGGGGCG	AATGTGTTCCGAGCAACAGAA	3,266
Hrpf-1	TGCGAATACCATGGAGAACG	AGTTTGAGTGAACGCTTGGG	4,694
Psf-1	ACTCCGAAAATTTGAGCCCG	CTCGATGCACACAACATTCC	6,209
Rsp-5	CGTGAGACAACTTCATCAGG	GTGTGAATAATACCATGTGC	1,665
Rsp-6	CTTTCAGAGTTGACGACTGC	CTCACAGAGCAAAAAGTCGC	2,154
Swp-1	TGCATTACTCAGGGAAACCC	CCAACTTCACCCACTTTTCG	4,830

Table 2. Details of the PCR reactions that worked for each of the six splicing factor genes *cyl-1*, *hrpf-1*, *psf-1*, *rsp-5*, *rsp-6*, and *swp-1*.

	Initiation	**Denaturing Step**	**Annealing Step**	**Elongation Step**	**Final Elongation**	**+5 µL $MgCl_2$**	**+20sec for last 28 cycles**
Cyl-1	93 ºC, 3min	93 ºC, 15 sec	57 ºC, 30 sec	68 ºC, 4 min	68 ºC, 3min	No	No
Hrpf-1	93º C, 3min	93 ºC, 15 sec	56-59 ºC, 30 sec	68 ºC, 5 min	68 ºC, 3min	No	No
Psf-1	93 ºC, 3min	93 ºC, 20 sec	60 ºC, 30 sec	68 ºC, 6 min	68 ºC, 3min	Yes	Yes
Rsp-5	95 ºC, 2min	95 ºC, 30 sec	55 ºC, 30 sec	72 ºC, 2.5 min	72 ºC, 10min	Yes	No
Rsp-6	95 ºC, 2min	95 ºC, 30 sec	60 ºC, 30 sec	72 ºC, 2.5 min	72 ºC, 10min	Yes	No
Swp-1	93 ºC, 3min	93 ºC, 15 sec	60 ºC, 30 sec	58 ºC, 5 min	68 ºC, 3min	Yes	No

Site-Directed Mutagenesis

The plasmids were mutagenized to insert an N-terminal epitope tag right after the start AUG for each transgene (Figure 2). We chose the FLAG tag because there are commercial antibodies available for it. A PsiI restriction site (TTATAA) was engineered within the FLAG tag without changing the resulting amino acid sequence. This was so we could more easily decipher if our mutagenesis worked by comparing PsiI restriction digests of the plasmid before and after the mutagenesis reaction to see if it was successful before confirming the mutagenesis by sequencing.

Mutagenesis reactions were performed with an XL site-directed mutagenesis kit (Agilent). Primers for mutagenesis contained the FLAG sequence (GAT TAT AAG GAT GAC GAC GAT AAG) flanked by 15 nt of complementary sequence to target insertion of this sequence right after the start codon in the plasmid (Figure 3). Since each mutagenesis primer was approximately 60 nucleotides long, each tube of oligonucleotide synthesized by Integrated DNA Technology (IDT) was resuspended in formamide dye and run on a 10% denaturing acrylamide gel in TBE to separate the primers from excess product at 300V for 1.5 hours. The band of primer was identified by UV shadowing and was cut out of the gel. Primers were purified from the gel band overnight in 0.5M ammonium acetate and the eluted primers were purified by phenol-chloroform extraction and ethanol precipitation.

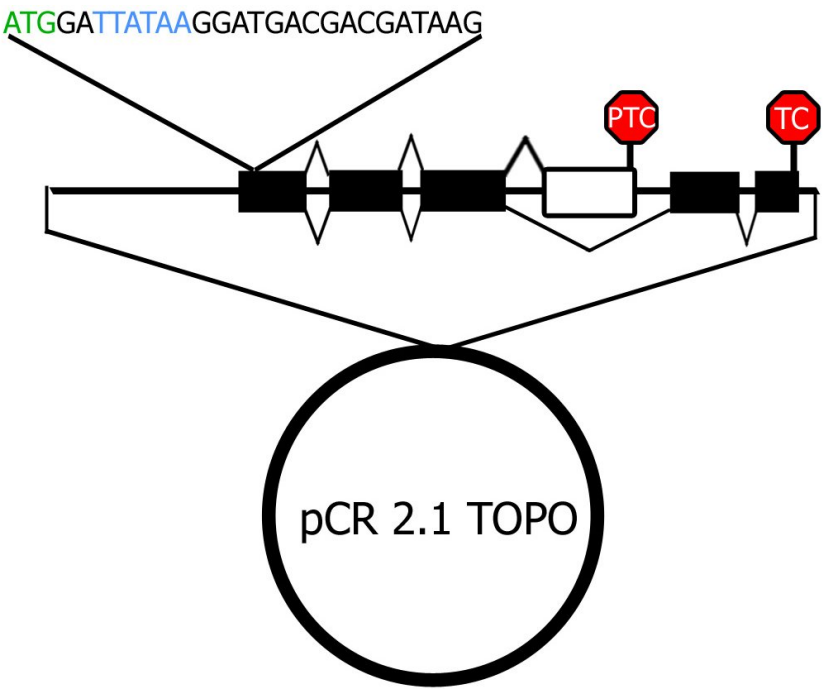

Figure 2. Map of transgenic plasmid created, showing where the PCR product is inserted into pCR 2.1 TOPO TA vector, and location of the N-terminal FLAG tag insert right after the start AUG. Alternative splicing patterns to produce PTC-containing and non-PTC-containing isoforms *rsp-6* are shown. A PsiI restriction site (blue) was engineered into the FLAG sequence without changing the protein coding sequence. This plasmid is representative of plasmids that include the genes *hrpf-1, cyl-1, psf-1, rsp-6* and *rsp-5.* Attempts to mutagenize the plasmid containing *swp-1* were unsuccessful.

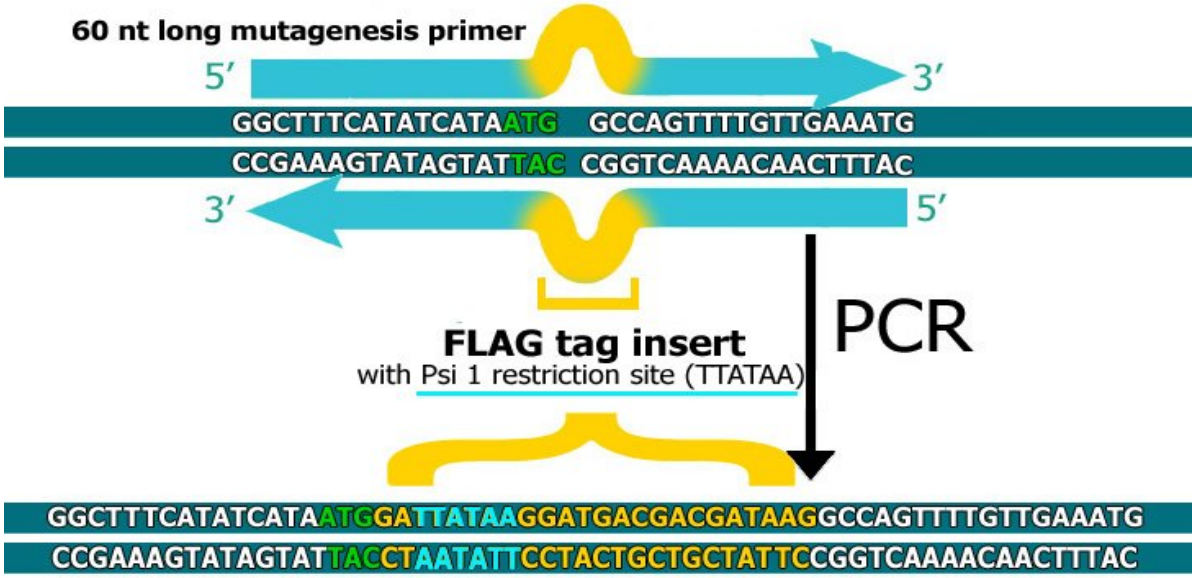

Figure 3. Site-directed mutagenesis primers designed with the FLAG-tag insert (yellow), complementary to the region of the plasmid surrounding the start codon (green). In this example, the plasmid contains *hrpf-1*. A Psi-1 restriction site (cyan) was engineered into the epitope tag. The resulting mutagenesis product is shown.

The approach we took presented two serious challenges to successful site-directed mutagenesis of these plasmids; the plasmids were very large (up to 10 kb in length) and 24nt were inserted by this procedure. Many variables were modified from the original QuickChange XL protocol in order to achieve successful site-directed mutagenesis. Several mutagenesis reaction variables, including denaturation times, elongation times, annealing temperatures, the use of $MgCl_2$ instead of Quick Solution, and the use of Pfu Turbo or Pfu Ultra, were modulated for each gene. Adding 5 µL of $MgCl_2$ instead of Quick Solution, increasing denaturation time, increasing the elongation time to 2.5min/kb, and allowing the extension time to increase by 20 seconds every cycle for the last 10 cycles, seemed to work in favor of a successful mutagenesis reaction.

Reaction products were digested for an hour or longer with DpnI to digest the parental strands, then ethanol precipitated to concentrate the mutagenized products. The pellet was resuspended in 5-10 µL of dH_2O, which was then used in the transformation step. Either 50 µL of XL-10 Gold Ultracompetent Cells from the kit or 200 µL of DH5α cells made competent with the lab's standard protocol, were transformed with 5 µL of DpnI treated DNA. For the few colonies that grew on LB ampicillin agar dishes, overnight LB-ampicillin liquid cultures were grown and the plasmids were isolated using a Miniprep Kit (Qiagen) and restriction-digested with Psi-I. Those plasmids that showed the presence of the new PsiI restriction site were sent to the Berkley DNA Sequencing Facility to confirm proper mutagenesis to include an N-terminal FLAG tag in the coding region.

Growth and Maintenance of *Caenorhabditis elegans*

Small (5-cm) or medium (10-cm) Petri dishes with a layer of hardened Nematode Growth Medium (NGM) agar were seeded with a lawn of OP50 *E.coli*. Hermaphrodite worms were allowed to grow and self-fertilize at 20ºC on these plates. As each plate started to reach its maximum sustainable population, 3-5 worms (small plates) or 15 worms (medium plates) were transferred to a new seeded plate with a platinum wire pick. Bristol N2 (wild-type) strains and CB4043 (*smg-2 (e2008))* strains were obtained from the Caenorhabditis Genetics Center at the University of Minnesota.

Creation of Transgenic Worms

The final recombinant plasmid constructs containing the FLAG-tagged genomic sequence for each splicing factor gene were mixed with genomic N2 DNA that had been digested with PvuII, and a plasmid containing the *sur*-5 promoter driving expression of GFP. The PvuII-digested N2 DNA helps to increase the complexity of the resulting extrachromosomal arrays as well as increase the ability of worms to maintain the array as a line, and the GFP serves as a visible marker for worms that have the extrachromosomal array. The injection mix is made to 10µL and contains 0.8µg of PvuII-digested N2 DNA, 0.3µg of a FLAG-tagged splicing factor construct, and 0.2µg of the sur-5::GFP marker plasmid.

Genetic Crosses

NMD mutant worms containing our mutagenized plasmid were generated through several genetic crosses (Figure 4). First, fluorescent hermaphrodite worms containing our extrachromosomal array were crossed with wild-type (N2) males. Male progeny carrying the extrachromosomal array (EX) were crossed with *smg-2 (e2008)* mutant hermaphrodite worms to generate transgenic hermaphrodite worms that were heterozygous for the smg-2 mutation. These were allowed to self, and 25% of the F2 generation should have the abnormally swollen hermaphrodite vulva that is characteristic of hermaphrodites homozygous for the mutation. It is these worms that are picked onto plates to be maintained as extrachromosomal array-carrying NMD-mutant worms.

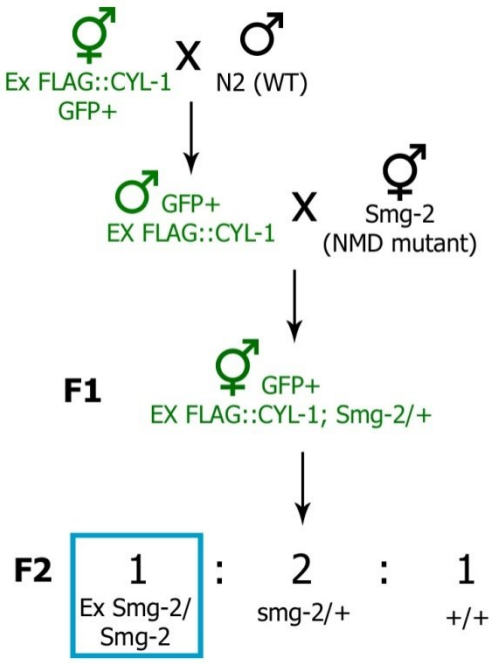

Figure 4. Genetic crosses performed to obtain NMD-mutant worms that express the transgene, indicated by the green fluorescence.

Collection of Worms

To obtain large quantities of worms for RNA isolations or protein extractions, several small or medium plates of worms were allowed to grow up until almost all of the food was gone. Worms were washed off of the plates in M9 buffer, transferred into 50 mL conical tubes with a glass pipet, and centrifuged at 4ºC for 5 min at 3000 RPM. The supernatant was removed, and the worms that formed the pellet were resuspended in 10 mL of M9 buffer. The worms were spun down again under the same conditions, excess M9 was removed, and worms were stored at

-75ºC until needed for RNA or protein isolations. Alternatively, the concentrated worms can be dropped slowly from a glass pipette into liquid nitrogen to make frozen worm balls, or "popcorn", which are stored at -75ºC until needed for nuclei extractions.

RNA extraction

Frozen worms (recovered from 3-10 small plates) were raised in 500µL TRIzol (Invitrogen), vortexed for 30 sec, then incubated for 10 min at room temperature. The sample was then pelleted in a microcentrifuge for 5 min at 13000 rpm, the supernatant transferred to a new tube, and 100µL chloroform added. The sample was vortexed for 20 sec, pelleted for 2 min at 13000 rpm, and the aqueous top layer was transferred to a new microcentrifuge tube. 350µL chloroform was added to the supernatant, vortexed, and pelleted again for 2 min at 13000 rpm. The aqueous layer was recovered to a new tube, mixed with 300µL isopropanol, and left to sit at -75ºC for an hour. The sample was then centrifuged for 20-30 min at 4ºC. The pellet was then rinsed with 70% ethanol and dried using a speed vac. The pellet was resuspended in 20µL dH2O, and the RNA concentration was measured using a spectrophotometer.

Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on both the full length and PTC-containing messages using the Superscript III One-step RT-PCR Kit with Platinum Taq (Invitrogen). The same 3' primer was used for both full-length and PTC-containing messages (Table 3). The 5' primer was either complementary to the epitope tag of our transgene, or complementary to the native gene, so that the relative steady-state ratios of either isoform could be compared. The RT-PCR is necessary to confirm that our transgene is being spliced to generate PTC-containing messages in the same

proportions as the native gene, and also to confirm that there is a higher percentage of PTC-containing product made in the NMD mutant worms.

Table 3. Primers used to reverse transcribe and amplify mRNA transcribed from either the native gene or the FLAG-tagged gene, and the expected mRNA sizes for each set of primers. The PTC-containing isoform is marked with a star (*). Data not available for *swp-1* construct.

	Forward Primer (5' → 3')	**Reverse Primer (5' → 3')**	**Estimated RT-PCR Product Size (bp)**
***Cyl-1* (native)**	TCCAGGACCTTTGGAGAATG	ACAGCTGCTGCTACTGGTCA	471* 419
***Cyl-1* (FLAG)**	GGATTATAAGGATGACGACG	TCCAGGACCTTTGGAGAATG	846* 794
***Hrpf-1* (native)**	TGATCCATACACGCAACCAG	CCATATGGTTCTGGCTTTGAA	556 307*
***Hrpf-1* (FLAG)**	GGATTATAAGGATGACGACG	CCATATGGTTCTGGCTTTGAA	1387 1138*
***Psf-1* (native)**	GCAGCTTCAAGACGTTCTAGC	GAAGATGTCGGCTTTTCGTC	777 275*
***Psf-1* (FLAG)**	GGATTATAAGGATGACGACG	GAAGATGTCGGCTTTTCGTC	1,150 548*
***Rsp-5* (native)**	TGGAAAAACCATGGAAGGAA	CCATCTCGGCTGTGACTTCT	416 686*
***Rsp-5* (FLAG)**	GGATTATAAGGATGACGACG	CCATCTCGGCTGTGACTTCT	583 853*
***Rsp-6* (native)**	TTCGAGTGAGAACGATCACG	GGATTTGCGTTCGTGGAATA	373 766*
***Rsp-6* (FLAG)**	GGATTATAAGGATGACGACG	GGATTTGCGTTCGTGGAATA	498 891*

Extraction of Nuclei

First, a mortar and pestle was cooled in a bucket of crushed dry ice by pouring liquid nitrogen over it. When the liquid nitrogen evaporated, frozen worm "popcorn" was ground to a fine powder. Periodically, a small pipette tip of the powder was mixed with DAPI solution and observed through a microscope to check for nuclei. Once the crushed worm parts gave way to

liberated nuclei, the frozen powder was scooped into siliconized microcentrifuge tubes. 1mL of a solution of PBS with protease inhibitors (1 Protease Inhibitor Cocktail Tablet (Roche) was added to 15 mL of PBS) were added to ~300 µL of powder. The sample was centrifuged at 500xg (2500 rpm) for 1 min at 4ºC. The supernatant with the nuclei was transferred to a new tube, and spun down at 5000xg (8000 rpm) for 5 min at 4ºC. The supernatant was removed with an aspirator, and the pellet resuspended in 1mL of PBS + protease inhibitors and spun down at 5000xg (8000 rpm) for 5 min at 4ºC. The pellet was resuspended in 50µL SDS-PAGE sample buffer.

Immunoblotting

To determine whether the PTC-containing isoforms of the tested genes are translated in wild type or NMD mutant backgrounds, we performed protein immunoblotting of the extracts probing with an antibody against the FLAG tag.

Proteins from nuclear extracts or from whole worms lysed into SDS-PAGE sample buffer were separated on a 10% acrylamide Laemmli separating gel at 200V for 2.5-3 hours. The protein gel was trimmed, and the proteins were transferred to a PVDF membrane in transfer buffer for 1 hour at 65V. The transfer buffer was made by dissolving 28.8 g glycine and 6.04 g tris base together in 1.6L dH_2O, then adding 200 mL methanol to make a 2L final volume. The PVDF was activated by pre-soaking it in methanol for 2 minutes before transfer. Once transferred to the PVDF membrane, the membrane was placed immediately into a 1:10 dilution of Blocking Solution (Roche Diagnostics) on a shaker table to swirl for 1 hour. The blocked membrane was then rolled up into a 50mL conical tube containing 4mL of a 1:20 dilution of Blocking Solution and 4µL of primary anti-FLAG M2 mouse monoclonal antibody (Sigma), a

1:1000 dilution of primary antibody. The 50mL conical tube was placed on a roller overnight at 4ºC. The following day, the membrane was removed and washed with TBS-T (200mL 1M Tris-Cl pH 8 and 35g NaCl in 4L ddH_2O + 2mL Tween-20) for 5 min at a time, 4-6 times. The blot was allowed to swirl for an hour in HRP-conjugated goat anti-mouse antibody (BIORAD), diluted 1:5000 in TBS-T. Then, the blot was washed in TBS-T for 5 min, 4-6 times. Then, the blot was swirled in 15 mL of SuperSignal West Pico Chemiluminescence (ThermoScientific) for 5 min to allow HRP to activate the chemiluminescence, and the blot was exposed to X-ray film in the dark room to image the bands.

To check that each lane was evenly loaded, a Commassie stain was performed on another protein gel with the same amount of sample in each lane.

Results

In order to determine whether or not the PTC-containing mRNA isoforms of splicing factors in wild-type and NMD deficient *C. elegans* are translated into truncated proteins, we inserted an epitope tag after the start codon for easy detection on a Western Blot. We chose six different splicing factor genes that can be alternatively spliced to include a PTC—*hrpf-1, cyl-1, swp-1, psf-1, rsp-5,* and *rsp-6*. All of the genes chosen have human homologs (Table 4). The first four of these genes showed inefficient NMD in the wild type worms, while the last two showed almost complete degradation of their respective PTC isoforms in wild type worms. In an NMD mutant background, the mRNA transcripts of the PTC isoforms of all six genes increased, as would be expected (Barberan-Soler et al., 2009).

Table 4. *C. elegans* genes *cyl-1, hrpf-1, psf-1, rsp-5, rsp-6,* and *swp-1*and their human homologs.

C. elegans gene	*Cyl-1*	*Hrpf-1*	*Psf-1*	*Rsp-5*	*Rsp-6*	*Swp-1*
Human homolog	Cyclin L	hnRNP F/H family of splicing factors	Polypyrimidine-tract-binding protein (PTB)-associated-Splicing Factor	SR protein family	SR protein family	Suppressor-of-white-apricot splice factor family

In order to check that our transgenes had similar alternative splicing patterns to the native gene, an RT-PCR was performed on both the native and transgenes in wild-type and NMD mutant backgrounds. The band corresponding to the PTC-containing isoform should become more intense in the NMD mutant backgrounds. For *cyl-1*, there is an increase in the ratio of PTC-containing isoform to normal isoform in the NMD (*smg-2*) mutants from the wild-type (Figure 5). In addition, when comparing the expression of the native gene to the transgene, both isoforms are expressed equally in the wild-type worms, and both show an increase in the amount of PTC-containing isoform in the NMD mutant background. Thus, for the *cyl-1* construct, our transgene is being spliced and regulated similarily to the endogenous gene, since it exhibits similar ratios of steady-state isoform in both wild-type and NMD mutant backgrounds. RT-PCR has been performed so far on *cyl-1, hrpf-1*, *psf-1*, *rsp-6, and rsp-5* (Data not shown).

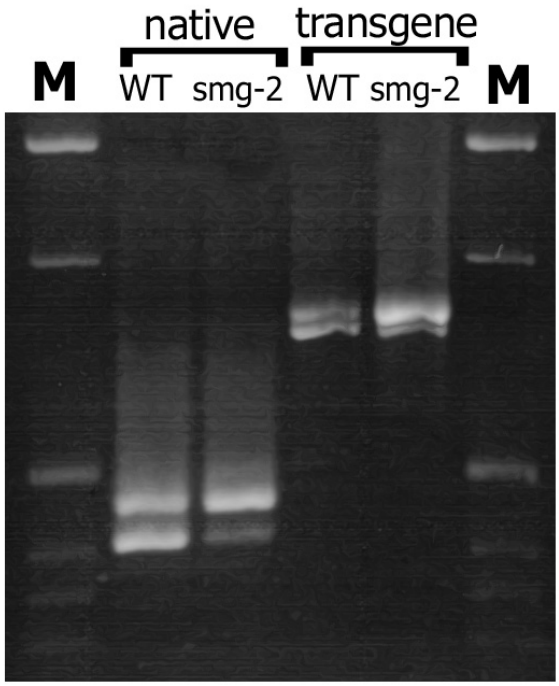

Figure 5. RT-PCR of the native and transgenes of *Ex FLAG::cyl-1* in a wild-type and NMD mutant (*smg-2*)

Once we established that our transgene splicing is under AS-NMD regulation in a manner similar to the native gene, we performed immunoblotting with an anti-FLAG antibody to detect proteins made from the transgenes, to see if the truncated splicing factor proteins are produced. We performed a general protein extraction from either wild-type or *smg-2* mutant worms that contained our constructs, ran the proteins out on a gel, and used an anti-FLAG antibody to look

for proteins. For CYL-1 and HRPF-1, the expected full-length protein sizes were 56 kD and 61.8 kD, respectively, but there were no proteins of that size detected in the immunoblot (Figure 6). Results were also inconclusive for RSP-5, as no full-length or truncated protein of the expected sizes were detected. The full-length PSF-1 protein (59.8 kD) was present in wild-type, *Ex FLAG::psf-1* worms, seen as an intense band on the Western blot. The full-length RSP-6 protein (20.5 kD) could be detected in *Ex FLAG::rsp-6* transgenic worms, and the truncated protein (16.4 kD) could be detected in *smg-2; Ex FLAG::rsp-6* transgenic worms.

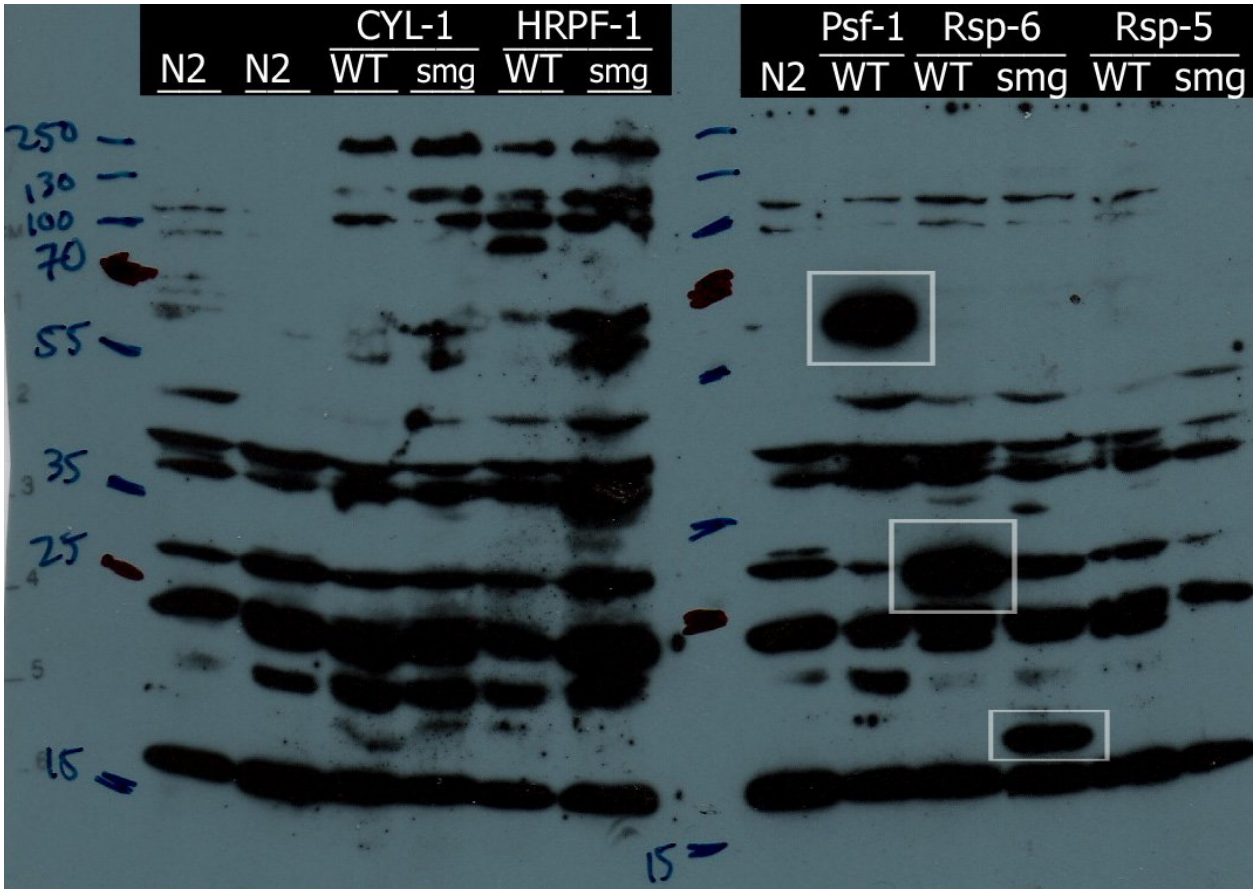

Figure 6. Immunoblot probed with monoclonal M2 anti-FLAG antibody. Whole worm extracts were used as the source of protein. Only full-length PSF-1 (59.8 kD) and full length RSP-6 (20.5 kD) could be detected in the wild-type background. Truncated RSP-6 (16.4 kD) could be detected in the NMD mutant background.

In order to get clearer results for HRPF-1, CYL-1, and RSP-5, we isolated nuclei from worms before extracting protein, to reduce the amount of background and enrich for nuclear proteins (Figure 7). The band corresponding to the PSF-1 protein can be seen clearly at around 59.8 kD, and CYL-1 at around 56 kD, after the nuclear extract.

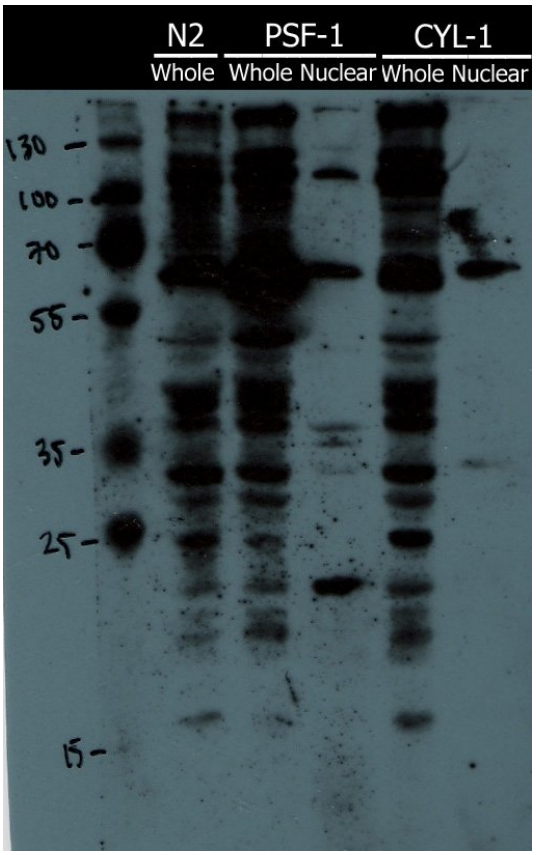

Figure 7. Comparison between protein obtained from whole worm extracts and nuclear extracts from Ex FLAG::psf-1 worms or Ex FLAG::cyl-1 worms in a wild-type background. Immunoblot was probed with monoclonal M2 anti-FLAG antibody.

Discussion

Establishing whether potential dominant-negative truncated forms of splicing factors produced by alternative splicing are translated in NMD mutant worms is the goal of this study. Since the PTC-containing mRNA encoding for RSP-6 is almost completely degraded in wild-type worms (Figure 1) (Barberan-Soler et al., 2009), we would not expect to detect the truncated version of this splicing factor in a wild-type background. However, with NMD absent, up to 20% of steady-state isoforms of *rsp-6* contain a PTC. The presence of truncated RSP-6 in *smg-2* mutant worms is evidence that without NMD there to degrade those PTC-containing isoforms, they are able to enter the polysome pool and be translated into truncated splicing factors.

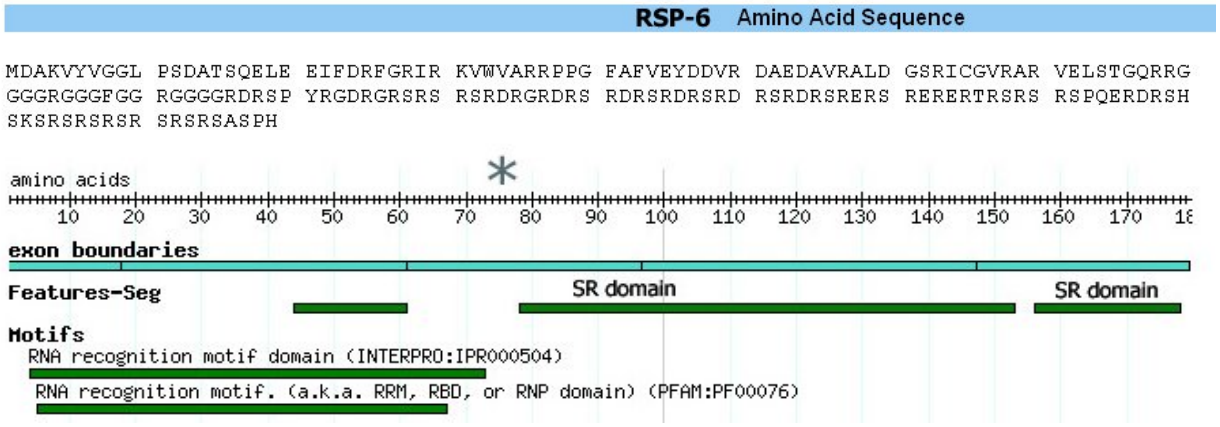

Figure 8. The amino acid sequence of RSP-6, with a map of the RRM and SR domains below. The SR domain contains many serine and arginine residues. The premature stop codon of the PTC-containing mRNA would fall between the RRM and SR domains, between amino acids 70 and 80 on the map (indicated by the asterisk).

Rsp-6 contains an RNA recogition motif domain, and two splicing activation domains called SR domains (Figure 8). The PTC in the mRNA regulated by NMD lies between the RRM and SR domains. The truncated splicing factor made from this PTC-containing mRNA would include the RRM domain and be missing the SR domain. Theoretically, this protein would retain

the ability to bind to RNA. However, since it lacks an SR domain, it would not be able to splice in the alternative exon. Therefore, it is possible that the competition between truncated and full-length RSP-6 in NMD mutants could effect splicing patterns of all of their mRNA targets, which could explain the secondary targets of NMD found by Barberan-Soler et al. in their 2009 paper.

Future Directions

We will continue the nuclear extractions and immunoblots to get clearer results for all genes in question. In addition, we plan to phosphatase-treat the proteins following their extraction from nuclei, because SR proteins tend to be phosphorylated heavily (Zahler et al., 1992). The extra phosphorylation would cause these proteins to run higher in the gel, so phosphatase-treating the proteins first would allow the proteins to run closer to their expected sizes in the gel.

We are also beginning to integrate the extrachromosomal arrays into the genome of the worms, so that we can do an RNA microarray to see how steady-state isoform ratios change when the truncated splicing factors are overexpressed. If global splicing patterns change significantly for some genes when the truncated splicing factors are overexpressed, that would mean that the truncated protein has the ability to interfere with the normal splicing patterns of the cell. In the future, it may also be possible to perform a CLIP assay with an anti-FLAG antibody to see what mRNAs these splicing factors bind to (Ule et al., 2005).

Acknowledgements

I want to thank everyone from the Zahler Lab, both past and present members, for their support and advice on lab techniques. I really enjoyed spending time with them in the lab. I also want to thank Rocio Nunez-Martinez from the Sanford Lab for taking the time to help me troubleshoot and improve my Western blotting technique.

I especially want to thank Dr. Alan Zahler for allowing me to join his lab as an undergraduate that had never even touched a micropipette before. I appreciate all of the time he put in to teach me not only the simple things like how to pipette or pour a gel, but also all of the other molecular biology skills I now know. I also want to thank Dr. Alan Zahler for his unwavering enthusiasm and support for both my project and anything that I wanted to take on, including writing this senior thesis. His enthusiasm in teaching cell biology sparked my interest in research, and without him I may never have realized my potential as a researcher, or even have considered a career in research.

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