Identifying Factors that Control Mechanoreceptor Neuron Development in *C. elegans*

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Abstract

Mechanosensation, or converting mechanical forces of external touch into electrical signals, is easily studied in Caenorhabditis elegans (C. elegans), a transparent nematode with a well-understood nervous system and a fully sequenced genome. The mechanosensory system mediates growth and development in most organisms; a lack of or defect in mechanosensory stimulation can lead to developmental delay in children, resulting in developmental disorders such as autism spectrum disorders (ASDs) and Attention Deficit Disorder (ADD). Studying touch in the animal model C. elegans can elucidate the underlying neuronal mechanisms of touch and their developmental effects. In C. elegans, mechanoreceptor neurons (MRNs) detect touch, with three types that require the gene mec-3 (one of the LIM-homeodomain transcription factors) for their proper differentiation: touch receptor neurons (TRNs) (gentle touch), FLP neurons (tip of the head touch) and PVD neurons (harsh touch). While these three MRNs differentiate by the same transcription factor, each cell type's differentiation produces distinct morphology and express different genes. FLPs exclusively express the sto-5 gene, while TRNs express the mec-17 gene. The genes that determine and distinguish FLP vs. TRN differentiation are currently limited to egl-44, egl-46 and alr-1. To identify additional genes needed for the differential expression of TRNs and FLPs, the wild-type strain (TU3813), with the FLP neurons labeled with sto-5p::gfp and the TRNs labeled with mec-17p::rfp, was mutated and animals with altered expression or neuronal morphology were isolated. While defects in both expression and morphology of the FLPs and TRNs were observed in six animals, only one animal's mutation affecting TRN appearance was true-breeding. This morphological mutant exhibits abnormal TRN axon morphology, due to an autosomal recessive mutation. The mutant strain will be sequenced to identify the mutated gene, and the role of this gene in TRN and FLP differentiation.

Introduction

Touch sensitivity via converting detected mechanical forces into electrical signals is a vital survival mechanism in most organisms, a behavior known as mechanosensation. To understand normal function of the mechanosensory system and its complex neuronal circuitry, defective genes can be studied in animals that are deficient in mechanosensation (touch insensitive). Touch insensitive mutants were first developed in the nematode Caenorhabditis elegans (C. elegans), which led to the discovery of a mechanosensory complex with the necessary components to allow certain cells to detect touch to the body. Mechanosensation is well studied as a basic behavioral component in C. elegans since the organism's entire genome has been mapped. Investigating mechanosensation is much more challenging in complex eukaryotes because the mechanosensing cells and their scarce transducing molecules are more difficult to isolate and observe (Chalfie and Bounoutas, 2007).

Development of touch sensitivity can be studied in C. elegans by analyzing genes expressed in a specialized set of 30 neurons that detect mechanosensation: mechanoreceptor neurons (MRNs). This experiment investigates expression mutants (with altered neuronal touch receptor-specific gene expression) and morphological mutants (with defects on genes that affect neuronal outgrowth, number, or branching patterns) of two types of MRNs (touch receptor neurons (TRNs) and FLP neurons) in order to better characterize MRN differentiation. TRNs are an important type of MRNs because they exhibit the greatest electrophysiological response to mechanical stimuli (Kamkin and Kiseleva, 2007). Two other types of MRNs in C. elegans are PVD neurons, a pair of posterior interneurons that mediate harsh touch, and FLP neurons, a pair of ciliated mechanosensory neurons that detect touch at the very tip of the head. Collectively, TRNs (six cells), FLPs (2 cells) and PVDs (2 cells) comprise one-third of all MRNs (Goodman, 2008) (Figure 1).

Several genes have been identified as necessary for differentiation of the six touch receptor neurons (ALML, ALMR, PLML, PLMR, AVM and PVM). mec-3 (mec for mechanosensory abnormal) and unc-86 (unc for uncoordinated) are two genes that encode transcription factors (MEC-3 and UNC-86, respectively), which form heterodimers (UNC-86::MEC-3, a complex with increased DNA-binding specificity) that regulate TRN development in C. elegans (Goodman, 2008). mec-3 encodes an LIM-type homeodomain protein (where LIM stands for the proteins Lin-11, Isl-1 and Mec-3) with an evolutionarily conserved role in neuronal differentiation, migration, and morphogenesis (Hobert and Westphal, 2000). LIM-type protein expression in the TRNs, FLPs, and PVDs activates the downstream transcription of genes vital for TRN, FLP, or PVD function, respectively, when its promoters bind to the UNC-86::MEC-3 heterodimer (Figure 2 displays the combinatorial action of these genes).

MRN differentiation is dependent on the combinatorial action of several genes: unc-86, mec-3, egl-44, egl-46 and lin-14. unc-86 encodes a POU-type (derived from the names of three transcription factors: the pituitary-specific Pit-1, the octamer-binding proteins Oct-1 and Oct-2, and the neural Unc-86 from C. elegans) homeodomain protein (from a highly conserved family of eukaryotic transcription factors) expressed in 59 cells, which is a key regulator in the development of cell lineages into TRNs, and activates expression of the mec-3 gene (Finney et al., 1988). The development of TRNs requires unc-86 to produce appropriate TRN lineages, and mec-3 for TRN differentiation (Way and Chalfie, 1989). In unc-86 mutants, the correct lineage of TRN precursors is disrupted, which prevents the six TRNs from being made. mec-3 mutants contain cells with the potential to become TRNs, but lack adequate differentiation and the typical features of TRNs. lin-14 is a gene that encodes a protein vital for regulating postembryonic cell division timing and acts as a switch between TRN and PVD differentiation: in the presence of lin-14, AVM and PVM differentiation occur, while the absence of lin-14 gives rise to PVDs. The combined action of these genes ultimately restricts the expression of TRN fate to the six neurons observable in wild-type animals (Mitani et al., 1993).

TRNs detect gentle touch to the body in a manner dependent on the expression of mec-3. In TRNs, combined action of mec-3 and unc-86 activates various other mec genes (mec-1, 2, 4, 7, 8, 9, 10, 12, 14, 15, 17, 18) (Chalfie and Au, 1989) and the alr-1 gene (Topalidou et al., 2011), which define TRN fate. These genes comprise the mechanoreceptor channel complex, encoding parts of the extracellular matrix, tubulins for the TRN specific 15-protofilament microtubules, and other proteins of unidentified function. Transduction of the touch stimulus itself is accomplished via proteins encoded by several mec genes (mec-2, 4, 6, 10) and unc-24 (Chalfie and Bounoutas, 2007).

FLP neurons express the gene sto-5, which encodes a stomatin-like protein that regulates ion permeability (Stewart et al., 1993). In the absence of mec-3, sto-5 is no longer expressed in FLPs, indicating that sto-5 expression in FLPs depends on mec-3 (Topalidou and Chalfie, 2011). Although all FLPs and TRNs are regulated by mec-3, they have different functions, which may be linked to how MRNs express their specific traits and acquire distinct cell fates (Way and Chalfie, 1988) (Figure 3 demonstrates the central role of MEC-3 in TRN and FLP differentiation).

Since both mec-3 and unc-86 are also expressed in FLP neurons and sto-5 is only expressed in FLPs, there must be other co-factors that promote or block TRN fate.

Although mec-3 is needed for sto-5 gene expression in FLPs (characteristic of FLP cell fate), FLPs do not express the alr-1 and other mec genes characteristic of TRNs. It is known that mec-3 activates the sto-5 gene directly (Topali-

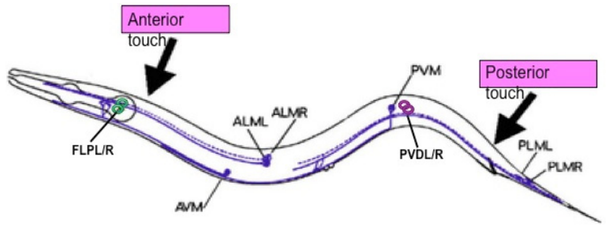

Figure 1 *Mechanosensory neurons in C. elegans*

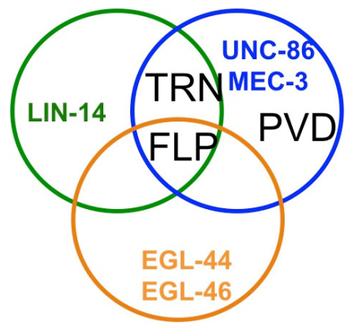

Figure 2 *Combinatorial MRN Differentiation*

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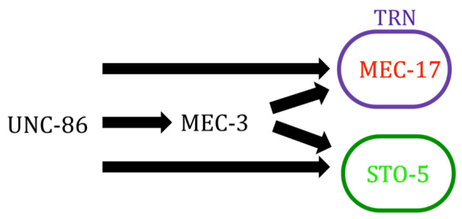


Figure 3 *unc-86 and mec-3 are both required for TRN and FLP differentiation, although they express different genes upon differentiation.*

dou and Chalfie, 2011), but it is not known why sto-5 is not expressed in the TRNs; this experiment aims to find genes that are involved in this process. Past studies have isolated egl-44 and egl-46 as two genes that restrict TRN fate in the FLP neurons: mutations of either gene cause the FLP neurons to transform into cells that resemble TRNs (Mitani et al., 1993) (Wu, Duggan and Chalfie, 2001).

However, the genes that restrict FLP fate in other cells remain unknown. We hypothesize that another gene or network of genes is restricting sto-5 expression to FLP neurons and is not allowing mec gene expression in the FLPs (which inhibits TRN fate). One morphological mutant was identified through this study, exhibiting irregularly wavy TRN axonal processes.

Materials and Methods

General methods and strains

To identify genes needed for the differential expression of TRNs and FLPs, wild-type animals (TU3813 strain) were mutated. These C. elegans strains were maintained on OP50 seeded agar plates at 20°C as outlined by Brenner (1974). The expression of sto-5 and mec-17 was observed in the wild-type strain using fluorescent protein tags: sto-5 was tagged with green fluorescent protein (sto-5p::gfp) and mec-17 was tagged with red fluorescent protein (mec-17p::rfp). sto-5 expression was used for FLP analysis since it is characteristic of FLP differentiation, and mec-17 was used for TRN observations since it is needed for sustained TRN differentiation. Since C. elegans are transparent, these fluorescently tagged genes are visible under a dissecting microscope. Gene expression was monitored under a dissecting microscope, looking for any changes in expression upon mutagenesis (Figure 4 & Figure 5 are images from the dissecting microscope of the fluorescent protein tagged FLP and TRN cells in a wild-type animal). The wild-type animals were synchronized to the same stage (L4) prior to mutagenesis using adecontaminating solution (20% bleach).

Fluorescence Imaging

The appearance of FLPs and TRNs in the wild-type strains were observed and characterized via a stereo-fluorescence dissecting microscope (Leica MZ12) powered by a UV light source (Kramer Scientific Corporation) coupled with the X-Cite 120 Fluorescence Imaging System. Axiovision 4.8.2 software was used to further characterize FLPs and TRNs.

EMS Mutagenesis

Mutagenesis was performed on the wild-type TU3813 strain with a standard protocol involving ethyl methanesulfonate (EMS) (Brenner, 1974): two to three plates of healthy TU3813 L4 animals (approximately 100-300 animals) were mutagenized, and 30 of the progeny animals (P0) were picked and plated individually, and stored at 25°C for three days. After three days, four animals from each P0 plate were plated individually (F1 animals) onto new plates and then stored at 25°C for three days (producing F2 animals). These F2 plates were manually screened three days later for any apparent abnormalities in FLP or TRN expression. F2 animals with noticeable abnormalities in either set of cells were picked individually onto new plates and their progeny were observed from subsequent generations to determine if the mutation is heritable (true-breeding) (Figure 6 schematically outlines the mutagenizing process).

Mutant screens via fluorescence imaging

The sto-5 promoter was expressed with gfp (sto-5p::gfp), and the mec-17 promoter with rfp (mec-17p::rfp) to observe and compare FLPs and TRNs among the wild-type and mutant strains. The fluorescent constructs are fu-

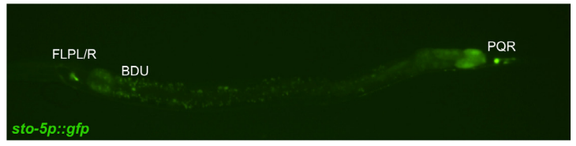


Figure 4 *sto-5p::gfp expression in wild-type TU3813 animals*

Figure 5 *mec-17p::rfp expression in wild-type TU3813 animals*

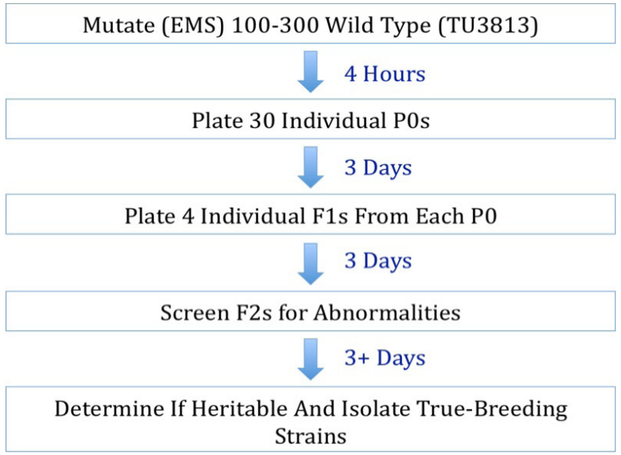


Figure 6 *Growth timeline and observation scheme for a standard EMS mutagenesis*

sions of promoters to gfp and rfp injected and integrated into the wild-type TU3813 animals. F2s were first screened abnormal expression of the gfp and rfp markers, indicating abnormal gene expression. Then, the morphology of FLPs and TRNs was characterized in identified mutant strains, noting the shape (axonal branching patterns), position, and changes in the number of FLPs or TRNs. Any animals with abnormal traits in these cells were isolated and screened for the abnormality over several generations. If the isolated mutant consistently produced progeny with the same abnormality, the mutation was further investigated through genetic crosses.

Characterizing mutants via genetic crosses

The isolated mutants were crossed with the N2 wild-type strain and then with the dpy-5 mutant strain to determine the nature of the mutation: whether it was dominant or recessive, and X-linked or autosomal. Two successive crosses were set up: the first was a cross between three hermaphrodite mutants and seven N2 (wild-type) males, and the second was a cross between seven of the male mutant progeny and three dpy-5 hermaphrodites. dpy-5 mutants are characterized by a short, "dumpy" body (Dpy phenotype) due to the disruption of the dpy-5 gene, which encodes a cuticle procollagen (Thacker, Sheps and Rose, 2006). The dpy-5 animals were used to distinguish hermaphroditic self-progeny (dpy/dpy genotype with Dpy phenotype) from hermaphroditic cross progeny (m/dpy genotype with normal phenotype, since dpy-5 is a recessive mutation). All animals crossed were in the L4 stage, a standard procedure used in order to enable mating between males and hermaphrodites and to minimize hermaphroditic self-progeny (Brenner, 1974).

Results

Six mutant strains exhibit abnormal FLP and TRN features

Seven mutageneses were performed on the TU3813 strain, yielding 710 observable F1 mutant animals. 32 candidate mutants were isolated from the mutant screens, six of which produced heritable FLP and/or TRN defects.

One mutant is true-breeding for abnormal TRN morphology

Among the six viable mutants, two mutants exhibited abnormalities in FLP neuron quantity and appearance. Observed morphological mutations included multiple and vertically moving FLP neurons, and abnormal TRN process morphology (wavy, crossed, or shortened). Expression mutations involved simultaneous FLP and TRN expression when observed in the GFP channel on the dissecting microscope.

Over successive generations, only one mutant (4A1) retained its mutant phenotype ('true-breeding'). The mutation is particularly observable along the processes of ALM and PLM neurons, producing irregularly wavy axons in these animals that are noticeably distinct from the straight axons in wild-type animals (Figure7-12 display side-by-side comparisons of TU3813 wild-type TRN morphology and 4A1 mutant TRN morphology from the anterior, mid-body, and posterior sections of the body).

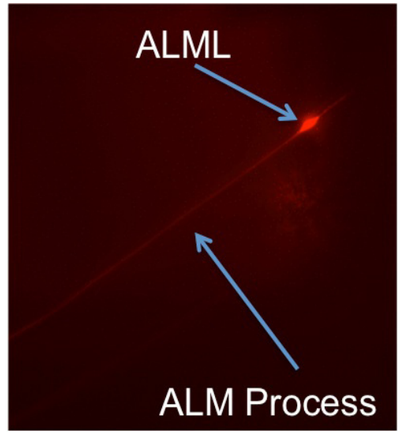


Figure 7 *Anterior view of TU3813 wild-type (WT) animal (left to right: ALM process, ALML). The ALM process is straight as it extends from the cell body. to-5p::gfp expression in wild-type TU3813 animals*

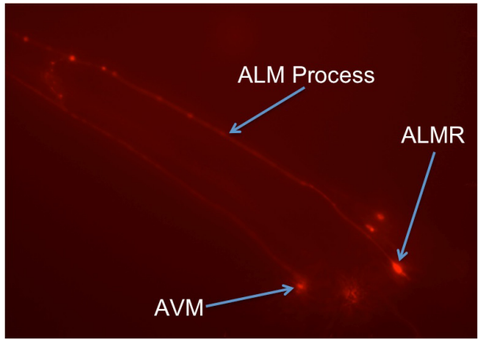


Figure 8 *Anterior view of 4A1 mutant (AVM), with irregularly wavy ALM process (left to right: ALM process, AVM, ALMR). The ALM process is wavy as it extends from the ALMR cell body*

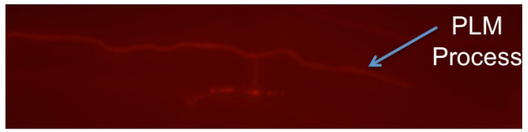


Figure 10 *Mid-body view of a 4A1 mutant's PLM process displays an irregularly wavy morphology throughout its midsection*

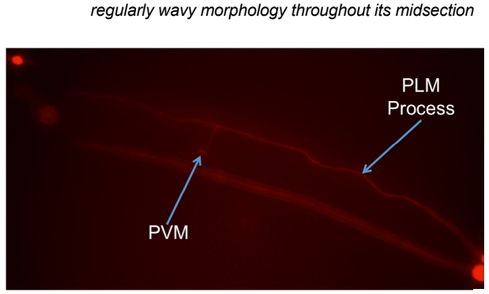
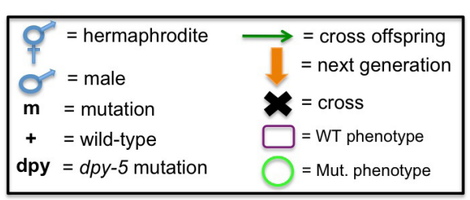


Figure 12 *4A1 mutant with irregularly wavy posterior process (PLM) (left to right: PVM, PLM process), exhibiting an apparent deviation from the WT process morphology (in Figure 11).*

Figure 13 *4A1 crosses for mutational characterization*



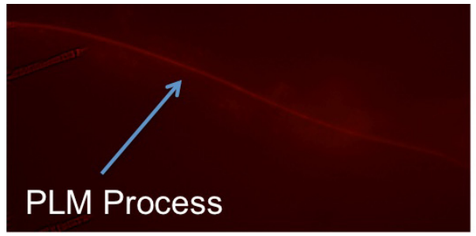


Figure 9 *Mid-body view of a TU3813 WT animal's PLM process displays its normally straight morphology.*

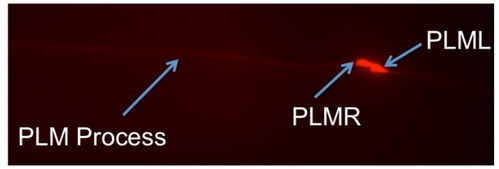
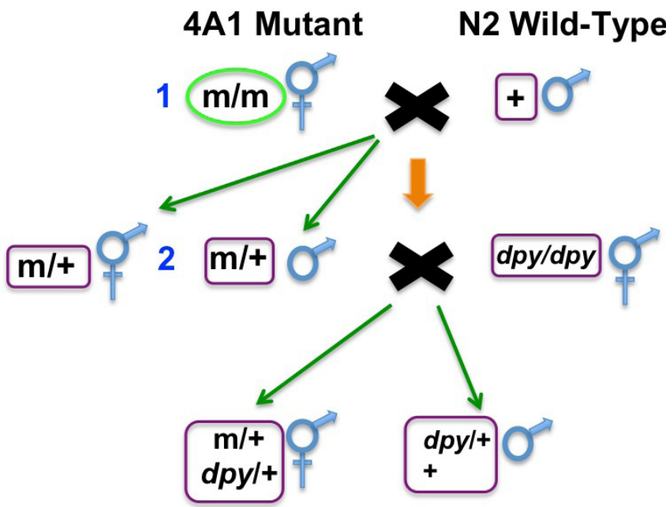


Figure 11 *Posterior view of a TU3813 WT animal's PLMR/L and normally straight PLM process (left to right: PLM process, PLMR/L).*

4A1 Mutation Characterization via Genetic Crosses

The first cross between hermaphrodite mutants and N2 (wild-type) males yielded male offspring and hermaphrodite offspring without the mutant phenotype (wild-type). The second cross between the male mutant progeny and dpy-5 hermaphrodites also produced wild-type male and hermaphrodite offspring (Figure 13 summarizes the cross).



Discussion

This study isolated genes that disrupt the function or development of gene expression, or morphology in the TRNs and FLP neurons (by isolating an observed phenotype and then identifying the responsible gene). Based on the specific abnormality observed in a mutant, the normal function of the mutated gene can be inferred from characteristics in its absence, and its role in MRN differentiation can then be established.

The seven mutageneses performed enabled screening of 710 F1 animals, yielding 32 candidate mutants, only one of which bred true. Typically, there is a 1/2000 probability of finding a mutant via mutagenesis, so an appropriate sample size would be screening 10,000 F1 animals, and their F2 progeny (Brenner, 1974). The F1 population size in our study was relatively small, and thus should be increased in the future by mutagenizing and screening more animals.

As mentioned above, this work has isolated one viable mutant via EMS mutagenesis (4A1). This mutant has displayed a consistent, morphological mutant phenotype in every successive generation, indicating that it is a true-breeding mutant. The 4A1 mutant phenotype, which involves irregularly wavy TRN morphology (along the ALM and PLM processes), suggests that the induced mutation may have disrupted the axonal development of the ALM and PLM neurons. Although none of the putative mutants with FLP abnormalities appeared to be true-breeding mutants, the only true-breeding mutant identified exhibits interesting TRN abnormalities. While finding a TRN or FLP morphological mutant was a secondary goal of the project (the primary goal was to find expression mutants), the mutant nonetheless appears to have a significant mutation that may reveal more about MRN functionality. Thus, the 4A1 mutant is worthy of further exploration and genetic characterization.

The results of the cross between 4A1 mutant hermaphrodites and N2 males, followed by the cross of the male offspring and dpy-5 hermaphrodites indicate that the mutation is autosomal recessive. The male offspring only inherited one X chromosome, from the mutant hermaphrodite. Since the cross with 4A1 mutant hermaphrodites and N2 males produced male offspring that did not exhibit the mutant phenotype, the mutation is not X-linked, which is further verified by the next cross. Furthermore, from both crosses the offspring do not show a mutant phenotype, which indicates that the mutation is recessive. An additional cross will be performed to reconfirm the mutant allele's mode of inheritance, by crossing dpy-5 (dpy/dpy) hermaphrodites with N2 (+/+) males. The heterozygous male offspring (dpy/+) will then be crossed with 4A1 mutant hermaphrodites (m/m), producing two types of heterozygous progeny (m/dpy and m/+). Since a recessive hermaphroditic allele was crossed with a male to produce heterozygote mutant offspring in the second generation, the cross further clarifies whether the mutation is dominant or recessive (based on the second cross male progeny's phenotype – if all males are mutant, the mutation is dominant; if the males are wild-type, the mutation is recessive).

Future studies will continue to follow the 4A1 mutant strain, specifically to determine on which chromosome the mutation is located. First, additional two-step crosses will be performed with mutants that have a known chromosomal location (i.e. dpy-5, with an autosomal recessive mutation on chromosome I). Such crosses will indicate which chromosome the 4A1 mutation is on, based on the progeny phenotypes. Then, complementation tests will be performed to determine whether the mutation is on a previously isolated gene, or an entirely unexplored gene. In addition, if it is a novel mutation for MRN differentiation (based on the chromosome and gene on which it is located), then the mutant strain will have its genome fully sequenced. Genomic sequencing of a 4A1 mutant animal can be compared with the wild-type genome in order to identify the gene that contains the mutation.

Additional mutageneses will also be performed (increasing F1 sample size) to find more genes potentially involved in the combinatorial regulation of MRN differentiation. Once these genes have been identified via genetic sequencing, they can be fused with GFP to further characterize their normal expression. With an understanding of the normal and abnormal function of these combinatorial genes, each gene's role in FLP and TRN differentiation can be determined. Other experiments can be performed to determine how these genes mechanistically function in the definition of MRN development or function. The irregularly wavy TRN processes observed in the 4A1 mutant could be due to a defect in axonal attachment. Another researcher in our lab has found a mutant with a cuticle attachment defect (from a mutation on the gene mec-5), so the 4A1 mutant will be compared to this other mutant's phenotype. Such comparisons can determine phenotypic similarities between mutants, and potentially identify genes that have a combinatorial interaction with one another in MRN differentiation. Once the combinatorial mechanisms regulating differentiation of the touch neurons is better understood, more insight into mechanosensory systems in higher organisms can be gained, due to the cross-organismal homology of touch.

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