Dissecting Interactions Across Gene Regulatory Layers in C. elegans

Morgan Taylor
Southern Methodist University, morganxtaylor@gmail.com

Follow this and additional works at: https://scholar.smu.edu/hum_sci_biologicalsciences_etds

Part of the Genetics Commons, and the Molecular Biology Commons

Recommended Citation
https://scholar.smu.edu/hum_sci_biologicalsciences_etds/20

This Dissertation is brought to you for free and open access by the Biological Sciences at SMU Scholar. It has been accepted for inclusion in Biological Sciences Theses and Dissertations by an authorized administrator of SMU Scholar. For more information, please visit http://digitalrepository.smu.edu.
DISSECTING INTERACTIONS ACROSS

GENE REGULATORY LAYERS IN C. ELEGANS

Approved by:

___________________________________
Dr. Adam D. Norris
Assistant Professor of Biological Sciences

___________________________________
Dr. Richard S. Jones
Chair and Professor of Biological Sciences

___________________________________
Dr. Zhihao Wu
Assistant Professor of Biological Sciences

___________________________________
Dr. Weihua Geng
Associate Professor of Mathematics
DISSECTING INTERACTIONS ACROSS
GENE REGULATORY LAYERS IN C. ELEGANS

A Dissertation Presented to the Graduate Faculty of the
Dedman College
Southern Methodist University
in
Partial Fulfillment of the Requirements
for the degree of
Doctor of Philosophy
with a
Major in Molecular and Cellular Biology
by
Morgan A. Taylor
B.S., Biology, Baylor University
M.S., Biology, Texas Christian University
December 17, 2022
Taylor, Morgan A.  
B.S., Biology, Baylor University  
M.S., Biology, Texas Christian University

**Dissecting Interactions Across**  
**Gene Regulatory Layers in C. elegans**

Advisor: Dr. Adam D. Norris

Doctor of Philosophy conferred December 17, 2022  
Dissertation completed October 31, 2022

The nematode *Caenorhabditis elegans* is a powerful tool for studying nervous system genetics. Though relatively simple compared to mammals, *C. elegans* boasts a remarkably well-conserved neuronal genome and proteome, and its utility in the characterization of neuronal genes has been well-established. However, gene expression is often controlled by complex interactions between multiple genes, and teasing apart the functions of individual genes within such networks remains a challenge. Dissecting these interaction networks is crucial in determining the multifaceted functions of important, conserved regulatory genes. Here we explore interactions between gene regulatory layers in the *C. elegans* nervous system, employing a synthetic genetic interaction (SGI) screen to identify interactions. In Chapter 1, conservation of Stomatin domain genes in sensory neurons of *C. elegans* and mice was investigated. We identify a novel conserved role of Stoml3/mec-2 in olfaction, asserting the high conservation of *C. elegans* neuronal genes. In Chapter 2, we characterize an alternative splicing event in the neuronal kinase *sad-1*. We find complex coordination between a set of transcription factors and a set of RNA binding proteins that together control alternative isoforms of *sad-1* in different neuron types. This prompted us to further investigate widespread coordination between neuronal TFs and RBPs. In Chapter 3, we embarked on a genetic interaction screen with the goal of identifying functionally-relevant interactions between neuronally-enriched TFs and RBPs. We
discovered a variety of genetic interactions which suggest novel roles for several TFs and RBPs. Most intriguing, we describe a novel role for two ALS-related RBPs, \textit{tdp-1} and \textit{fust-1}, in facilitating \textit{C. elegans} fertility. \textit{tdp-1} and \textit{fust-1} mutants do not exhibit a strong phenotype, but \textit{tdp-1; ceh-14} and \textit{fust-1; ceh-14} double mutants present a striking loss of fertility, coinciding with a decline in gamete functionality and apparent defects in gonad development. RNA-seq analysis of double mutants identifies \textit{ceh-14} as the main controller of transcript levels, while \textit{fust-1} and \textit{tdp-1} control splicing through a shared role in exon inhibition. We identify a cassette exon in the polyglutamine-repeat protein \textit{pqn-41} which \textit{tdp-1} inhibits. Loss of \textit{tdp-1} causes the \textit{pqn-41} exon to be aberrantly included, and forced skipping of this exon in \textit{tdp-1; ceh-14} double mutants rescues fertility. In sum, using a systematic combinatorial genetic interaction screen, we identify a novel shared physiological role for \textit{fust-1} and \textit{tdp-1} in promoting \textit{C. elegans} fertility in a \textit{ceh-14} mutant background and reveal a shared molecular function of \textit{fust-1} and \textit{tdp-1} in exon inhibition. Together, these results highlight the importance of dissecting interaction networks to elucidate functions of individual genes.
TABLE OF CONTENTS

LIST OF FIGURES..............................................................................................................................x

ACKNOWLEDGEMENTS..............................................................................................................................xii

INTRODUCTION........................................................................................................................................13

SPECIFIC AIMS........................................................................................................................................16

CHAPTER 1..............................................................................................................................................19

1.1 Background......................................................................................................................................19

1.2 Stomatin genes in olfactory neurons of mice..................................................................................20

1.3 Stoml3 is required for olfactory behavior in mice.........................................................................22

1.4 Discussion.......................................................................................................................................24

1.5 Methods.........................................................................................................................................25

1.5a RNA Seq data...............................................................................................................................25

1.5b Mice...............................................................................................................................................25

1.5c Genotyping....................................................................................................................................26

1.5d Innate olfactory attraction test ....................................................................................................26

1.5e Buried cereal test..........................................................................................................................26

1.5f Habituation/dishabituation test ......................................................................................................27

1.5g Block test......................................................................................................................................28
CHAPTER 2.................................................................................................................................29

2.1 Background ........................................................................................................................................29

2.2 Alternative splicing of the neuronal kinase sad-1 in specific cell types ........................................31

2.3 Forward genetic screen identifies three fate-determining TFs affecting sad-1 splicing in the ALM neuron .......................................................................................................................34

2.4 A pair of RNA binding proteins regulates sad-1 splicing in the ALM neuron ..................................36

2.5 TFs affect sad-1 splicing by controlling RBP expression in the ALM neuron ..................................38

2.6 RBPs directly mediate sad-1 exon inclusion through interactions with surrounding introns .................................................................................................................................41

2.7 sad-1 splicing in other neuron types is controlled through both distinct and overlapping mechanisms ..............................................................................................................................42

2.8 Discussion ........................................................................................................................................46

2.8a Neuron-specific regulation of sad-1 splicing ..................................................................................46

2.8b Coordinated splicing regulation across layers of gene expression .................................................46

2.9 Methods .........................................................................................................................................47

2.9a Strain maintenance ..........................................................................................................................47

2.9b Mutant generation and genetic screening .....................................................................................47

2.9c Microscopy .....................................................................................................................................48

2.9d Generation of splicing reporters .....................................................................................................48

CHAPTER 3.........................................................................................................................................49

3.1 Background .......................................................................................................................................49

3.2 Genetic interaction screen identifies synthetic fitness effects in several double mutants ..................51

3.3 aptf-1; fox-1 double mutants exhibit developmental delay ...............................................................55
3.4 Reproductive defects in double mutants of TF ceh-14 and ALS-associated RBPs fust-1 or tdp-1
3.5 Gonad development defects in fust-1; ceh-14 and tdp-1; ceh-14 double mutants
3.6 Gamete defects in fust-1; ceh-14 and tdp-1; ceh-14 double mutants
3.7 Expression of TDP-1, FUST-1, and CEH-14 overlaps in the spermatheca
3.8 Distinct transcriptional and post-transcriptional networks in double mutants
3.9 tdp-1 and fust-1 co-inhibit exon inclusion
3.10 Aberrant exon inclusion in pqn-41 contributes to fertility defect of tdp-1; ceh-14
3.11 Discussion
3.11a Novel genetic interactions across regulatory layers
3.11b fust-1 and tdp-1 interact with ceh-14 to affect C. elegans fertility
3.11c fust-1 and tdp-1 co-inhibit exon inclusion
3.12 Methods
3.12a C. elegans strains and maintenance
3.12b Competitive fitness assays
3.12c Larval growth assay
3.12d Fluorescence microscopy
3.12e DAPI gonad imaging
3.12f Uterine egg retention
3.12g Lifetime egg-laying and brood size assays
3.12h Male mating efficiency
3.12i Paired brood size assay
3.12j RNA sequencing and analysis
3.12k Reverse Transcription PCR
LIST OF FIGURES

Figure 1 Stomatin domain genes expressed in mouse neurons, and Stoml3 knockout mice........21
Figure 2 Olfactory assays for wild-type and Stoml3 knockout mice......................................................23
Figure 3 sad-1 is alternatively spliced in single neurons.................................................................33
Figure 4 Genetic screen identifies neuronal TFs affecting sad-1 splicing in the ALM neuron.....35
Figure 5 Two neuronal RBPs combinatorially control sad-1 splicing in ALM neurons...........36
Figure 6 Canonical RBP alleles of mec-8 and mbl-1 affect sad-1 splicing similarly to CRISPR deletions of mec-8 and mbl-1..................................................................................................................37
Figure 7 Neuronal TFs establish expression of both mec-8 and mbl-1 to mediate splicing of sad-1 in ALM neurons.........................................................................................................................39
Figure 8 mbl-1 and mec-8 affect sad-1 splicing by direct interaction with sad-1 introns........41
Figure 9 sad-1 splicing in motor neurons of the ventral nerve cord is controlled by mbl-1 and msi-1 RBPS........................................................................................................................................43
Figure 10 Phenotypic convergence at the level of splicing regulation.................................45
Figure 11 Identifying genetic interactions with competitive fitness assays..........................54
Figure 12 Synthetic fitness in aptf-1; fox-1 double mutants.........................................................55
Figure 13 aptf-1; fox-1 double mutants exhibit slowed rates of egg laying and pharyngeal pumping compared to single mutants........................................................................................................56
Figure 14 aptf-1; fox-1 double mutants exhibit developmental delay........................................57
Figure 15 Negative synthetic fitness effects in both fust-1; ceh-14 and tdp-1; ceh-14 double mutants........................................................................................................................................59
Figure 16 Defects in reproduction in fust-1; ceh-14 and tdp-1; ceh-14......................................60
Figure 17 fust-1; ceh-14 and tdp-1; ceh-14 exhibit defects in adult hermaphrodite gonad........63
Figure 18 Defects in sperm and oocytes in fust-1; ceh-14 and tdp-1; ceh-14.................................65

Figure 19 Spermatheca of tdp-1; ceh-14 and fust-1; ceh-14 double mutants are morphologically similar to wild-type.................................................................67

Figure 20 ceh-14 controls transcriptional networks in tdp-1; ceh-14 and fust-1; ceh-14..............69

Figure 21 Distinct post-transcriptional regulation in tdp-1; ceh-14 and fust-1; ceh-14.............71

Figure 22 tdp-1 and fust-1 inhibit exon inclusion.................................................................72

Figure 23 pqn-41 sequences and CRISPR/Cas9 exon deletion..............................................75
ACKNOWLEDGMENTS

Grad school was lot. A lot of time, energy, successes, defeats, and growth. I am lucky to have had the support system I did, and there are many people to thank. Thank you to my advisor, Dr. Adam Norris, for the continued support and guidance. Thank you for your patience and encouragement along every step of the way. Thanks also go to my committee members, Dr. Jones, Dr. Wu, and Dr. Geng, for being a source of ideas and advice. A lot of work, both in print here and behind the scenes, went into this dissertation. I am grateful for the guidance I had in these professors.

Thanks to my friends and fellow graduate students, without whom my tenure at SMU would not have been nearly as enjoyable. Special thanks to Kelsey and Lauren for always commiserating when grad school was tough, celebrating the high points, and knowing that sometimes you just have to leave, get your mind off of science, and revisit in the morning. Thank you to my fellow members of the Norris Lab, all of whom made coming to work fun.

Thank you to Meagan, Michaela, Mom, and Dad, for being supportive as I decided to go back to school yet again. I promise this is my last graduation! Thank you to Gypsy, Archie, Syl, and Salazar for being a captive audience when I needed to practice presentations. Thanks to my wonderful husband Sean for always being there for all of the highs and lows of grad school, and for genuinely wanting to know how my research was going even though I sometimes struggled to translate. Thank you for being the most supportive partner through it all.

No, they’re not earthworms.
INTRODUCTION

One of the challenges faced by geneticists is determining the precise function of an individual gene. Multiple genes function together to carry out molecular events, and many essential genes even have redundant roles. A visible phenotype is often due to the interacting effects of more than one gene. One strategic method of dissecting the functions of individual genes within complex networks is to conduct a genetic interaction screen in which a series of organisms bearing a single mutation are crossed with each other to generate double mutants. If the two mutated genes interact, then the mutation of one gene will modify the effects of the other mutation\(^1\). If the characteristics of each single mutant are well-defined, genetic interactions can be identified in the double mutants by screening for unexpected phenotypes. Genetic interaction screens have been extensively conducted in budding yeast, and recently a comprehensive genetic interaction network was constructed for all yeast genes\(^2\), but it remains more of a challenge to conduct such extensive screens in multicellular organisms.

The nematode *Caenorhabditis elegans* is a relatively simple but powerful eukaryote model system; an ideal candidate for such complex genetic interaction screens. *C. elegans* has been extensively used as a model for studying the function of conserved genes, with many biological processes and pathways conserved. A previous proteomics study estimated that 83% of the *C. elegans* proteome has human homologs\(^3\). *C. elegans* is an especially valuable tool for neuronal studies. It contains a well-annotated nervous system comprised of 302 neurons, produced through a series of invariant cell divisions which has been extensively studied\(^4,5\).
Though the *C. elegans* nervous system is relatively simple, there is a remarkably high level of conservation of neuron types and neuronal genes\(^6\). This, along with the organism’s short lifespan and transparent body, makes it an ideal model organism for nervous system genetics. Previous genetic interaction screens have been conducted mainly with the use of RNA interference (RNAi) of multiple targets at once\(^7,8\). These studies have been useful for establishing interaction networks in *C. elegans*, but have limitations. RNAi only knocks down expression of a gene rather than deleting it entirely. Furthermore, RNAi experiments are more difficult to conduct on neuronal genes, as most *C. elegans* neurons are resistant to RNAi\(^9,10\).

Recent advances in genome editing have made it possible to selectively knock out genomic targets with the use of clustered regularly interspaced palindromic repeat (CRISPR) RNA and their associated nucleases (Cas). In *C. elegans*, CRISPR/Cas9 has been established as a method to introduce targeted heritable mutations in the germline, generating stable mutant strains and providing an ideal alternative to RNAi-knockdown\(^11–13\). Our lab has successfully employed CRISPR/Cas9 to knock out target genes and use homology-guided replacement to insert GFP fluorescent markers in place of the deleted gene, allowing for visible verification of recombinant progeny\(^14\). Additionally, we have previously used these CRISPR/Cas9-generated deletion mutants to conduct a genetic interaction screen of neuronal RNA binding proteins (RBPs), revealing novel interactions and identifying new combinatorial roles for several RBPs\(^14\).

As part of my dissertation work I conducted a similar genetic interaction screen to dissect interactions between conserved, neuronally-enriched RBPs and transcription factors (TFs). In Chapter 1, we discuss the conservation of stomatin genes in specific sensory neurons. We identify a conserved role for *mec-2/Stoml3* in olfactory behavior in both *C. elegans* and mice, highlighting the remarkable level of conservation of *C. elegans* neuronal genes. In Chapter 2, we
investigated alternative splicing in a neuronal kinase, sad-1. We identified a network of transcription factors and RNA binding proteins that coordinated alternative splicing, providing novel evidence of cross-talk between transcription and RNA processing. In Chapter 3, I sought to identify further evidence of cross-talk between gene regulatory layers. I embarked on a screen to identify physiologically-relevant interactions between conserved transcription factors and RNA binding proteins in *C. elegans*. This work uncovered physiological roles for several neuronal RBP-TF interaction pairs. Importantly, these findings identify shared novel roles for the ALS-related RBPs *fust-1* and *tdp-1* in facilitating reproduction in *C. elegans*.
SPECIFIC AIMS

Chapter 1: Determine whether a mouse homolog of mec-2 has conserved function in olfaction.

Recent work in our lab uncovered a novel role for mec-2 in *C. elegans* olfactory behavior. *Stoml3*, the mouse homolog of mec-2, was previously known to have a conserved role in mechanosensation in mice and worms. We generated *Stoml3* knockout mice and investigated the effect on olfaction. We find that *Stoml3* KO mice can detect odors, but they cannot efficiently discriminate between different odors. This suggests that, in addition to a conserved function in mechanosensation, *Stoml3/mec-2* also has a conserved role in olfaction. These findings represent a noteworthy example of the high level of conservation of the *C. elegans* neuronal genome.

Chapter 2: Investigate neuron-specific expression of alternatively spliced isoforms

Aim 2.1. Identify alternative splicing in single neurons.

We employed a two-color splicing reporter to investigate alternative splicing of an alternatively-spliced exon in the synaptic gene *sad-1* in individual neurons. While many neurons express both the exon-skipped and exon-included isoforms of *sad-1*, we identified neuron types that strictly express either one or the other isoform. We discover distinct isoform expression in two related neurons, the BDU and ALM neurons, suggestive of strict neuron-specific splicing regulation.
Aim 2.2. Investigate neuron-specific regulators of sad-1 alternative splicing.

We conducted a genetic screen to identify regulators of sad-1 alternative splicing in the BDU and ALM neurons. We found three TFs and two RBPS that are required for proper sad-1 splicing. It was revealed that the three TFs control cell-specific expression of the two RBPs, which themselves directly bind to sad-1 sequence and mediate alternative splicing.

Chapter 3: Identify functionally-relevant interactions between transcription and RNA processing

Aim 3.1. Identify novel interactions between neuronal RBPs and TFs that affect fitness.

The complex TF-RBP coordination we identified in sad-1 motivated further exploration of interactions between the regulatory layers of transcription and RNA processing. Specifically we sought to uncover TF-RBP interactions that had measurable consequences on the overall physiology of the organism, denoting functionally significant genetic interactions. Using deletion mutants of conserved, neuronally-enriched TFs and RBPs, we generated 110 TF; RBP double mutants and screened them for synthetic effects on C. elegans fitness. Synthetic effects in the double mutant implies a genetic interaction between the constituent TF and RBP that were mutated.

Aim 3.2. Determine specific phenotypes contributing to strong synthetic fitness effects in double mutants.

Several novel TF-RBP interactions were uncovered in the synthetic genetic interaction screen. Follow-up experiments investigated which of a wide variety of C. elegans behaviors contributed to the synthetic fitness effect that was measured. We describe a developmental delay in fox-1 aptf-1 double mutants that conferred a strong negative effect on fitness. We also highlight a synthetic negative interaction that tdp-1 and fust-1 share with the transcription factor ceh-14. We find that tdp-1; ceh-14 and fust-1; ceh-14 exhibit severe defects in reproduction.
Importantly, *tdp-1, fust-1*, and *ceh-14* single mutants do not exhibit this defect, suggesting a novel role for *tdp-1* and *fust-1* in *C. elegans* fertility in the context of the *ceh-14* mutant background.

**Aim 3.3. Identify underlying mechanisms in *tdp-1; ceh-14* and *fust-1; ceh-14* causing decline in fertility.**

We wanted to determine how three neuronally-enriched genes contributed to *C. elegans* fertility. We investigated expression of these genes and found interesting overlap of expression in the hermaphrodite spermatheca. We next asked whether defects in the oocytes or the sperm caused the decline in fertility of double mutants. Interestingly, we found that both oocytes and sperm function, but at significantly reduced efficiency. We also investigated the transcriptomes of *tdp-1; ceh-14* and *fust-1; ceh-14* double mutants. We found distinct transcriptional and post-transcriptional networks in both double mutants. We identify a set of spermatheca-specific genes regulated by *ceh-14*, and find a shared role for *fust-1* and *tdp-1* in exon inhibition.
CHAPTER 1

A CONSERVED ROLE FOR STOMATIN DOMAIN GENES IN OLFACTORY BEHAVIOR

These data are available online in preprint – Liang & Taylor et al., 2022, bioRxiv

1.1 Background

Olfaction is a remarkable sensory system, enabling animals to detect and distinguish among a considerable range of odors, and at very low concentrations. A number of cellular and molecular aspects of the olfactory systems of various animals have been well characterized, including odorant-receptor interactions, signal transduction cascades within olfactory neurons, and downstream neuronal circuits. However, many factors that are highly expressed in olfactory neurons remain completely uncharacterized or with poorly defined function.

In a recent study from our lab, we employed single-cell transcriptomics to identify neuron type-specific functions of mec-2, the C. elegans homologue of Stomatin. mec-2/Stomatin is a conserved component of mechanosensory channels, previously understood to be required for touch sensation. Researchers from our lab confirmed the mechanosensory function for the canonical mec-2 isoform expressed in C. elegans touch neurons, and identified a non-canonical short isoform of mec-2 which is expressed in olfactory neurons. It was found that the mec-2 short isoform is required for olfactory behavior in C. elegans, and worms which lack this isoform in chemosensory neurons do not appropriately chemotax to attractive odors. These findings prompted us to ask whether the olfactory role of mec-2 is conserved in mammals. Here, we investigate the role of Stoml3, a mouse homologue of C. elegans mec-2, in olfaction. We
generate a *Stoml3* knockout mouse and demonstrate that it is required for proper olfactory behavior in mice. *Stoml3* KO mice are able to detect, but not efficiently distinguish between, odors. Therefore, in addition to their conserved roles in mechanosensory behavior, we identify an additional conserved role in olfactory behavior for *mec-2* and *Stoml3*.

**1.2 Stomatin genes in olfactory neurons of mice**

We recently showed that the *C. elegans* stomatin domain protein MEC-2 is expressed in olfactory neurons in addition to its previously-described expression in mechanosensory neurons, and demonstrated that *mec-2* mutants are deficient in both mechanosensory and olfactory behaviors\(^\text{22}\). Follow-up studies in mice established a role for *Stoml3*, the mouse homolog of *mec-2*, in mammalian mechanosensory behavior\(^\text{23,25}\). We similarly wished to determine whether the role for *mec-2* in olfaction represents a novel evolutionarily-conserved function for stomatin domain genes.

We first tested whether specific stomatin domain genes are selectively expressed in mouse olfactory receptor neurons (ORNs). To do so we analyzed RNA Seq data in which mouse olfactory receptor neurons were GFP labeled and FACS sorted, and compared this with similar data obtained from mouse motor neurons\(^\text{21,26}\) (Fig 1A-D). We confirmed that marker genes for the respective neuron classes are expressed in the expected cell types (Fig 1A, C), and found that stomatin domain genes exhibit distinct expression patterns (Fig 1B, D). We observed high expression of both *Stoml3* and *Stomatin* in ORNs, but not in motor neurons, while *Stoml1* and *Stoml2* are expressed in motor neurons but not olfactory neurons (Fig 1A-B). Because *Stoml3* displays the highest level of expression in ORNs, and because of a previous report demonstrating striking localization of *Stoml3* to ORN sensory cilia\(^\text{20}\), we prioritized *Stoml3* for further genetic and behavioral analysis.
Figure 1: Stomatin domain genes expressed in mouse neurons, and Stoml3 knockout mice. (A-D) Cell-specific sequencing of mouse olfactory neurons (A-B) and motor neurons (C-D). Omp is used as a positive marker for olfactory neurons, and Chat as a positive marker for cholinergic motor neurons. (E) Stoml3 gene in mouse, indicating the region we deleted in orange, and the primer sets used to detect both the wild-type and mutant genomic DNA (black arrows). (F) Sanger sequencing confirming the ~5.4 kb deletion. (G) Genotyping gels for identifying homozygous Stoml3 mutants and their wild-type littermates.
1.3 Stoml3 is required for olfactory behavior in mice

We used CRISPR/Cas9 to remove four exons and generate a Stoml3 KO mouse (Fig 1E-G). The deletion resulted in the loss of the conserved stomatin domain in Stoml3 as well as the introduction of downstream premature stop codons. To test whether Stoml3 mutant mice are defective in olfactory behavior, we performed a number of standard olfactory behavioral assays on Stoml3-/- mice and their wild-type littermates. We first tested the ability to detect and respond to olfactory stimuli that elicit innate behavioral responses in wild-type mice. We measured time spent sniffing attractive odors compared to controls and found that both wild-type and Stoml3 mutant mice spend more time sniffing attractive odors (Fig 2A-B), suggesting that Stoml3 mutant mice retain the ability to detect odors. We next measured latency to find and eat a buried cereal pellet, as mice with impaired olfaction take longer to uncover buried food. As with the innate olfactory assays, we found no differences between Stoml3 and wild-type mice in latency to uncover the buried pellet (Figure 2C). Together these results suggest that loss of Stoml3 does not affect innate olfactory response to attractive stimuli such as food odors.

To test whether Stoml3 knockout mice are able to distinguish between different odors, we performed a habituation/dishabituation test in which mice are habituated with one scent for six consecutive trials, then switched to a novel scent on the seventh trial. We measured time spent sniffing a scented odor cartridge during each trial. Both wild-type and Stoml3 mutant mice display an initial interest in the odor, with sniffing time peaking during the second trial, as previously described. On the seventh trial, the odor was changed to a novel scent. Wild-type mice spend more time sniffing the novel scent compared to the familiar scent (Fig 2D), as previously described. In contrast, Stoml3 mutant mice do not exhibit an increased interest in the
Figure 2: Olfactory assays for wild-type and Stoml3 knockout mice. (A-B) Innate olfactory attraction tests. Mean time sniffing a scented block in wild-type mice (n=16) and Stoml3 KO mice (n=11) during the 3-min test period. Water was used as a control scent. Peanut butter and mouse urine were used as attractive scents. Both wild-type and Stoml3 KO mice showed attraction response to peanut butter and mouse urine. (C) Buried cereal test. Mean time in seconds wild-type (n=14) and Stoml3 KO mice (n=10) took to find the pellet. Surface pellet test is a positive control, confirming that the cereal pellet is attractive to the mice. ns represents no significant difference between wild-types and Stoml3 KO. (D) Habituation/dishabituation test. Mean time sniffing a scented cartridge in wild type mice (n=16) and Stoml3 KO mice (n=12) during 30-sec test period across 7 trials. Almond extract was used on trial 1-6 and banana extract was introduced as a novel scent on trial 7. Significant difference of sniffing time in wild-types was observed when novel scent was introduced on trial 7 (***represents p< 0.01), while no significant difference between trial 6 and 7 was observed in Stoml3 KO mice. (E) Block test. Mean time sniffing home cage blocks (A, B and C) and novel block (E) in wild-type mice (n=16) and Stoml3 KO mice (n=12). Wild-types spent more than 2-fold time exploring novel block (E) than Stoml3 KO mice (***represents p< 0.01, Mann-Whitney U test). (F) Time spent sniffing the blocks is minimal on trial 6 of the block test, where no novel odor is present.
novel scent in the seventh trial and spend the same amount of time investigating the novel scent in trial 7 as with the habituated scent in trial 6. This suggests that Stoml3 mice, unlike wild-types, are unable to discriminate between familiar and novel scents (Fig 2D).

Further evidence for the hypothesis that Stoml3 mice are unable to discriminate between olfactory cues comes from experiments on social odors in the “block test.” In this test, we housed mice individually with several wooden blocks for 24 hours, allowing for the blocks to acquire the odors of the home cage. The next day, we removed and reintroduced these home cage blocks for six consecutive trials to measure investigation time of the familiar blocks. Mice became habituated to the presence of the blocks and spent minimal time investigating them by the sixth trial (Fig 2F). On the seventh trial, one of the blocks was swapped with a block from the cage of a different mouse, thus introducing a block with novel social odors. Wild-type mice spend a substantial amount of time sniffing of the novel-odor block, as previously described (Fig 2E). However, Stoml3 mutants spend significantly less time sniffing the novel-odor block (Fig 2E), suggesting a deficit in distinguishing between self- and non-self social odors.

Together, these experiments demonstrate that Stoml3 knockout mice do not completely lose the ability to detect odors (Fig 2A-C). Rather, loss of Stoml3 impairs the ability to discriminate between odors, including familiar versus novel odors (Fig 2D), and distinction between self and non-self odors (Fig 2E).

1.4 Discussion

We have demonstrated a conserved role of mec-2/Stoml3 in olfaction. We previously demonstrated that in worms, mec-2 is required for chemotaxis. Here, we show that Stoml3 KO mice are able to detect odors, but unable to efficiently distinguish between odors. These
experiments suggest that in addition to their well-established roles in mechanosensory behavior, mec-2 and Stoml3 have an additional shared role in olfactory behavior.

In recent related work, electrophysiological recordings from olfactory epithelium slices revealed that Stoml3 KO in olfactory sensory neurons (OSNs) yielded modest reductions in spontaneous firing frequency. Odor-evoked firing frequency is not affected, but spike duration and frequency are modestly reduced. These electrophysiological properties of Stoml3 KO OSNs could underly the deficits in olfactory behavior we describe here.

The precise biochemical role of stomatin domain proteins remains somewhat mysterious. A common theme is the modulation of ion channel function, but the molecular mechanisms have yet to be fully elucidated. A model consistent with our data is that mec-2 and Stoml3 modulate ion channel activity in olfactory neurons in a manner required for their normal levels of activity. It will be interesting to test whether mec-2/Stoml3 exhibit similar or distinct interactions with ion channels or other membrane proteins in mechanosensory versus olfactory neurons.

1.5 Methods

1.5a RNA Seq data

Raw fastq files were downloaded from the NCBI SRA for C. elegans and mouse neuron-specific sequencing. Reads were mapped using STAR and alternative splicing mapped using JUM.

1.5b Mice

Stoml3 -/- mice were generated by deleting exon 2-5 of Stoml3 using CRISPR/Cas9 genome editing. gRNAs used to generate deletion. Upstream: CACATATGCGGGATGGTTTG & TAAACACCACATATGCGGGA. Downstream: GAGCCAAGACTCCCCAGCCC & AGTACAGCTATCCCTGGGCT. Mice were housed in a temperature and light controlled room.
(12 h dark/light cycle) and all animal experiments were conducted in accordance with policies of NIH guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee (IACUC) of Southern Methodist University. Adult mice (> 6-month-old) were used in this study, and both sexes of animals were used unless otherwise noted.

1.5c Genotyping

DNA was extracted from tails of 19-21 days old mice. Wild-type and mutant mice were determined by PCR using primer set (Stoml3 common forward primer 5’ - tgttctccacatgacacc - 3’, Stoml3 wild-type reverse primer 5’ - ggaccctcattagatgcccc - 3’, Stoml3 mutant reverse primer 5’ – ggcatcagttctctggaac – 3’). Primer’s location on gene Stoml3 is illustrated in Figure 3F.

1.5d Innate olfactory attraction test

Olfactory assays were conducted as previously described, with some modifications. Mice were isolated and habituated with a block one day before the test day. On the test day, mice were transferred to a clean cage with a thin layer of bedding for at least 10 minutes for habituation. Bedding is essential in this step to help mice reduce the fear of open space. After habituation, a scented wood block with different test odor was introduced. Animals were video recorded from the front side of the cage for 3 minutes. Sniffing time was defined as nasal contact with the block and was measured afterwards by analyzing the video. Odorants used were water (control, 80uL), peanut butter (10% w/v, 80 uL) and mouse urine (80 uL). Mouse urines were collected freshly from different litters and mixed well before use.

1.5e Buried cereal test

Food restricted mice (90% of body weight) were used in buried cereal test to ensure mice were motivated to seek food. Mice were individually separated, and body weight was monitored every day before testing. A sweetened cereal was given to the tested mice before testing to
overcome food neophobia. On all test days, mice were habituated for 1 hour in their cages without water or food. Clean cages with ~3 cm evenly distributed bedding were prepared, and 1 piece of sweetened cereal was buried 0.5 cm below the bedding. Mice were transferred from the habituation cage to the prepared new cage. A 5-minute timer was started when the mouse was introduced in the testing cage, and time was recorded when the mouse found the cereal and began eating it. If the mouse cannot uncover the pellet within 5 min, 300 sec was recorded for that mouse. Buried cereal test was performed 5 days in a row, surface test was performed on the 6th test (cereal was put on the surface of bedding). Time to uncover the buried pellet from day 3-5 was averaged and then compared between wild-type and mutant mice using a Mann-Whitney U test; similarly, time to uncover the surface pellet from 6th day was compared between groups.

1.5f Habituation/Dishabituation test

The habituation/dishabituation test was performed as previously described27 to assess the ability to discriminate two non-social odors. Mice were isolated in a clean cage overnight before testing. A tissue cartridge holding a non-scented cotton ball was placed in the cage to let mice get used to the novel item. On the testing day, mice were moved to the testing area without water and food for 1-hour habituation. After habituation, a scented tissue cartridge (noted as odor 1 in Fig 2D) was placed into cage. Animals were video recorded from front side of the cage for 30 sec. This was repeated for 6 consecutive trials with odor 1, with inter-trial intervals of 5 minutes. On the 7th trial, a novel odor (noted as odor 2 in Fig 2D) was introduced. Time sniffing was measured on each trial during the 30 sec test period. Almond extract (5 μL) was used as odor 1, banana extract (5 μL) was used as odor 2. Almond and banana scent were selected because they were considered as neutral odors for mice38.
1.5g Block test

To measure ability to discriminate social odors, we performed the block test as previously described\textsuperscript{30}. Each mouse was individually housed in a clean cage with bedding and 5 wood blocks labeled A-E at least 24 hours before testing. On testing day, all five blocks were removed from cage and placed into a sealed bag. Mice were transferred to testing area without food and water for 1-hour habituation. After habituation, blocks A-D from the same mouse were placed back into the cage. Mice were video recorded from front side of the cage for 30 sec. This procedure was repeated 6 times with at least 5 min interval between trials. On the 7\textsuperscript{th} trial, the same procedure was performed, but block D was replaced with block E from another mouse’s cage so that A, B, C were home-cage blocks and block E was from other mouse’s cage. Sniffing time of each block during the 30 sec test period from 7\textsuperscript{th} trial was measured.
CHAPTER 2

SPlicing IN A SINGLE NEURON IS COORDINATELY CONTROLLED BY RNA BINDING PROTEINS AND TRANSCRIPTION FACTORS

These are published data – Thompson et al., 2019, eLife

2.1 Background

Gene expression guides the identity of an individual cell, determining the complement of genes which control its function and development. Transcriptional regulation is a major component of gene expression, establishing which genes are expressed at key developmental time points and in specific tissue types. Post-transcriptional regulation further shapes and diversifies cellular attributes through mechanisms such as alternative splicing. Recent molecular studies have characterized networks of transcription factors which shape gene expression in single neurons. However, less is known about post-transcriptional regulation, and the associated RNA binding proteins (RBPs) which mediate this regulation, especially at the level of individual neurons. It is also unknown to what extent transcriptional and post-transcriptional gene regulatory networks are coordinated in single cells. A number of studies have identified individual RBPs that affect the splicing of a TF, altering the activity or specificity of that TF\textsuperscript{39–41}. This suggests that extensive cross-talk occurs between these two regulatory layers, adding another measure of regulation and specificity to establishing the gene expression network in an individual cell.
The nematode *Caenorhabditis elegans* is a powerful model for neuronal transcriptomics, with all 302 of its neurons identified and many neuronal genes conserved in humans. *C. elegans* has been used extensively as a model to reveal underlying principles by which TFs shape the transcriptomes of individual neurons. The worm’s invariant cell lineage, coupled with genetic tools and a transparent body, enables systematic in vivo analysis of gene expression in single neurons, and identification of TFs responsible for cell-specific gene expression. This type of analysis has revealed a number of gene regulatory principles, including the concept of a ‘combinatorial code’ of TFs which can be re-used in different neuron types, with particular combinations of TFs determining specific cell fates\(^{42-44}\). Another example is the concept of phenotypic convergence, by which various neurons express similar gene networks but the TFs driving the networks are different for each neuron type\(^{42,44}\). These principles appear to apply to the nervous systems of other organisms as well\(^{45}\). However, it remains unknown whether similar mechanistic principles apply to post-transcriptional regulation by RBPs in the nervous system.

RBPs bind and associate with RNA in the cell, functioning in many different aspects of RNA biology including pre-mRNA processing, mRNA export and localization, and translation\(^{46,47}\). A major function of RBPs is their role in alternative splicing of pre-mRNA. Through alternative splicing, a single transcript of RNA can be rearranged to yield a wide variety of final transcripts and even multiple distinct functional proteins. This is essential for generating the diverse and complex proteome of eukaryotes and is particularly significant in the nervous system, where a finite number of neuronal genes are carefully regulated and alternatively processed throughout development to yield neurons with diverse functions\(^{48-51}\). RBP dysfunction is especially detrimental in the nervous system and has been implicated in neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and Huntington’s disease\(^{52-55}\). Other
neuronal diseases such as spinal muscular atrophy, which affects motor neurons, are caused by splicing errors\textsuperscript{56–58}. Understanding how RBPs, particularly those enriched in the nervous system, regulate gene expression through splicing and other RNA modifications is essential for developing therapies that can treat these kinds of diseases.

Here we investigate the cell-specific splicing of \textit{sad-1}, a conserved neuronal kinase. We take advantage of the transparent body of \textit{C. elegans}, employing single-cell in vivo fluorescent splicing reporters to visualize splicing patterns in specific neuron populations. The \textit{C. elegans} \textit{sad-1} gene encodes two isoforms that differ in their ability to interact with the F-actin binding protein NAB-1/Neurabin\textsuperscript{59}, and have different roles in synapse formation and development\textsuperscript{60}. We discovered unique splicing patterns of \textit{sad-1} in different neuron types, including expression of distinct isoforms in two closely related neurons, the ALM and BDU. We investigated the regulation of \textit{sad-1} splicing in the ALM and BDU and found a network of three cell-fate determining TFs and two RBPs required for proper splicing. We determined that the three TFs regulate cell-specific expression of the two RBPs, which themselves directly bind to \textit{sad-1} intronic regions to mediate exon inclusion in the ALM neuron. Finally, we find similar principles in other neuron types, with unique neuron-specific combinations of TFs and RBPs regulating \textit{sad-1} alternative splicing. These results indicate a combinatorial code of RBPs and TFs that shape neuron-specific splicing patterns, and demonstrate phenotypic convergence through which different RBPs mediate similar outcomes in various neuron types.

\textbf{2.2 Alternative splicing of the neuronal kinase \textit{sad-1} in specific cell types}

To identify alternative splicing regulation in individual neuronal cell types, we created two-color splicing reporters that provide a fluorescent readout of splicing regulation \textit{in vivo} in single cells\textsuperscript{61,62}. A minigene representing an alternative splicing event of interest is cloned.
upstream of a dual GFP/RFP cassette (Fig 3A-B). The GFP and RFP coding sequences reside in distinct reading frames. The alternative exon is engineered to shift the reading frame by +1 nucleotide such that splicing of the alternative exon determines the reading frame, and therefore the translation of GFP versus RFP. Application of two-color fluorescent reporters to transparent organisms such as *C. elegans* enables in vivo imaging of alternative splicing in individual cells. We have created reporters for splicing events in a number of neuronal genes, and uncovered a rich variety of splicing patterns in single neurons\(^{63}\).

One intriguing example of neuron-specific alternative splicing is in the conserved neuronal kinase *sad-1*, which plays important roles in neuronal development in both worms and mice\(^{64,65}\). In *C. elegans*, *sad-1* is encoded by seventeen exons, and the fifteenth exon is an alternative cassette-type exon (Fig 3A). Alternative splicing of this exon changes the coding sequence and length of the *sad-1* C-terminus\(^{60}\). This presents an interesting parallel with mice and human genomes, which encode two separate genes homologous to *sad-1* (SAD-A and SAD-B) that are nearly identical except for their C-terminal coding sequence and length.

A two-color splicing reporter for *sad-1* in *C. elegans* revealed that many neurons express both the skipped and included isoforms. For example, motor neurons in the ventral nerve cord express both isoforms of *sad-1* (Fig 3C). On the other hand, the ALM touch-sensing neuron expresses only the included isoform, while the BDU neuron, which is the sister cell to the ALM neuron, expresses only the skipped isoform (Fig 3C-F). While different neurons exhibit differences in *sad-1* splicing, the splicing pattern in a given neuron is reproducible and invariant from one animal to the next, suggesting that *sad-1* splicing in various neurons is under strict regulatory control. These results led us to ask how ALM and BDU neurons, which are
Figure 3: sad-1 is alternatively spliced in single neurons. (A) The sad-1 gene. Alternative cassette exon in blue. (B) Two-color splicing reporter schematic for sad-1 cassette exon. The cassette exon encodes a + 1 nt frameshift so that when skipped, GFP is produced with an in frame stop codon. When skipped, GFP is read out of frame without stop codons, followed by in-frame translation of RFP. (C) Whole worm fluorescent micrograph demonstrating both exon inclusion (RFP) and skipping (GFP) in many neurons, while certain neurons express only the included (ALM) of skipped (BDU) isoforms. (D–E) Higher magnification focusing on ALM and BDU neurons. (F) BDU and ALM are both paired neurons present on the left and right side of the worm. Each BDU neuron is a sister cell to an ALM neuron, derived from the same neuroblast. Scale bar represents 10 µm.
developmentally related (Fig 3F) and share a number of anatomical and gene-expression features, specify opposite splicing regimes.

2.3 Forward genetic screen identifies three fate-determining TFs affecting sad-1 splicing in the ALM neuron

To identify regulators of sad-1 splicing in the ALM touch neuron, we performed an unbiased forward genetic screen. Parental worms harboring the sad-1 splicing reporter were mutagenized with EMS. We then screened for F2 animals (potential homozygotes) with aberrant expression of the skipped (GFP) isoform in the ALM neuron (Fig 4A). This screen identified three distinct loci that transform the splicing pattern from the ALM neuron pattern (full exon inclusion) to resemble the pattern in their BDU sister cells (full exon skipping).

Whole-genome resequencing of the mutant strains identified loss-of-function mutations in three conserved TFs: unc-86, mec-3, and alr-1 (Fig 4B-F). All three genes have previously been identified as key regulators of touch-neuron cell fate43,66. The three TFs function in a transcriptional cascade ensuring cell-specific expression of mec-3 in touch neurons, which then results in expression of a battery of touch-neuron specific genes (Fig 4G). Loss of the TF mec-3 results in touch neurons (ALMs) adopting certain gene-expression characteristics of their sister cells (BDUs)43, mirroring our observation that loss of mec-3 transforms sad-1 splicing from an ALM (exon 15 included) to a BDU (exon 15 skipped) pattern.

Previous work demonstrates that the MEC-3 TF is expressed only in touch neurons, while UNC-86 and ALR-1 are expressed in various neuron types66. However, we find that unc-86 and alr-1 mutants affect sad-1 splicing only in the touch neurons (Figure 4D–F). This is in accordance with previous work indicating that a major function of unc-86 and alr-1 in touch neurons is to combinatorially ensure appropriate expression of mec-3, and that all three TFs are
Figure 4: Genetic screen identifies neuronal TFs affecting sad-1 splicing in the ALM neuron. (A) Schematic of forward genetic screen to identify regulators of sad-1 splicing in the ALM touch neuron. (B–F) ALM neurons (dashed boxes) shift from complete inclusion (RFP) to skipping (GFP) in unc-86(e1416), mec-3(e1338), or alr-1(oy42) TF mutants. Splicing phenotypes fully penetrant (n = 50 animals) (G) Previously-identified roles of the three TFs in a transcriptional cascade to control touch neuron gene expression. Scale bar represents 10 µm.
needed for proper differentiation of touch neurons\textsuperscript{66}. We therefore conclude that the combinatorial activity of all three TFs is required for proper \textit{sad-1} splicing in the ALM neuron.

\textbf{2.4 A pair of RNA binding proteins regulates \textit{sad-1} splicing in the ALM neuron}

We were surprised to identify TFs, but not RBPs, in our forward genetic screen for regulators of \textit{sad-1} alternative splicing. We hypothesized that multiple RBPs might co-regulate \textit{sad-1} alternative splicing in the ALM neuron and therefore mutations in individual RBPs might result in mild splicing defects. We therefore examined the sequence surrounding the \textit{sad-1} alternative exon for conserved \textit{cis}-elements corresponding to known in vitro RBP sequence preferences\textsuperscript{67}. We identified three candidate elements: one corresponding to the \textit{mbl-1}/Mbnl1 consensus binding motif, and two corresponding to the \textit{mec-8}/RBMS motif (Fig 5A-C).

\textbf{Figure 5: Two neuronal RBPs combinatorially control \textit{sad-1} splicing in ALM neurons.} (A) Conservation scores in the introns surrounding \textit{sad-1} exon 15, basewise phyloP26way comparison of 26 nematode species\textsuperscript{68}. Numbers 1–3 indicate consensus binding motifs for \textit{mbl-1} and \textit{mec-8} displayed in B–C. (B–C) \textit{cis}-elements matching consensus binding motifs for \textit{mbl-1} and \textit{mec-8}. (D–F) \textit{mec-8} and \textit{mbl-1} mutants both cause a partial loss of \textit{sad-1} exon inclusion. (G) \textit{mec-8}; \textit{mbl-1} double mutants cause complete loss of exon inclusion, phenocopying the TF mutants. Splicing phenotypes fully penetrant (\(n = 50\) animals) Scale bar represents 10 \(\mu\text{m}\).
To test whether these RBPs affect *sad-1* alternative splicing, we created deletions for each gene with CRISPR/Cas9\(^{14}\). Both *mec-8* and *mbl-1* mutants result in aberrant *sad-1* splicing in the ALM neuron, displaying partial skipping and partial inclusion (Fig 5D-F). As in the case of the TF mutants, *mec-8* mutants affect *sad-1* splicing specifically in the ALM neurons, whereas *mbl-1* mutants affect *sad-1* splicing in ALM neurons as well as specific neurons in the ventral nerve cord (see Figure 8, below). To verify that the phenotypes of our CRISPR mutants were on-target effects, we crossed the *sad-1* splicing reporter into existing alleles for *mec-8* (*e398*, premature stop codon\(^{69,70}\)) and *mbl-1* (*wy560*, large deletion affecting multiple genes including *mbl-1*\(^{71}\)). We found these alleles to affect splicing of *sad-1* exactly as our CRISPR mutation (Fig 6).

![mec-8(e398) and mbl-1(wy560)](image)

**Figure 6:** Canonical RBP alleles of *mec-8* and *mbl-1* affect *sad-1* splicing similarly to CRISPR deletions of *mec-8* and *mbl-1*. *mec-8* (*e398*) premature stop codon mutation and *mbl-1* (*wy560*) large deletion. Splicing phenotypes are fully penetrant (n = 50 animals). Both canonical RBP mutants cause a partial loss of *sad-1* exon inclusion.

Whereas TF mutants result in full skipping of the *sad-1* alternative exon, RBP mutants result in only partial skipping. This provides a probable explanation for not identifying these RBPs in our genetic screen: partial exon skipping leads to dim GFP expression, which is not sufficiently bright to be noticed upon brief visual inspection. We therefore tested whether
simultaneous loss of both RBPs recapitulates the full skipping of sad-1 exon 15 observed in TF mutants. We created mec-8; mbl-1 double mutants expressing the sad-1 splicing reporter. These double mutants result in complete loss of sad-1 exon inclusion in the ALM neuron, recapitulating the splicing phenotype of the single TF mutants (Fig 5G). These results led us to hypothesize that the TFs identified in our screen exert their effects on sad-1 splicing by controlling expression of both mec-8 and mbl-1.

2.5 TFs affect sad-1 splicing by controlling RBP expression in the ALM neuron

To examine whether the neuronal TFs alter expression of mec-8 and mbl-1 RBPs in the ALM neuron, we created reporter lines for each RBP. To this end, each RBP was C-terminally tagged in a fosmid containing large regions of surrounding genomic context\(^{71,72}\) (Fig 7A-E). Compared to traditional transgenic reporters, fosmids are more likely to contain all regulatory information needed to drive normal expression of the gene in question. This is demonstrated in the case of the mec-8 RBP. The classical mec-8::GFP promoter fusion drives expression in a number of cells, but not in the ALM neuron\(^73\). On the other hand, we detected expression of the mec-8 fosmid reporter in many of the same cells, both neuronal and non-neuronal, plus strong expression in the ALM neuron (Fig 7A-B). A similar fosmid reporter for mbl-1 likewise exhibits expression in the ALM neuron, as well as many other neurons in the nervous system (Fig 7D). This is in line with previous reports on mbl-1 expression\(^71\).

We tested expression of our reporters in the context of a mec-3 mutant to determine whether expression of mec-8 and mbl-1 in ALM neurons depends on the TF cascade uncovered in our screen. The mec-3 TF is expressed only in touch neurons, and therefore we would expect mec-3 mutants to affect RBP expression only in the touch neurons. Indeed, in mec-3
Figure 7: Neuronal TFs establish expression of both mec-8 and mbl-1 to mediate splicing of sad-1 in ALM neurons. (A–B) A mec-8 translational GFP fosmid reporter reveals strong expression in ALM neuron (strong expression in 28/31 = 90% of animals inspected). (C) In a mec-3 TF mutant, mec-8 expression is absent specifically in ALM (no detectable expression in 43/50 = 86%, dim expression in 7/50 = 14% of animals inspected). (D) mbl-1 translational RFP fosmid reporter is expressed in ALM neuron (strong expression in 19/20 = 95% of animals inspected). (E) In a mec-3 mutant, mbl-1 expression is absent specifically in ALM (no detectable expression in 19/21 = 90%, dim expression in 2/21 = 10% of animals inspected). (F–G) Aberrant splicing of sad-1 in alr-1 TF mutants is partially rescued by over-expression of either mec-8 (6/6 animals examined) or mbl-1 (6/7 animals examined) RBPs (H–I). Scale bar represents 10 µm.
mutants, expression of both mec-8 and mbl-1 RBPs are abolished in the ALM neuron, while expression in the surrounding neurons and tissues remains unchanged (Fig 7B-E). Together these results indicate that the expression of mec-8 and mbl-1 RBPs are under the control of neuron subtype-specific TFs.

The observations that (1) mec-8; mbl-1 RBP double mutants recapitulate the phenotype of the TF mutants, and (2) the TFs are necessary for expression of both RBPs in the ALM neuron, together suggest that the splicing defects in the TF mutants are mediated by effects on expression of the two RBPs. Further support for this hypothesis arose indirectly in the course of crossing TF and RBP mutants together. We found that while TF or RBP mutant heterozygotes exhibit normal sad-1 splicing in the ALM neuron, double heterozygotes (for example alr-1/+; mbl-1/+ or mec-3/+; mec-8+/+) exhibit partial exon skipping in ALM, similar to the RBP single mutants. Such ‘non-allelic non-complementation’ is often interpreted to mean that the two genes function in the same complex, or in the same pathway. This indirect evidence further suggests that the TFs and RBPs affect sad-1 splicing as part of the same molecular pathway.

If sad-1 splicing is controlled in a linear pathway as suggested by the above series of experiments, with upstream TFs affecting RBP expression in the ALM neuron, then over-expressing an RBP in the context of a TF mutant should partially restore splicing in ALM. To test this hypothesis we created a strain over-expressing a mec-8 transgene specifically in the touch neurons (pmec-3::mec-8). When introduced into an alr-1 mutant, this transgene partially rescues the splicing of sad-1 in the ALM neuron (Fig 7F-H). Likewise, over-expression of mbl-1 in an alr-1 mutant partially rescues splicing in the ALM neuron (Fig 7I). These results further support a linear gene regulatory pathway in which neuronal fate-determining TFs control neuron-specific expression of RBPs, which then control alternative splicing of sad-1 (Fig 7J).
2.6 RBPs directly mediate *sad-1* exon inclusion through interactions with surrounding introns

To test whether *mec-8* and *mbl-1* directly affect splicing by binding to the *sad-1* pre-mRNA, we created two-color splicing reporters in which the putative *mec-8* or *mbl-1* cis-elements are mutated (Fig 5A and Fig 8). If the RBPs act directly by binding the cis-element, then mutation of the cis-element should affect the splicing pattern in a manner resembling the wild-type splicing reporter in the context of the RBP deletion mutant. If the RBPs act indirectly, mutating the cis-element should have no effect on the splicing pattern.

**Figure 8:** *mbl-1* and *mec-8* affect *sad-1* splicing by direct interaction with *sad-1* introns. (A–B) Mutation of *mbl-1* consensus sequence in *sad-1* splicing reporter results in aberrant splicing in ALM neurons that phenocopies an *mbl-1* mutant. (C–E) Mutation of either *mec-8* binding motif, or both simultaneously, likewise results in aberrant *sad-1* splicing in ALM neurons. ALM splicing phenotypes fully penetrant (n = 25 animals) Scale bar represents 10 µm.
Mutation of the \textit{mbl-1} cis-element resulted in ALM neurons with altered \textit{sad-1} splicing in which the exon is partially skipped and partially included (Fig 8A-B). This recapitulates the phenotype of \textit{mbl-1} null mutations (Fig 5F), suggesting that \textit{mbl-1} exerts its effects on splicing directly through binding a conserved \textit{cis}-element in the upstream intron.

We identified two consensus \textit{mec-8} binding motifs in conserved regions in the intron downstream of the cassette exon. We therefore created splicing reporters mutant for both \textit{cis}-elements as well as for each element individually. The splicing reporter mutant for both elements recapitulates the splicing phenotype of \textit{mec-8} null mutants (Fig 8E). Likewise, mutating either \textit{mec-8} binding site in isolation recapitulates a \textit{mec-8} null mutation (Fig 5E and Fig 8C-D), suggesting that \textit{mec-8} binding to both \textit{cis}-elements is required for appropriate \textit{sad-1} splicing.

We tested whether mutation of a putative \textit{cis}-element could be rescued by over-expression of its cognate RBP, and found that \textit{cis}-element mutants were not rescued by RBP over-expression, providing further evidence that the RBPs act directly on the \textit{sad-1} pre-mRNA. Together these results indicate that \textit{mec-8} and \textit{mbl-1} RBPs combinatorially ensure \textit{sad-1} exon inclusion in ALM neurons through direct interactions with the neighboring introns.

\textbf{2.7 \textit{sad-1} splicing in other neuron types is controlled through both distinct and overlapping mechanisms}

Having identified regulatory mechanisms controlling \textit{sad-1} splicing in the ALM neuron, we next wondered whether similar principles apply in other neuron types. Most neurons besides the ALM and BDU neurons express both skipped and included \textit{sad-1} isoforms. This could represent the neuronal ‘ground state’ of splicing in the absence of cell-specific splicing regulators. On the other hand, our observations that loss of both \textit{mec-8} and \textit{mbl-1} in the ALM neuron results in full exon skipping suggest that the ground state may be complete exon
skipping. This hypothesis predicts that other neurons in which sad-1 is partially included express one or more RBPs mediating exon inclusion.

In the course of examining sad-1 splicing in ALM neurons, we noticed that mbl-1 mutants affect sad-1 splicing not only in ALM, but also in the excitatory cholinergic motor neurons of the ventral nerve cord (Fig 9A-D). Whereas mbl-1 mutants cause a change in sad-1 splicing from full inclusion to partial inclusion in ALM neurons, in excitatory motor neurons mbl-1 mutants shift from partial inclusion to no inclusion (Fig 9C-D). On the other hand, the inhibitory motor neurons remain unaffected in mbl-1 mutants, expressing both the included and skipped isoforms (Fig 9D, arrowheads). This is consistent with our mbl-1 gene expression reporter, which reveals expression of mbl-1 in the excitatory motor neurons, but not in the inhibitory motor neurons.

Figure 9: sad-1 splicing in motor neurons of the ventral nerve cord is controlled by mbl-1 and msi-1 RBPs. (A–C) In wild-type worms, sad-1 is partially included in both excitatory and inhibitory motor neurons. (D) In mbl-1 mutants, exon inclusion is lost in excitatory motor neurons, but remains in inhibitory motor neurons (arrowheads). (E) msi-1 mutants lose exon
inclusion in inhibitory motor neurons (arrowheads) but not in excitatory motor neurons. (F) *mbl-1; msi-1* double mutants lose exon inclusion in all motor neurons in the ventral nerve cord. Splicing phenotypes in ventral nerve cord invariant (n = 15 animals) (G) Conservation scores (determined as in Figure 3A) in the introns surrounding *sad-1* exon 15. Number one indicates consensus binding motifs for *msi-1*. (H) *cis*-elements matching consensus binding motifs for *msi-1*. Asterisk indicates anterior-posterior position of ALM neuron as anatomical reference. Splicing phenotypes fully penetrant (n = 50 animals). Scale bar represents 10 µm.

We did not detect *mec-8* expression in motor neurons of the ventral nerve cord, and *mec-8* mutants had no effect on splicing of *sad-1* in motor neurons (Fig 5E and Fig 7B). It therefore seems that in neurons expressing *mbl-1* such as excitatory motor neurons, the presence of *mbl-1* mediates partial exon inclusion. In neurons expressing both *mbl-1* and *mec-8* such as ALM touch neurons, the two RBPs together mediate full inclusion.

In *mbl-1* mutants, *sad-1* exon inclusion is lost in excitatory neurons but remains in inhibitory motor neurons. We therefore wondered whether there was an additional RBP expressed in inhibitory motor neurons mediating *sad-1* inclusion. *mec-8* was ruled out because it is not expressed in inhibitory motor neurons and does not affect *sad-1* splicing in the nerve cord. On the other hand, the RBP *msi-1*/Musashi has been reported to be expressed in inhibitory but not excitatory neurons of the nerve cord⁷⁵, which is a mutually exclusive pattern with *mbl-1*. We therefore tested *msi-1* as a candidate for the RBP mediating *sad-1* exon inclusion in the inhibitory motor neurons. We generated a *msi-1* deletion mutant, which shows loss of *sad-1* inclusion specifically in the inhibitory motor neurons (Fig 9E). Furthermore, *msi-1; mbl-1* double mutants result in complete loss of exon inclusion in the ventral nerve cord (Fig 9F). These results indicate that *mbl-1* and *msi-1* act in distinct cell types to achieve partial *sad-1* exon inclusion throughout the ventral nerve cord.

We suspect that *msi-1*, like *mbl-1* and *mec-8*, directly affects *sad-1* splicing by binding in the intronic regions surrounding the alternative exon. *in vitro* experiments have identified a UAG
motif (Fig 9H)\textsuperscript{67}, usually in bipartite form (\textit{e.g.} UAGNNUA\textit{G})\textsuperscript{76}, as the consensus binding motif for \textit{msi-1}. There is a conserved bipartite UAG motif in the intron downstream of the \textit{sad}-\textit{l} cassette exon (Fig 9G-H), and we hypothesize that \textit{msi-1} binds there to mediate exon inclusion in inhibitory motor neurons.

Together the results from three different neuronal cell types (ALM neuron, excitatory motor neurons, and inhibitory motor neurons) constitute an example of phenotypic convergence, in which phenotypic similarity between cells is generated by distinct molecular mechanisms. Substantial evidence of such phenotypic convergence exists for TFs controlling neuronal properties in worms and flies\textsuperscript{42,44,45}. Our results now extend this principle to RBPs and their control of alternative splicing, revealing phenotypic convergence in which similar splicing patterns (\textit{i.e.} \textit{sad}-\textit{l} exon inclusion) are generated in various neurons by diverse RBPs acting in specific neuronal subtypes (Fig 10).

\textbf{Figure 10: Phenotypic convergence at the level of splicing regulation.} Different RBPs act in different neuron types to carry out the common function of mediating \textit{sad}-\textit{l} exon inclusion.
2.8 Discussion

2.8a Neuron-specific regulation of sad-1 splicing

In this study we find that sad-1 splicing undergoes precise regulation in numerous neuronal types. Although ALM and BDU neurons are sister cells, express many of the same genes, and share a number of cell-specific TFs, they have opposing patterns of sad-1 splicing. This highlights the fact that post-transcriptional control can further diversify attributes of single cells on top of the more well-known role of transcriptional control.

Our results demonstrate that sad-1 splicing is regulated according to a combinatorial RBP code, with different splicing outcomes depending on whether a cell expresses zero, one, or two neuron-specific RBPs (Fig 10). This suggests that the ‘default’ outcome of sad-1 splicing is full skipping of the cassette exon, as observed in the BDU neuron which does not express any of the sad-1-regulating RBPs. Only cells with at least one RBP mediating exon inclusion express sad-1 included isoforms. Cells with multiple such RBPs (e.g., the ALM neuron) express only the included isoform.

2.8b Coordinated splicing regulation across layers of gene expression

The importance of TFs controlling gene expression networks in single neurons is well established, and the importance of RBPs controlling post-transcriptional networks in single cells is gaining wider appreciation. How these two modes of regulation might interact remains understudied. Here we show that the two modes of regulation interact in a traditional linear type of pathway. A combination of cell-specific TFs establishes a transcriptional network in a single neuron type. This network includes a specific combination of neuronal RBPs, and the particular combination of RBPs in a given neuron then establish a unique post-transcriptional gene
regulatory network in that neuron. Multiple layers of regulatory control can thus increase the diversity of single neuron transcriptomes and fine-tune the properties of individual neurons.

Here, we have identified a linear pathway in which TFs influence the expression of RBPs, which then influence alternative splicing in single neurons. This adds to a substantial body of literature finding that RBPs can affect the function of specific TFs by modulating their alternative splicing. It will be interesting next to determine whether additional regulatory logics exist between TFs and RBPs. Single-neuron TF combinations have been identified with a variety of feedback and feedforward mechanisms resulting in interesting regulatory properties, and in principle TFs and RBPs could likewise interact in complex ways, leading to an even greater array of diversification strategies. Together this study highlights the importance of considering neuron-specific ‘combinatorial codes’ not only from the perspective of TF combinations, but the specific complement of both TFs and RBPs shaping the transcriptome of a given neuron.

2.9 Methods

2.9a Strain maintenance

C. elegans were maintained under standard conditions at 20°C on nematode growth media (NGM) plates seeded with OP50 E. coli bacteria.

2.9b Mutant generation and genetic screening

The forward mutagenesis screen was performed on animals harboring the sad-1 exon 15 splicing reporter with EMS at 47 mM for 4 hours. F1s were picked onto new plates, 10 F1s per plate. After 3-4 days of growth, F2s were screened by eye on the Zeiss Axiozoom.V16 for touch cells appearing in the GFP channel (representing aberrant exon skipping) and were then verified for a concomitant loss of RFP (representing loss of exon inclusion). Such worms were picked
individually onto a new plate to verify the phenotype in the F3 generation and to establish a clonal population. After outcrossing, strains were subjected to whole-genome resequencing (Illumina, 1 x 75 bp) and potential causative mutations were identified using the CloudMAP workflow on the Galaxy web platform\textsuperscript{83}.

Targeted mutant strains were generated using CRISPR/Cas9 as previously described\textsuperscript{13,84}, such that the gene of interest is deleted and is replaced with a heterologous GFP reporter construct. Seamless gene replacement was verified by PCR amplification and Sanger sequencing of both junction boundaries.

2.9c Microscopy

Images were obtained with a Zeiss Axio Imager.Z1 and processed in ImageJ.

2.9d Generation of splicing reporters

sad-1 minigenes were created using the following primers: Forward 5’

GATAAAACTGAAACAACTTCTGC and Reverse 5’

GGGGTTGGCGATTTGTATGAGaTAGC. Restriction sites were appended to both the forward primer (XhoI) and reverse (NotI) primers to facilitate cloning into a Gateway-compatible vector as previously described\textsuperscript{63}. Mutant versions of the splicing reporter were synthesized \textit{de novo} then cut with XhoI and NotI and cloned as above.

Some strains were provided by the Caenorhabditis Genome Center, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). Other strains were provided by the National BioResource Project (Tokyo).
CHAPTER 3

DISSECTING INTERACTIONS ACROSS GENE REGULATORY LAYERS: FUST-1, TDP-1, AND CEH-14 COORDINATE FERTILITY IN C. ELEGANS

3.1 Background

Eukaryotic gene expression requires coordination across multiple layers of regulatory control, including transcription, RNA processing, and translation. Two major classes of proteins responsible for this gene expression regulation are transcription factors (TFs) and RNA binding proteins (RBPs). Regulatory activities for individual TFs and RBPs have been well described, and a growing body of recent evidence demonstrates extensive crosstalk between transcriptional and post-transcriptional factors. Our previous work lent further evidence to this crosstalk, identifying a set of TFs and RBPs that coordinate sad-1 alternative splicing. Nevertheless, how TFs and RBPs coordinately control gene expression across multiple regulatory layers has remained unclear.

RBPs regulate many aspects of RNA processing including pre-mRNA splicing, mRNA export and localization, and translation. RBP dysfunction is especially notable in the nervous system, and mutations have been implicated in multiple neurodegenerative diseases. For example, the RBPs TDP-43 and FUS are primarily found in the nucleus and are involved in several RNA-related functions, including transcription, splicing, and RNA transport.
Mutations in either RBP are directly linked to amyotrophic lateral sclerosis (ALS) and cause their mislocalization and aggregation in the cytoplasm, leading to progressive degeneration of neurons. The disease-associated roles of RBPs such as TDP-43 and FUS have been extensively studied, but in many cases the physiological functions for these RBPs remain unresolved. Understanding how RBPs play a role in essential cellular functions and in the context of global gene expression coordination will be key for understanding and treating these kinds of diseases.

To identify functionally important TF-RBP gene expression coordination, we set out to systematically test for genetic interactions between TF and RBP mutants. Screens for genetic interactions, in which a phenotype occurs in a double mutant that is not predicted based on the single mutant phenotypes, have a rich history of identifying genes with related activities and/or redundant functions. In single-celled organisms such as bacteria and yeast, genetic interaction analysis has been carried out at genome-wide scale, revealing hundreds of thousands of interactions in which a double mutant has a fitness greater than expected (positive interaction) or less than expected (negative or synthetic interaction) based on the single mutant fitness phenotypes.

In the nematode C. elegans, we recently employed CRISPR/Cas9 to systematically knock out neuronally-enriched RBPs using homology-guided replacement to insert heterologous GFP fluorescent markers in place of the deleted gene. These CRISPR/Cas9-generated RBP mutants enabled us to conduct a systematic pairwise genetic interaction screen across neuronal RBPs in C. elegans. We identified multiple novel synthetic interactions and revealed previously-unexplored physiological functions for several RBPs. Here, we employed a similar synthetic
genetic interaction (SGI) screen between neuronally-enriched RBPs and TFs to investigate coordination of gene expression across regulatory layers.

To do so, we generated all possible double-mutant TF-RBP combinations of 10 RBP and 11 TF gene deletion mutants, creating a total of 110 double mutants. We identified significant novel phenotypes in several double mutants, revealing extensive functional interactions between TFs and RBPs. One striking synthetic phenotype was reduced fertility in \textit{tdp-1; ceh-14} and \textit{fust-1; ceh-14} double mutants. \textit{tdp-1} and \textit{fust-1} are the \textit{C. elegans} homologs of TDP-43 and FUS, and mutations in both RBPs have been implicated in ALS\textsuperscript{89,95,98}. Both \textit{tdp-1; ceh-14} and \textit{fust-1; ceh-14} double mutants exhibit reduced egg production, decreased sperm efficacy, and gonad migration defects. As \textit{tdp-1}, \textit{fust-1}, and \textit{ceh-14} single mutants do not exhibit the same striking fertility phenotype as these double mutants, our findings identify a potential coregulatory role for these genes in gonad and sperm development. We find a shared role of \textit{fust-1} and \textit{tdp-1} in inhibiting exon inclusion, and identify a cassette exon in \textit{pqn-41}, inhibited by \textit{tdp-1}, that contributes to the fertility defects in \textit{tdp-1; ceh-14} double mutants. Our findings thus uncover novel physiological functions for \textit{fust-1} and \textit{tdp-1}, in the specific context of a \textit{ceh-14} mutant background, and shed light on their shared molecular roles.

\textbf{3.2 Genetic interaction screen identifies synthetic fitness effects in several double mutants}

To identify regulatory crosstalk with important functional consequences, we performed a genetic interaction screen between TF and RBP mutants in \textit{C. elegans}. We focused on evolutionarily-conserved RBPs and TFs expressed in the nervous system, and took advantage of existing deletion alleles to generate all possible double-mutant combinations of 10 RBPs and 11 TFs. To identify genetic interactions, we measured relative fitness using a simple and quantitative competitive fitness assay\textsuperscript{14,84}. In this assay, equal numbers of stage-matched mutant
and wild-type worms are grown together on a single growth plate. The worms are given five days to develop, eat available food, and reproduce for multiple generations (Fig 11A). Then the relative proportions of mutant and wild-type worms are quantified, assigning a value to the fitness of each genotype. A mutant with an identical fitness to wild-type would grow and reproduce at the same rate as wild-type worms, yielding a population of 50% mutants and 50% wild-type. This would yield a fitness value of 1 (Fig 11A), and increased or decreased fitness would result in values greater than or less than 1, respectively. Competitive fitness assays can identify mutants with a variety of underlying phenotypes, including lethality, developmental defects, reproductive defects, and behavioral defects.14,97

Each RBP and TF single mutant strain was first assayed against wild-type worms to establish their respective relative fitness values (Fig 11B-C). The fitness values of the 10 RBPs and 11 TFs we assayed ranged from strong decreases in fitness to mild increases in fitness (Fig 11B-C). Several mutants with known behavioral defects, including alr-1, unc-86, and mec-870,99,100, showed significantly lower fitness than wild-type. We identified a few single mutants with novel fitness phenotypes not predicted by previously-described phenotypes, for example a reduction of fitness in tab-1 mutants (Fig 11B). We also found that a few mutants, including aptf-1 and fox-1, outperformed wild-type worms in our assays and have fitness values greater than 1.

After establishing baseline fitness values for single mutants, each RBP mutant was crossed to each TF mutant to obtain all possible RBP; TF mutant combinations, yielding a total of 110 RBP; TF double mutants. These crosses were facilitated by the use of either existing deletion alleles which can be genotyped by simple PCR, or by CRISPR/Cas9-mediated deletions.
A 5 days at 25°C (2 generations)

% mutant
% expected (50%) = Relative fitness value

B RBP relative fitness

C TF relative fitness

D msi-1; unc-86 relative fitness

E exc-7; ets-5 relative fitness

F RNA Binding Proteins

Transcription Factors

Synthetic fitness effect (c)
in which a traceable fluorescent maker was inserted into the deletion locus, enabling *in vivo* monitoring of the genotype (see methods for list of genotypes used). To systematically identify genetic interactions between the RBPs and TFs, we conducted competitive fitness assays in which each double mutant was assayed with one of its constituent single mutants. Assuming no genetic interaction, the fitness value of the double mutant would be expected to equal that of the single mutant that was not being assayed. For example, when *msi-1*; *unc-86* double mutants were competed against *unc-86* mutants, the expected fitness value was equal to the relative fitness of *msi-1* single mutants competed against wild type. In this case, there was no significant difference between the expected and observed fitness of the double mutant (Fig 11D). Any deviation in the observed fitness from the expected fitness value is quantified as a synthetic fitness effect. As an example, *exc-7*; *ets-5* double mutants were competed with *ets-5* single mutants. The expected outcome was for *exc-7*; *ets-5* double mutant fitness to be similar to *exc-7* fitness when competed with wild type, but instead *exc-7*; *ets-5* double mutants have much lower fitness values (Fig 11E). To restrict our focus to interactions with the strongest physiological consequences, we set a threshold of an absolute value of 0.4 or greater change in fitness. As expected, most RBP; TF double mutants do not exhibit a significant synthetic fitness effect, suggesting that most TFs and RBPs do not act synergistically in ways that result in changes in fitness. However, we identified eight RBP; TF double mutants with strong synthetic effects (Fig 11F).
3.3 *aptf-1; fox-1* double mutants exhibit developmental delay

Relative fitness is a broad parameter, and there are a variety of underlying phenotypes that contribute to the organism’s overall fitness. Performance in the competitive fitness assay depends on the ability of worms to develop, survive, reproduce, and consume food, competing for resources with other worms on the plate. Therefore, some of the key phenotypes that could directly correlate with an individual’s performance in competitive fitness assays are its ability to feed, move, and lay eggs. For each double mutant that generated a significant synthetic fitness effect, we followed up with multiple assays to determine underlying phenotypes that contributed to a decrease or increase in fitness.

In one interesting case, we found that *aptf-1; fox-1* double mutants exhibited a strong negative synthetic fitness effect, where the measured fitness is much lower than expected (Fig 12). The RBP *fox-1* is a key regulator of *C. elegans* sex determination, while *aptf-1* is a neuronal TF important for sleep behavior\(^{101,102}\). Both factors are highly conserved, but the loss of either *fox-1* or *aptf-1* in a single mutant did not result in a measurable fitness deficit (Fig 11B-C).

![Graph showing fitness relative to wild-type and expected fitness](image)

**Figure 12: Synthetic fitness in *aptf-1; fox-1* double mutants.** Relative fitness data reveals synthetic negative effect in *aptf-1; fox-1* double mutant. Asterisk indicates observed fitness of *aptf-1; fox-1* is significantly lower than expected, p<0.05.
A variety of assays were conducted to determine underlying causes of the reduced fitness of aptf-1; fox-1 double mutants. No significant changes were measured in locomotion-related phenotypes, such as thrashing and escape from touch stimulus (Fig 13A-B). However, we measured moderate but significant reductions in egg-laying rate and pumping rate compared to aptf-1 and fox-1 single mutants (Fig 13C-D).

![Graphs showing reduced egg laying and pharyngeal pumping in double mutants](image)

**Figure 13: aptf-1; fox-1 double mutants exhibit slowed rates of egg laying and pharyngeal pumping compared to single mutants.** (A-B) Number of thrashes per 30 seconds (A) and touch response (B) were measured in L4 worms to assess locomotion. No significant differences were observed. (C) Number of pharyngeal pumps in 20 seconds was counted. Asterisk indicates a significant reduction in pumps in aptf-1; fox-1 compared to fox-1 single mutants, p < 0.05. (D) Early adult worms were individually plated and given 24 hours to lay eggs. Asterisk indicates significantly reduced eggs were counted on aptf-1; fox-1 plates compared to aptf-1, p < 0.05.

Upon further investigation, we noted that aptf-1; fox-1 worms exhibit slower than normal growth. We measured larval development time, from L1 stage to L4 stage, and confirmed that aptf-1; fox-1 double mutants experience a significant delay in developmental timing compared to wild-type and single mutant worms (Fig 14A-B). 48 hours after hatching, when wild-type worms have reached L4 stage, the majority of aptf-1; fox-1 double mutants are still at L3 (Fig 14A).
These effects are synthetic and not additive, as the constituent single mutants exhibit normal developmental timing. Therefore, although single mutants for aptf-1 and fox-1 display fitness equal to or greater than wild type (Fig 11B-C), when simultaneously lost they result in substantial fitness defects due to defects in developmental timing. Together, this implicates a novel role for fox-1 and aptf-1 in coordinating development.

Figure 14: aptf-1; fox-1 double mutants exhibit developmental delay. (A) Developmental timing delay in aptf-1; fox-1 double mutants. Larval stage was assessed 48 hours after L1 worms were plated, and aptf-1; fox-1 worms were still at L3 stage while wild-type and single mutant worms had reached L4. Asterisks indicate significant differences between aptf-1; fox-1 and wild-type, p<0.05. (C) Representative images of aptf-1; fox-1, single mutants, and wild-type. Upper panel shows comparison of L3 and L4 worms. Arrow indicates characteristic white patch found in mid-body of L4, which was used to score larval stage. Lower panels show comparison of plates after 48 hours at 20°C when assays were scored.
### 3.4 Reproductive defects in double mutants for TF *ceh-14* and ALS-associated RBPs *fust-1* or *tdp-1*

Two of the strongest negative synthetic effects we identified were between the TF *ceh-14* and the RBPs *tdp-1* and *fust-1* (Fig 11E, 15). *tdp-1* and *fust-1* are the *C. elegans* homologs of human TDP-43 and FUS, both implicated in ALS\(^{103,104}\). The roles of both TDP-43 and FUS in the context of disease have been well-studied since their discovery\(^{105–107}\). In ALS, mutations in TDP-43 and FUS cause them to mislocalize and aggregate in the cytoplasm, depleting them from the nucleus and disrupting their function\(^{108–110}\). However, their roles under non-diseased conditions are less understood. TDP-43 and FUS have many structural and functional commonalities and are known to share roles in many processes such as transcription, splicing, and DNA damage repair\(^{111–113}\), but much remains unclear about the molecular nature of these functions, especially their cytoplasmic roles. A better understanding of these molecular functions is key to understanding how they are disrupted in ALS.

In mammals, loss of function of either TDP-43 or FUS is fatal, demonstrating that both genes are essential for survival but making it difficult to study their function\(^{114,115}\). In *C. elegans* the loss of *tdp-1* or *fust-1* alone does not cause a strong phenotype, and indeed our *tdp-1* and *fust-1* mutants have fitness values indistinguishable from wild-type (Fig 11B). This gives us the opportunity to investigate the molecular functions of *tdp-1* and *fust-1* in our deletion mutants, and to explore their shared interaction with the transcription factor *ceh-14*.

*ceh-14* mutants display a slight decrease in fitness compared to wild-type, but have no strong visible phenotype (Fig 11C). Only when *tdp-1* or *fust-1* mutations are combined with the *ceh-14* mutation does a strong fitness defect occur (Fig 15). We wanted to further explore the interaction that *fust-1* and *tdp-1* share with *ceh-14* and hoped to learn more about how these regulatory genes coordinate to have such a strong physiological effect.
Figure 15: Negative synthetic fitness effects in both fust-1; ceh-14 and tdp-1; ceh-14 double mutants. (A-B) Both fust-1; ceh-14 (A) and tdp-1; ceh-14 (B) exhibit strong negative synthetic effects on relative fitness. Asterisk indicates observed fitness is significantly reduced from expected fitness, p<0.05.

One readily discernible commonality between fust-1; ceh-14 and tdp-1; ceh-14 double mutants was a reduced progeny count, based on the observation that plates of these strains appeared less crowded and took longer to starve. We quantified progeny produced per worm at both 20°C and at the mildly stressful temperature 25°C\textsuperscript{116-118}. While there is a moderate decrease in brood size at 20°C, the defect becomes more pronounced at 25°C (Fig 16A-B). None of the constituent single mutants exhibit this strong defect (Fig 16A-B). These results therefore suggest that fust-1; ceh-14 and tdp-1; ceh-14 double mutant fertility is susceptible to a mild increase in temperature. To confirm that the nature of this interaction between tdp-1 or fust-1 and ceh-14 is due to on-target mutations and not background effects, we generated double mutants using alternate alleles\textsuperscript{14,119}. These new double mutants recapitulated the fertility defect, confirming the synergistic phenotypes in fust-1; ceh-14 and tdp-1; ceh-14 are due to on-target TF and RBP mutations (Fig 16C). To further characterize the reproductive rate in double mutants, progeny produced per day was measured. In wild-type worms reproduction peaks at day 1 and day 2 of
adulthood, then drops off and ceases by day 5 (Fig 16E). We found that *tdp-1; ceh-14* and *fust-1; ceh-14* have a consistently lower rate of reproduction, with significantly fewer progeny produced on day 2 of adulthood compared to wild type (Fig 16E).
Figure 16: Defects in reproduction in \textit{fust-1; ceh-14 and tdp-1; ceh-14}. (A-B) At 20°C, differences in brood size are subtle (A), but at 25°C both \textit{fust-1; ceh-14} and \textit{tdp-1; ceh-14} produce significantly smaller brood sizes than wild type, while \textit{fust-1 tdp-1} double mutants do not exhibit a reproductive defect (B). Asterisks indicate significant differences from wild-type brood size, p < 0.05. (C) Both double mutants were generated using \textit{ceh-14 (ot900)}, which contains a full deletion of \textit{ceh-14}. No significant differences in brood size were measured between double mutants generated with the original \textit{ceh-14 (ch3)} allele and those generated with \textit{ceh-14 (ot900)}. (D) \textit{fust-1 tdp-1; ceh-14} triple mutant did not exhibit a worsened brood size defect when compared to \textit{fust-1; ceh-14 and tdp-1; ceh-14} double mutants. Asterisk indicates significant difference from wild type, p<0.05. \textit{fust-1 tdp-1; ceh-14} brood size is not significantly different from that of wild type. (E) Progeny were counted every day, and brood sizes for \textit{tdp-1; ceh-14 and fust-1; ceh-14} are consistently lower than wild type. Asterisk indicates egg production of both \textit{fust-1; ceh-14 and tdp-1; ceh-14} was significantly lower than that of wild-type worms at day 2 of adulthood, p < 0.05. (F) TDP-1 and FUST-1 interact with CEH-14, but not with each other, to affect \textit{C. elegans} brood size.

tdp-1; ceh-14 and fust-1; ceh-14 double mutants have fertility defects, but tdp-1 fust-1 double mutants do not (Fig 16B, D). Furthermore, fertility was not worsened in \textit{fust-1 tdp-1; ceh-14} triple mutants (Fig 16D). Together, this indicates that \textit{tdp-1} and \textit{fust-1} genetically interact with \textit{ceh-14}, but not with each other, to coordinately affect reproduction in \textit{C. elegans} (Fig 16F). This suggests that the reproductive defects of both double mutants might stem from a shared underlying dysfunction in which the activity of both \textit{tdp-1} and \textit{fust-1} are required in conjunction with that \textit{ceh-14}. In sum, we find that \textit{tdp-1} and \textit{fust-1}, whose human counterparts are implicated in shared disease states, also share similar physiological roles in \textit{C. elegans}. This role in promoting fecundity is only revealed in the context of the \textit{ceh-14} mutant background.

3.5 Gonad development defects in \textit{fust-1; ceh-14 and tdp-1; ceh-14} double mutants

A reduction in progeny could be attributed to number of underlying issues, including a physical inability to push eggs out of the vulva, a defect in gonadogenesis, or deficient gametes. We tested each possibility to determine the underlying causes of the double mutant phenotype. Worms with a mechanical defect in egg-laying retain eggs in the uterus\textsuperscript{120,121}. We quantified

61
uterine eggs in two-day-old adults and found no significant difference between double mutant and wild-type worms (Fig 17A).

We next examined the gonad to see if there were any differences in development, using DAPI (4′,6-diamidino-2-phenylindole) nuclear stain to visualize gonads within whole worms. During normal gonad development the distal tip cell (DTC) guides migration of each of the two symmetrical C. elegans gonad arms. The DTC guides the developing gonad out from the ventral midbody, then makes one dorsal turn, followed by a second turn towards the midbody\textsuperscript{122,123} (Fig 17B). This migrating tissue receives a signal to stop when the DTC crosses the midbody and reaches the vulva\textsuperscript{124,125}. The uterine cells undergo a characteristic outgrowth, expanding and setting up the uterus centered around the vulva\textsuperscript{126–128}. In a fecund adult, there is typically a visible gap between the DTC of anterior and posterior arm, and the DTCs do not overlap (Fig 17C). When maintained at 25°C, about 30% of fust-1; ceh-14 and tdp-1; ceh-14 adults exhibit overmigration of the distal gonad tip, with the anterior and posterior tips overlapping or even passing each other (Fig 17C-D). We found no discernable differences in gonad morphology in larval stage worms, and overgrown distal arms are not observed until adulthood (Fig 17D). This suggests that rather than a dysfunction during larval gonad development, there may be a disruption in stop signaling as the distal tip reaches the vulva. Previous studies have identified similar overmigration defects in C. elegans in response to development under stress, including under conditions of changing temperature\textsuperscript{129}, which could be related to the temperature-sensitive gonad development phenotypes observed in our double mutants.
Figure 17: *fust-1; ceh-14* and *tdp-1; ceh-14* exhibit defects in adult hermaphrodite gonad. (A) Double mutants do not exhibit egg retention. (B) Representative images of DAPI-stained larval hermaphrodites. (C) Representative images of DAPI-stained wild-type and double mutant adult hermaphrodites. *fust-1; ceh-14* and *tdp-1; ceh-14* exhibit overlapping distal tips. Arrows
indicate path of gonad development. Arrow heads denote location at midbody where vulva is present in adult. Green brackets indicate approximate location of distal tip. (D) Quantification of gonad defects. Double mutants do not differ from wild-type at younger larval stages, but exhibit increased defects at adulthood. Asterisk indicates significant difference from wild-type, p<0.05.

3.6 Gamete defects in *fust-1; ceh-14* and *tdp-1; ceh-14* double mutants

*C. elegans* hermaphrodites are self-fertile, and a reduction in progeny could stem from defects in male gametes, female gametes, or both. To determine the functionality of the male and female gametes, we conducted reciprocal mated brood assays. First, we mated mutant males with wild-type hermaphrodites and quantified the proportion of cross-progeny versus self-progeny to determine the efficiency of the mutant male's sperm. If there are no sperm defects in the mutant males, the proportion of cross-progeny produced will be similar to that produced by wild-type males. Under normal maintenance conditions at 20°C, about 40% of total progeny from wild-type male crosses are cross progeny (Fig 18A). When males are maintained at 25°C, the proportion of cross progeny is reduced to 15% (Fig 18A). Mating efficiencies for double mutant males were first assayed at 25°C. At this temperature, both *fust-1; ceh-14* and *tdp-1; ceh-14* had a mating efficiency close to 0% (Fig 18A). Additional experiments were performed at 20°C, and mating efficiency at this lower temperature was improved to near 20% (Fig 18A). Notably, *tdp-1; ceh-14* double mutant males still exhibited significantly lower mating efficiency at 20°C compared to wild-type males (Fig 18A). These results suggest that *tdp-1; ceh-14* and *fust-1; ceh-14* double mutant sperm exhibit temperature-sensitive defects, with almost no cross-progeny produced at 25°C.
Figure 18: Defects in sperm and oocytes in *fust-1; ceh-14* and *tdp-1; ceh-14*. (A) Male mating efficiency assays were performed at both 20°C and 25°C. *tdp-1; ceh-14* males produced significantly fewer cross-progeny than wild-type at 20°C. At 25°C, both *tdp-1; ceh-14* and *fust-1; ceh-14* produced almost no cross progeny. (B) Male mating was also measured with *fog-2* (*q71*) feminized germline mutants. Double mutant males produced smaller brood sizes than wild-type, and differences are more apparent at 25°C. (C) Wild-type males were mated with either wild-type or double mutant hermaphrodites. Mating with a male significantly increases brood
size for wild-type, but does not increase brood size in either \textit{tdp-1; ceh-14} or \textit{fust-1; ceh-14}. Asterisk indicates \( p < 0.05 \). (D-F) Representative images show expression of CEH-14 (D), TDP-1 (E), and FUST-1 (F) in the anterior half of adult hermaphrodites. Solid outline indicates location of gonad, and dotted outline indicates location of spermatheca. Bright streak of green in (D) is gut autofluorescence. Rightmost panels show spermatheca at higher magnification. CEH-14, TDP-1, and FUST-1 are expressed in the cells making up the bag-like structure of the spermatheca. Scale bar represents \( 50 \mu m \)

In the above crosses, double mutant male sperm had to compete with wild-type sperm harbored by the self-fertile hermaphrodite. When wild-type males are crossed to hermaphrodites, male sperm outcompetes hermaphrodite self-sperm for fertilization of oocytes\textsuperscript{130,131}. To test whether double-mutant male sperm is fertile in the absence of competition from hermaphrodite sperm, we mated double mutant males with feminized \textit{fog-2 (q71)} mutant hermaphrodites which are unable to generate sperm\textsuperscript{132}. Therefore, all progeny produced when \textit{fog-2} hermaphrodites are paired with males will be cross progeny. Similar to the findings from the previous male mating assays, there was a marked reduction in brood size between double mutants and wild-type males, with the difference becoming more pronounced at \( 25^\circ C \) (Fig 18B). These findings indicate that double mutant male sperm is defective even in the absence of competition from wild-type hermaphrodite sperm.

To determine oocyte viability, wild-type males were mated with double mutant hermaphrodites. In self-fertile hermaphrodites, the total number of progeny produced is limited by the number of sperm generated. Therefore, if hermaphrodites are mated with males, the increased availability of sperm from the male will significantly increase the total progeny produced\textsuperscript{133}. Indeed, we observed that mating with a male more than doubles wild-type brood size (Fig 18C). However, when double mutants were paired with males, there was no significant increase in brood size (Fig 18C). This indicates that \textit{fust-1; ceh-14} and \textit{tdp-1; ceh-14} double mutants are defective in both male (sperm) and female (oocyte) gametes.
3.7 Expression of TDP-1, FUST-1, and CEH-14 overlaps in the spermatheca

To visualize where these genes are expressed in *C. elegans*, we used CRISPR/Cas9 to tag the C-terminus of each gene. *fust-1* and *tdp-1*, both tagged with RFP, exhibit ubiquitous nuclear expression. The expression of *ceh-14*, which we tagged with GFP, was more limited. As previously described, it is primarily expressed in a subset of neurons including several in the head and tail\textsuperscript{134}. Within the gonad, *ceh-14* exhibits nuclear expression in the membrane of the spermatheca (Fig 18D). *fust-1* and *tdp-1* are also expressed in the nuclei of these cells (Fig 18E-F). We hypothesize that this overlapping expression in the spermatheca could be the source of their combinatorial effect on reproduction.

To investigate the development and morphology of the spermatheca in *fust-1; ceh-14* and *tdp-1; ceh-14*, we crossed each double mutant with a strain containing a spermatheca GFP reporter. We did not see any physical defects in the spermatheca of either double mutant; they appeared similar to that of wild-type worms grown under the same conditions (Fig 19). This suggested that the defect in reproduction observed in our double mutants could not be explained by defective spermatheca development or morphology.

![Figure 19: Spermatheca of *tdp-1; ceh-14* and *fust-1; ceh-14* double mutants are morphologically similar to wild-type.](image)

UX993 worms containing spermatheca GFP and germline RFP were crossed into *tdp-1; ceh-14* and *fust-1; ceh-14* double mutants to visualize spermatheca morphology\textsuperscript{135}. No differences in morphology or development of spermatheca were detected in either double mutant. Representative images show day 1 adults of wild-type and double mutants containing the UX993 transgenes.
3.8 Distinct transcriptional and post-transcriptional networks in double mutants

To investigate the gene regulatory networks controlled by the three factors, we analyzed the transcriptomes of *tdp-1; ceh-14* and *fust-1; ceh-14* double mutants, as well as the constituent single mutants. At the level of gene expression, double mutants display changes in the expression of hundreds of genes compared to wild-type animals (Fig 20A). Such gene expression changes could be the result of (1) losing a single regulatory factor, (2) additive effects of losing both factors, or (3) synthetic/synergistic effects of losing both factors. To distinguish among these possible scenarios, we first compared gene expression changes between single mutants and double mutants. Linear regressions show that *ceh-14* accounts for the majority of gene expression changes observed in *tdp-1; ceh-14* double mutants (Fig 20B), while *tdp-1* accounts for very few gene expression changes in the double mutant (Fig 20C). Likewise, *ceh-14* accounts for the majority of gene expression changes observed in *fust-1; ceh-14* mutants (Fig 20D), while *fust-1* accounts for comparatively few changes (Fig 20E).

Since most gene expression changes in the double mutant are accounted for by *ceh-14* regulation, this suggests that very few gene expression changes are regulated in an additive or synergistic manner by *tdp-1* and *ceh-14*. One notable exception is the gene *clec-190*, whose expression is unchanged in single mutants, but strongly downregulated in *tdp-1; ceh-14* and modestly downregulated in *fust-1; ceh-14* mutants (Fig 20F). Given the strong synergistic regulation of *clec-190*, we wondered whether loss of *clec-190* expression might contribute to the synergistic double mutant phenotypes. To test this, we generated a *clec-190* null mutant in which the entire coding sequence was deleted, but found that the mutant results in no discernible fertility defects (Fig 20G). Therefore, although *clec-190* represents an interesting example of combinatorial regulation, it does not on its own contribute to the double mutant phenotypes.
Figure 20: *ceh-14* controls transcriptional networks in *tdp-1; ceh-14* and *fust-1; ceh-14*. (A) Gene expression changes of *tdp-1; ceh-14* compared to wild-type. (B-C) Linear regression showing gene expression changes for genes dysregulated (|log2fold-change| >2 and q<0.01) in *tdp-1; ceh-14* mutants, compared to *ceh-14* (B) and *tdp-1* (C) mutants. (D-E) Linear regression showing gene expression changes for genes dysregulated (|log2fold-change| >2 and q<0.01) in *fust-1; ceh-14* mutants, compared to *ceh-14* (D) and *fust-1* (E) mutants. (F) qRT-PCR shows unique downregulation of *clec-190* in *tdp-1; ceh-14* double mutants. (G) *clec-190* deletion mutants do not have a fertility defect. (H) Significant tissue and gene ontology enrichment in downregulated genes of *tdp-1; ceh-14* double mutants. (I) Similar spermatheca gene dysregulation is seen in *fust-1; ceh-14* and *ceh-14* mutants.
To test whether functional classes of genes are dysregulated in \textit{tdp-1; ceh-14} double mutants, we performed Gene Ontology and Tissue Enrichment analysis. Upregulated genes have no statistically significant (q<0.01) enrichment categories, but downregulated genes are enriched in a few categories, including genes expressed in the spermatheca (Fig 20H). This is notable given the co-expression of all three factors in the spermatheca (Fig 18D-F) and the central role played by the spermatheca in fertilization. We examined all spermatheca-annotated genes with dysregulated expression in \textit{tdp-1; ceh-14} and found that most are downregulated in the double mutant, and that \textit{fust-1; ceh-14} double mutants have similar patterns of dysregulated gene expression (Fig 20I). Moreover, we found that \textit{ceh-14}, but not \textit{fust-1} or \textit{tdp-1}, is the main driver of these changes, as \textit{ceh-14} mutants display similar gene expression patterns to the double mutants (Fig 20I). Together these data indicate that \textit{ceh-14} is necessary for stimulating the expression of a network of genes in the spermatheca, and motivate future work to determine whether these genes play a role in the double mutant fertility phenotypes.

Analysis of alternative splicing reveals a contrasting regulatory landscape to that of gene expression. \textit{tdp-1} accounts for the majority of splicing changes observed in \textit{tdp-1; ceh-14} mutants, while \textit{ceh-14} accounts for very few splicing changes (Fig 21A-C). Likewise, \textit{fust-1} is largely responsible for the splicing changes observed in \textit{fust-1; ceh-14} mutants (Fig 21D-E). As with gene expression, we observe very little additive or synergistic regulation of alternative splicing. We also observe very little overlap between genes with altered splicing regulation and genes with altered expression levels in the double mutants. Together, these data indicate that dysregulated genes in double mutants are either regulated transcriptionally by \textit{ceh-14} or post-transcriptionally by \textit{fust-1} and/or \textit{tdp-1}. 
Figure 21: Distinct post-transcriptional regulation in *tdp-1; ceh-14* and *fust-1; ceh-14*. (A) Analysis of splicing changes in *tdp-1; ceh-14* compared to wild-type. (B-C) Linear regression showing splicing events dysregulated in *tdp-1; ceh-14* ($|\Delta\text{PSI}|>10$, $q<.01$) compared to *ceh-14* (B) and *tdp-1* (C) mutants. (D-E) Linear regression showing splicing events dysregulated in *tdp-1; ceh-14* ($|\Delta\text{PSI}|>10$, $q<.01$) compared to *ceh-14* (D) and *fust-1* (E) mutants.

3.9 *tdp-1* and *fust-1* co-inhibit exon inclusion

Loss of *tdp-1* or *fust-1* results in many types of dysregulated splicing, including 5’ and 3’ splice site selection, cassette exon inclusion, and intron retention (Fig 22A). One notable feature we observed is that the effect of *tdp-1* or *fust-1* mutation on cassette exons is almost exclusively an increase in exon inclusion (Fig 22B). This is in contrast with many other RNA binding proteins, which both stimulate and inhibit exon inclusion, in a context-specific manner. Therefore we conclude that *tdp-1* and *fust-1* function specifically to inhibit exon inclusion.

We next asked whether *tdp-1* and *fust-1* inhibit expression of overlapping or distinct alternative exons. Strikingly, we found that half of *fust-1*-regulated cassette exons are also regulated by *tdp-1* (Fig 22C), and that the direction of splicing change is always concordant, with
Figure 22. *tdp-1* and *fust-1* inhibit exon inclusion. (A) Splicing dysregulation in *tdp-1* and *fust-1* mutants (ΔPSI>10, q<0.01). (B) For cassette exons dysregulated in either single mutant (ΔPSI>10, q<0.01), increased exon inclusion in the mutant is much more common than increased skipping. (C) Overlap of cassette exon dysregulation between *tdp-1* and *fust-1* mutants (ΔPSI>10, q<0.01). (D-E) Increase of inclusion of *sav-1* cassette exon in both *tdp-1* and *fust-1*...
increased exon inclusion in both mutants. An example of an exon repressed by both factors is in
the gene sav-1, which harbors an unannotated cassette exon. In wild-type or ceh-14 mutants, this
exon is predominantly skipped, but in either fust-1 or tdp-1 mutants, the exon becomes
predominantly included (Fig 22D-E). In fust-1 tdp-1 double mutants, the exon is included at
levels similar to either single mutant (Fig 22E), suggesting that tdp-1 and fust-1 do not act
synergistically, but rather are both simultaneously required to repress sav-1 exon inclusion.

Given the striking concordance of inhibition of exon inclusion by fust-1 and tdp-1, we
next tested whether such activity is an evolutionarily-conserved attribute of the two RNA
binding proteins. This would be of particular interest given both factors’ prominent links to the
human neuronal disorders ALS and FTD\textsuperscript{137}. To this end we re-analyzed data in which either of
the mouse homologues (FUS or TDP-43) was knocked down in mouse brains and splicing
analyzed by microarray\textsuperscript{113}. Focusing on cassette exons, we found a substantial overlap between
the regulatory activity of FUS and TDP-43 (Fig 22F). As in our C. elegans experiments, exons
co-regulated by both FUS and TDP-43 in mouse brain tend to be inhibited by both factors (Fig
22G). Thus, tdp-1 and fust-1 have a propensity to coordinately inhibit exon inclusion both in C.
elegans and in mouse brain.
3.10 Aberrant exon inclusion in *pqn-41* contributes to fertility defect of *tdp-1; ceh-14*

One intriguing example in which *C. elegans* *tdp-1* (but not *fust-1*) inhibits exon inclusion is found in *pqn-41*, a gene encoding a polyglutamine-containing protein. This gene harbors an alternative cassette exon which in wild-type conditions is primarily skipped, but in *tdp-1* mutants is primarily included (Fig 22H-J). *pqn-41* was previously shown to be important for proper developmental cell death of the linker cell in the gonad of male *C. elegans*\(^{138}\), which prompted us to ask whether *pqn-41* might contribute to the gonad development or fertility defects observed in *tdp-1; ceh-14* mutant hermaphrodites. We obtained a potentially null deletion allele, *pqn-41(ok3590)*\(^{139}\), and tested fertility. We found that brood sizes of *pqn-41* mutants are significantly lower than wild-type worms, and that these fertility defects are particularly pronounced at higher temperatures (Fig 22K). Therefore, loss-of-function *pqn-41* mutants have similar temperature-sensitive fertility defects to *tdp-1; ceh-14*.

We next generated a *pqn-41* mutant using CRISPR/Cas9 in which the alternative exon is removed and the flanking exons are fused together, thereby forcing expression of the exon skipped version (Fig 23A-B). We crossed this *pqn-41* exon-deletion mutant into a *tdp-1; ceh-14* background, thus restoring *pqn-41* to the isoform most abundant under wild-type conditions (exon skipped). Remarkably, these triple mutants (*tdp-1; pqn-41[exon-skipped]; ceh-14*) exhibit a strong rescue of brood size at 25°C compared to *tdp-1; ceh-14* double mutants (Fig 22L). This suggests that aberrant *pqn-41* exon inclusion plays a major role in the fertility defects observed in *tdp-1; ceh-14* double mutants.

In contrast with *tdp-1; ceh-14* double mutants, *fust-1; ceh-14* double mutants do not exhibit *pqn-41* splicing defects. Likewise, crossing the *pqn-41[exon-skipped]* into the *fust-1; ceh-14* double mutant does not cause an increase in brood size (Fig 22L), suggesting that the
Figure 23: *pqn*-41 sequences and CRISPR/Cas9 exon deletion. (A) *pqn*-41 mRNA sequence. Alternative cassette exon is circled. (B) CRISPR/Cas9 was used to delete the alternative exon and surrounding introns, generating *pqn*-41(syb6090). Flanking exons, shown in green, were fused together. (C) *pqn*-41 amino acid sequence. Approximate locations of glutamine are highlighted in red, and Q-rich region is shown downstream of the circled alternative exon. (D) *pqn*-41 amino acid composition reveals a bias towards glutamine.

rescue of *tdp-1; ceh-14* by *pqn*-41(exon-skipped) is mechanistically linked to the mis-splicing of *pqn*-41 caused by *tdp-1* loss of function. Together these results highlight a new role for the polyglutamine gene *pqn*-41 in fertility, and indicate that *pqn*-41 mis-splicing is a major cause of the fertility defects observed in *tdp-1; ceh-14* double mutants.

In sum, using a systematic combinatorial genetic interaction screen, we found that two RBPs, *fust-1* and *tdp-1*, are both required in the context of a *ceh-14* mutant background to maintain fitness and fertility in *C. elegans*. These two RBPs have both been implicated in ALS and FTD in humans, and we now identify a common physiological role for both RBPs in *C.*
*elegans*. Both RBPs have overlapping roles in inhibiting exon inclusion, pointing to shared molecular activities and a potential molecular basis for the physiological roles for the two RBPs described here. Failure to inhibit exon inclusion in the *pqn-41* gene is a major cause of the fertility defects in *tdp-1; ceh-14* double mutants, thus providing a mechanistic link between the molecular activity of the RBP TDP-1 and the fertility phenotype observed in *tdp-1; ceh-14* double mutants.

### 3.11 Discussion

#### 3.11a Novel genetic interactions across regulatory layers

We took a systematic genetic interaction approach to identify cross-regulatory genetic interactions in which a TF and RBP are combinatorially required for phenotypes affecting organismal fitness. This screen revealed a number of TF-RBP pairs required for phenotypes including fitness, development, and fertility. The strongest of these genetic interactions involves the homeodomain TF *ceh-14* and either of the ALS-associated RBPs *tdp-1* or *fust-1*.

Transcriptome analysis revealed distinct regulatory networks, in which *ceh-14* regulates transcription, and *tdp-1/fust-1* regulate splicing, with few genes additively or synergistically regulated and few genes regulated at both transcriptional and post-transcriptional levels. Therefore, it seems likely that the synthetic fertility phenotypes result from the combination of distinct gene dysregulation events. We identify one such dysregulation in the alternatively-spliced exon in *pqn-41*. Mis-splicing of this exon is a major contributor to the phenotype, as restoring exon skipping rescues fertility defects of *tdp-1; ceh-14* double mutants. Mis-splicing of this exon in isolation does not cause fertility defects, as *tdp-1* single mutants mis-splice *pqn-41* at the same levels as *tdp-1; ceh-14* mutants, but do not have fertility defects. We therefore speculate that mis-splicing of *pqn-41*, in combination with altered expression of one or more
*ceh-14* target genes, results in fertility defects. A number of genes with spermatheca expression dependent on *ceh-14* (Fig 20I) represent promising candidates.

These results highlight the utility of the systematic reverse-genetic interaction approach both for understanding relationships between regulatory factors and for understanding the roles of individual factors whose regulatory roles are only apparent when redundant or compensatory pathways are simultaneously perturbed\(^2\). In this case, a shared molecular and physiological role for *tdp-1* and *fust-1* is revealed by their shared genetic interaction profile. Future studies characterizing additional genetic interactions identified here may shed light on novel physiological roles for additional RBPs and TFs.

### 3.11b *fust-1* and *tdp-1* interact with *ceh-14* to affect *C. elegans* fertility

The hermaphrodite spermatheca is a key site of overlapping expression for *tdp-1*, *fust-1*, and *ceh-14*, and double mutants have reduced sperm efficiency. Signaling from somatic gonad cells such as spermathecal cells is required for germline development and function, as ablation of specific spermatheca cells results in defective germ cell function, and even sterility\(^141\). We hypothesize that faulty signaling between spermathecal cells and germ cells might explain the defects in fertility and gonad development observed in our double mutants. The double-mutant fertility phenotype is particularly pronounced at 25°C, which is a mildly stressful temperature for wild-type worms, causing modest defects in fertility and gonad development\(^118,129,142\). Temperatures higher than 25°C result in damage to sperm and strong fertility defects\(^143,144\). We hypothesize that *tdp-1*; *ceh-14* and *fust-1*; *ceh-14* double mutants are deficient in their heat stress responses\(^116,117\), and are therefore unable to maintain normal homeostasis under a mild temperature increase. Therefore, temperatures that cause mild reductions in reproduction in wild-type animals result in strong defects in double mutants.
We identified an alternative cassette exon within the gene encoding *C. elegans* polyglutamine-containing protein PQN-41 which is regulated by *tdp-1*. This exon is primarily skipped in wild-type conditions, but exhibited a predominant switch to inclusion in *tdp-1* and *tdp-1; ceh-14* mutants. Strikingly, we found that deletion of this alternative exon in a *tdp-1; ceh-14* double mutants rescued fertility, indicating that the aberrant inclusion of this *pqn-41* exon contributes to the reproductive defect of these double mutants.

The PQN-41 protein is abundant with glutamines, and it contains a particularly polyglutamine-rich domain at the C-terminus\(^{138}\). Notably, this domain starts almost immediately downstream of the alternative exon we investigated (Fig 23C-D). We hypothesize that, like other polyglutamine proteins such as the Huntington protein, PQN-41 is subject to pathogenic aggregation\(^{145}\). If the exon-included isoform of PQN-41 is particularly prone to aggregation, and if stressful conditions such as higher temperatures further increase the likelihood of aggregation, this could lead to temperature-sensitive defects. Indeed, there is evidence that PQN-41 forms aggregates *in vivo*\(^{138}\), but a full mechanistic test of this hypothesis awaits further investigation. It will be interesting in future studies to investigate whether there is a related prion-like polyQ protein underlying the similar fertility defects of *fust-1; ceh-14* double mutants.

### 3.11c *fust-1* and *tdp-1* co-inhibit exon inclusion

The identification of shared phenotypes between *tdp-1; ceh-14* and *fust-1; ceh-14* double mutants led to the observation that *tdp-1* and *fust-1* also have shared effects on the transcriptome. Most notably, they both act to inhibit inclusion of alternatively-spliced cassette exons, including many inhibited by both RBPs. For exons that are targeted by both RBPs, *fust-1* and *tdp-1* do not appear to act redundantly, as *fust-1 tdp-1* double mutants do not result in increased exon inclusion compared to either of the single mutants. Rather, *fust-1* and *tdp-1* are both
simultaneously required for inhibition of these exons. One plausible explanation for this finding is that both RBPs bind together to specific pre-mRNAs where they act in concert to prevent aberrant exon inclusion.

The activity of *C. elegans tdp-1* and *fust-1* in inhibiting exon inclusion is also a shared feature of mammalian TDP-43 and FUS. We find that knockdown of TDP-43 or FUS in mouse brain results in aberrant exon inclusion, and that many of these exons are co-inhibited by both TDP-43 and FUS. This is interesting in light of recent findings suggesting a pathologically-relevant role for TDP-43 in inhibiting cryptic exons. Exons are sometimes classified as cryptic if they exhibit low inclusion levels, lack of evolutionary conservation, and/or propensity to disrupt the function of the gene they reside in. TDP-43 has been identified as an inhibitor of cryptic exons, and recent evidence implicates aberrant inclusion of two different cryptic exons in the genes *STMN2* and *UNC13A* as potential causative mechanisms underlying TDP-43 pathology in ALS.

Many of the exons identified as targets of *tdp-1* and/or *fust-1* in *C. elegans* have attributes of cryptic exons as well. For example, the alternative exons in *sav-1* and *pqn-41* are expressed at low levels in wild-type (Fig 22), and in the case of *sav-1* the exon is unannotated. In the case of *pqn-41*, failure of *tdp-1* to inhibit exon inclusion leads to detrimental phenotypes (fitness and fertility defects). This is an interesting parallel to the pathogenic consequences of TDP-43 failing to inhibit cryptic exon inclusion in the *STMN2* or *UNC13A* genes, and suggests that inhibition of aberrant exon inclusion may be an evolutionarily-conserved feature of *tdp-1/TDP-43* and *fust-1/FUS*.

Our findings are consistent with a role for *tdp-1/TDP-43* in inhibiting aberrant exon inclusion, and we extend this observation to also include a role for *fust-1/FUS* in inhibiting exon
inclusion. This leads us to speculate whether FUS-related pathogenesis might also be mechanistically linked to inappropriate inclusion of exons inhibited by FUS. Previous work on mammalian TDP-43 and FUS has concluded that the two RBPs share many common RNA targets, but also have considerable non-overlapping regulatory functions\textsuperscript{113,152}. We focused here on the regulation of cassette exons, and found substantial overlap between the RBPs in inhibiting exon inclusion. It will therefore be interesting to ask whether aberrant exon inclusion underlies FUS-mediated pathology in an analogous way to that of TDP-43-mediated pathology.

3.12 Methods

3.12a *C. elegans* strains and maintenance

All *C. elegans* strains were cultured on Nematode Growth Media (NGM) plates seeded with *E. coli*. Strains were maintained at 20°C unless otherwise stated. Some strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440). Double mutant strains were created and confirmed by visualization of GFP markers and by PCR.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADN267</td>
<td>mec-8(csb23)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-3::GFP marker</td>
</tr>
<tr>
<td>ADN264</td>
<td>unc-75(csb20)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-2::GFP marker</td>
</tr>
<tr>
<td>ADN283</td>
<td>fox-1(csb39)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-2::GFP marker</td>
</tr>
<tr>
<td>ADN273</td>
<td>exc-7(csb29)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-3::GFP marker</td>
</tr>
<tr>
<td>ADN274</td>
<td>mbl-1(csb30)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-2::GFP marker</td>
</tr>
<tr>
<td>ADN265</td>
<td>fust-1(csb21)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-2::GFP marker</td>
</tr>
<tr>
<td>ADN282</td>
<td>tdp-1(csb38)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-3::GFP marker</td>
</tr>
<tr>
<td>ADN268</td>
<td>msi-1(csb24)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-2::GFP marker</td>
</tr>
<tr>
<td>ADN279</td>
<td>tiar-3(csb35)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-3::GFP marker</td>
</tr>
<tr>
<td>ADN270</td>
<td>hrpf-1(csb26)</td>
<td>Norris Lab, SMU</td>
<td>deletion with myo-2::GFP marker</td>
</tr>
<tr>
<td>TB528</td>
<td>ceh-14(ch3)</td>
<td>CGC, University of Minnesota</td>
<td>1277 bp deletion</td>
</tr>
<tr>
<td>OH7160</td>
<td>vtls1, ets-5(tm866)</td>
<td>CGC, University of Minnesota</td>
<td>deletion; vtls1 construct contains a rol phenotype and was removed by backcrossing for the purpose of this study</td>
</tr>
<tr>
<td>VC1669</td>
<td>aptf-1(gk794)</td>
<td>CGC, University of Minnesota</td>
<td>655 bp deletion</td>
</tr>
<tr>
<td>VC1605</td>
<td>tab-1(gk753)</td>
<td>CGC, University of Minnesota</td>
<td>820 bp deletion</td>
</tr>
<tr>
<td>PY1598</td>
<td>alr-1(oy42)</td>
<td>CGC, University of Minnesota</td>
<td>deletion</td>
</tr>
<tr>
<td>CB1416</td>
<td>unc-86(e1416)</td>
<td>CGC, University of Minnesota</td>
<td>deletion</td>
</tr>
<tr>
<td>VC369</td>
<td>pag-3(ok488)</td>
<td>CGC, University of Minnesota</td>
<td>1188 bp deletion</td>
</tr>
<tr>
<td>VC2396</td>
<td>mec-3(gk1126)</td>
<td>CGC, University of Minnesota</td>
<td>774 bp deletion</td>
</tr>
<tr>
<td>CB1170</td>
<td>unc-55</td>
<td>CGC, University of Minnesota</td>
<td>unc-slow, tends to coil</td>
</tr>
<tr>
<td>VC1444</td>
<td>unc-42</td>
<td>CGC, University of Minnesota</td>
<td>1430 bp deletion</td>
</tr>
<tr>
<td>JY359</td>
<td>lim-4</td>
<td>CGC, University of Minnesota</td>
<td>located on X chromosome</td>
</tr>
<tr>
<td>EG4883</td>
<td>oxis318; unc-119(ed3)</td>
<td>CGC, University of Minnesota</td>
<td>oxis318 [spe-11p::mCherry::histone + unc-119(+)]. Wild type. Dim mCherry expression in hermaphrodite sperm.</td>
</tr>
<tr>
<td>UX993</td>
<td>jnSi12; ezls2; ltl37</td>
<td>CGC, University of Minnesota</td>
<td>jnSi12 [peel-1p::htas-1::mCherry::tbb-2 3'UTR + Cbr-unc-119(+)] II. ezls2 [fkh-6::GFP + unc-119(+)] III. ltl37 [pie-1p::mCherry::his-58 + unc-119(+)] IV. GFP expression in spermatheca. mCherry expression in germline nuclei.</td>
</tr>
<tr>
<td>CB4108</td>
<td>fog-2(q71)</td>
<td>CGC, University of Minnesota</td>
<td>Male/female strain. XX females and XO males</td>
</tr>
<tr>
<td>RB2578</td>
<td>pqn-41(ok3590)</td>
<td>CGC, University of Minnesota</td>
<td>Approximately 700 bp deletion</td>
</tr>
<tr>
<td>AN74 PHX6090</td>
<td>pqn-41(syb6090)</td>
<td>SunyBiotech</td>
<td>1809 bp deletion of pqn-41 exon 3</td>
</tr>
<tr>
<td>AN28 PHX3345</td>
<td>clec-190(syb3345)</td>
<td>SunyBiotech</td>
<td>deletion</td>
</tr>
</tbody>
</table>
3.12b Competitive fitness assays

Competitive fitness assays were performed as previously described to establish the relative fitness for each single mutant. Briefly, four L4 larvae of each genotype were placed together on a seeded NGM plate and incubated for 5 days at 25°C. The fraction of each mutant on the plate after 5 days was calculated to generate a fitness value relative to wild-type fitness for each mutant using the formula \( F = \frac{(# \text{ mutant} / # \text{ total})}{50\%} \).

To determine double mutant fitness each double mutant was assayed with the transcription factor mutant used in the cross. Expected fitness was equivalent to the fitness of the RNA binding protein mutant in the cross. To identify double mutants with unexpected fitness, the expected fitness value for each double mutant was subtracted from its observed fitness. Synthetic fitness effects (\( \varepsilon \)) in the double mutants were calculated by \( F_{\text{obs}} - F_{\text{exp}} \). Our threshold for significance was \(|\varepsilon| > 0.4\), and all assays were completed in triplicate.

3.12c Larval growth assay

Worms were synchronized by standard bleaching procedure to obtain a population of L1 worms for each genotype. Synchronized L1 larvae were then plated onto seeded NGM plates and cultured for 48 hours at 20°C. Developmental stages of the worms on each plate were then assessed.

3.12d Fluorescence microscopy

Images were obtained on a Zeiss Axio Imager.Z1 microscope. Images were processed using ImageJ.

3.12e DAPI gonad imaging

Worms were collected into microcentrifuge tubes in batches at each larval stage and at day 1 of adulthood. Whole worms were then fixed and stained with 4’,6-diamidino-2-
phenylindole (DAPI). Worms were washed three times with 0.01% Tween-PBS, then frozen in 1 mL of methanol at -20°C for 5 minutes. Then, methanol was removed and worms rinsed with 1mL of 0.1% Tween-PBS. 1μL of 100ng/mL DAPI was then added to the worms in Tween-PBS, and incubated in the dark at room temperature for 5 minutes. Tubes were then rinsed with 0.1% Tween-PBS once more. Tubes were centrifuged and all solution removed from worms, then 10μL of 75% glycerol added. Worms in glycerol were then placed on agar pads on microscope slides for imaging.

3.12f Uterine egg retention

Egg retention was measured in hermaphrodites on day 1 of adulthood. Worms were placed in 4°C refrigerator for 5-10 minutes to slow movement down, then examined under the microscope. Total eggs present in the uterus were counted.

3.12g Lifetime egg-laying and brood size assays

Six L4 worms were placed on a seeded NGM plate and incubated at either 20°C or 25°C. The following day, and every subsequent day until egg production stopped, the 6 adults were transferred to new seeded plates. The number of progeny on each plate was counted and divided by the total number of adults to determine average egg production per day per worm. Total brood size was quantified as the sum total of eggs produced over lifespan per worm.

3.12h Male mating efficiency

To assay male mating efficiency, four young adult males were paired with four L4 hermaphrodites, and the worms were kept at 20°C for 24 hours to allow time for mating to occur. After 24 hours the adults were removed from the plate, and progeny were given another 48 hours to develop. Progeny were then counted, and percent of male-produced cross progeny out of the total progeny were scored. Mutant males with CRISPR deletions express GFP in either the body
wall muscle or the pharynx, so this fluorescence was used to identify cross progeny. For wild-type assays, males carrying myo-2::RFP which expresses bright RFP in the pharynx were used to allow scoring of cross progeny.

To measure male mating in the absence of wild-type hermaphrodite sperm, four young adult males were instead paired with four L4 fog-2 (q71) hermaphrodites with feminized germlines and a complete lack of sperm. For these assays, mutant males were paired with fog-2 hermaphrodites until egg production stopped, and adults were transferred to new plates to prevent overcrowding and starvation as breeding continued. After hermaphrodites no longer continued to produce eggs, adults were removed and the total number of progeny was counted. This total was divided by the number of adult hermaphrodites present on the assay (4) to give the average brood size produced per worm. As a control, these assays were simultaneously conducted with wild-type males.

3.12i Paired brood size assay

Four L4 wild-type males were paired with four L4 mutant hermaphrodites on a single plate, and pairs were kept paired for multiple days until egg-laying was complete. Every day, adults were transferred to new plates, and progeny left on each plate was counted to measure the total brood produced. As a control, unpaired brood assays were carried out at the same time.

3.12j RNA sequencing and analysis

Total RNA was extracted from L4 worms using Tri reagent according to manufacturer’s protocol (Sigma Aldrich). Three biological replicates were extracted per genotype. mRNA was purified from each sample using NEBNext® Poly(A) mRNA Magnetic Isolation Module, and cDNA libraries were prepared using NEBNext® Ultra™ II RNA Library Prep Kit for Illumina, following kit protocols.
3.12k Reverse Transcription PCR

Relative abundances of splicing isoforms of sav-1, and pqs-41 were determined by RT-PCR to confirm RNA seq results, using qScript® XLT One-Step RT-PCR Kit. The kit and reagents were used following the kit reaction protocol.
CONCLUSION

Overall, my findings highlight the importance of considering complex interactions when characterizing gene functions, demonstrating the utility of a synthetic genetic interaction screen for dissecting such interactions. Additionally, I underscore the value of *C. elegans* as a model of the nervous system. In Chapter 1, I identified a novel conserved role for *mec-2/Stoml3* in olfactory behavior of both *C. elegans* and mice. It was previously known that *mec-2/Stoml3* had an important role in mechanosensation, and our findings expand upon the conserved functions of *mec-2* and *Stoml3*. Previous work had also highlighted the impressive level of conservation of neuronal genes in *C. elegans*. Here, we find additional evidence of this conservation in our own hands, emphasizing the value of *C. elegans* as a model organism for nervous system genetics.

In Chapter 2, I investigated an alternative splicing event in a neuronal kinase, *sad-1*. Our initial screen identified three cell-specific TFs which regulated this splicing event. This was surprising as RBPs are known for their roles in alternative splicing, and we expected to identify RBPs rather in TFs in our screen. Follow-up experiments determined that the three TFs we identified regulated alternative splicing through their transcriptional control of a pair of RBPs that directly facilitate *sad-1* splicing. A growing body of recent work has suggested extensive coordination between regulatory layers is important for gene expression. Here, I present evidence of transcriptional and post-transcriptional cross-talk. I identify a specific incidence of this cross-talk that regulates cell-specific splicing of the neuronal kinase *sad-1*. 
This finding in *sad-1* prompted the question of how often this crosstalk between transcription and RNA processing occurs, and what biological processes such interactions coordinately control. In Chapter 3, I conducted a screen to identify physiologically-relevant instances of coordination between conserved neuronal genes in *C. elegans*. I employed a CRISPR/Cas9-based synthetic genetic interaction screen, a technique previously established in our lab as a valuable method for uncovering genetic interactions and identifying novel functions of neuronal RBPs\(^\text{14}\). Of particular interest, I identified a novel shared role for *tdp-1* and *fust-1* in facilitating *C. elegans* reproduction. This function came to light only in the *ceh-14* mutant background, suggesting *tdp-1* and *fust-1* share an interaction with *ceh-14* to affect fertility. I found additional evidence of a shared molecular role of *tdp-1* and *fust-1* in inhibition of exons during alternative splicing, and identify a *tdp-1*-regulated splicing event in *pgn-41* as a potential effector of fertility. The molecular and physiological roles of human TDP-43 and FUS remain elusive. Understanding the physiological functions of essential genes such as these is a crucial step in identifying, and eventually correcting, their dysregulation in diseases like ALS. Here, I provide evidence of a shared role of their *C. elegans* homologs, in addition to novel functions for other important neuronal RBPs and TFs. These findings emphasize the importance of dissecting interactions between gene regulatory layers in order to fully understand the complex, multifaceted functions of neuronal genes.
REFERENCES


