MICROTUBULES, ACETYLATION, AND MEC-3 REGULATED GENES IN C. ELEGANS

MECHANOSENSATION

Charles C. Keller

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ABSTRACT

The ability to transform physical energies from internal and external environments into neuronal signals underlies the senses of hearing and touch as well as many aspects of body self-awareness. The small soil dwelling nematode Caenorhabditis elegans has proven to be a useful system in which to study neuronal mechanosensation and the development of specialized neuronal subtypes. Response to gentle body touch in C. elegans is mediated by the six Touch Receptor Neurons (TRNs). TRN cell fate is specified by the LIM-Homeodomain transcription factor MEC-3. Gene expression profiling has revealed a set of putatively MEC-3 dependant transcripts, which may represent the set of genes necessary to establish a TRN fate. I characterized this set of putatively MEC-3 regulated transcripts confirming the MEC-3 dependant expression of several previously unrecognized MEC-3 targets providing insight into TRN development and function and identifying the CCT chaperonin complex as being needed for TRN function. LIM-HD transcription factors play important developmental roles across phyla particularly in neurons. The data presented in this thesis provide insight into LIM-HD function in general and provide targets for further research in C. elegans TRNs as well as neurons from higher organisms.

Microtubules (MTs) play crucial roles in the majority of eukaryotic cells where they are required for cell division, intracellular transport, morphological stability and a variety of other
functions. The TRNs of *C. elegans* are characterized by unique large-diameter, heavily acetylated microtubules (MTs), which are required for mechanosensation. The TRNs thus present a valuable model for the study of neuronal MTs and MT acetylation, which is a poorly understood but widespread MT modification particularly in post-mitotic neurons. I employed TRN-specific RNAi to identify MT-associated proteins not previously known to be involved with mechanosensation including the CCPP-1 tubulin deglutamylase, the *C. elegans* homolog of the MT severing enzyme katanin, and several other proteins. I also shed light on the significance of α-tubulin acetylation by investigating the acetyltransferases responsible. MEC-17 and ATAT-2 are α-tubulin acetyltransferases found in the TRNs, which illustrate mechanosensory defects when mutated. I found that *mec-17* mutants exhibit a drastic decrease in TRN MT number as well as an elimination of the unknown electron-dense material found in the lumen of TRN MTs and other MTs across phyla. I also found that eliminating MEC-17 activity changed TRN morphology, resulting in ectopic sprouting and process growth. These results suggest that acetyltransferase activity is required for TRN MT integrity, mechanosensory function, and the maintenance of TRN morphology.
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CHAPTER I

INTRODUCTION
Mechanosensation

The transduction of physical force into neuronal signaling events forms the basis of the senses of hearing and touch. Force transduction is also essential for the maintenance of posture, balance, musculo-skeletal positioning, and the regulation of crucial homeostatic parameters such as blood pressure. Higher organisms have evolved a suite of mechanosensory receptor neurons responsible for perceiving and differentiating between the wide range of physical stimuli present in their external and internal environments. An understanding of how these specialized neurons develop and function would provide insight into crucial sensory systems and their associated pathologies. The functional diversity exhibited by mechanosensory neurons also provide a model for understanding how subtle differences in gene expression can lead to the dizzying array of neuronal subtypes exhibited in higher nervous systems.

Metazoan mechanosensory neurons share several common features. Physical force perception requires mechanosensory neurons to transform physical perturbances to their local cellular environment into the fundamental currency of neuronal communication, which is based on ion flux across their membranes. Most neurons generate ion flux through the selective opening and closing of ligand-gated channels which respond to the presence or absence of chemical signals in the area. Physical force does not convey chemical-signaling elements however, and mechanosensory neurons must rely upon mechanically gated ion channels to turn force into electrical signals. Mechanically gated channels must be able to open in response to distension of the cell membrane in which they reside. This requirement has resulted in specializations to mechanosensory neurons not observed in other neuronal sub-types such as force transducing cilia, unique extracellular matrix or tethering elements, unique cytoskeletal compositions, and the location of the cells themselves in close association with tissues subject
to force such as ligaments and hair follicles. Examples of mechanosensory specializations observed in human skin are shown in Figure 1.

Figure 1. Examples of mechanosensory neuron diversity in cutaneous mechanosensory fibers and structures. Differences in mechanosensory neuron position, shape, and extracellular support structures result in a suite of similar neurons with subtle differences in gene expression and functionality. The sum of such mechanosensory neuron heterogeneity results in the ability to differentiate the wide spectrum of physical forces present in the environment. Modified from Tsunozaki et al., (2009)

The specializations allowing mechanosensory neurons to perform their specialized tasks present challenges to the study of their development and function. Multiple specialized mechanoreceptors differentiating between mechanosensory sub modalities are found in close proximity to each other in dorsal root ganglia, autonomic ganglia, auditory structures, or the trigeminal nerve complicating the isolation of specific mechanoreceptor subtypes from higher metazoans. Cell body location also complicates direct electrophysiological recording from identifiable mechanosensory neurons. Functional heterogeneity between specialized mechanoreceptor subtypes complicates the identification of genes and proteins specific to particular modalities. The relative rarity of mechanosensory neurons in any tissue type and the
small number of actual mechanosensory channel proteins in each cell has confounded efforts to obtain pure cultures of mechanosensory neurons and biochemical efforts to isolate and characterize proteins required for mechanosensation.

Reconstitution of a functional mechanosensory channel apparatus in heterologous expression systems would help identify the exact components necessary for mechanotransduction. Understanding gained by expression of putative mechanosensory components in cell culture is confounded by the nature of mechanosensory channel gating, which seems to require multiple protein components in addition to the channel itself. Whereas a ligand-gated channel can often be expressed in cell culture and activated by simple addition of its ligand to the culture media, known mechanosensory channels require several supporting gene products and it has not been possible to fully reconstitute a functional mechanosensory complex in an orthologous expression system though constitutively open mechanosensory channel mutant proteins have been expressed and recorded from in frog oocytes (Goodman et al., 2002).

Genetic approaches in model organisms have provided much of our understanding of the genetic and molecular basis of mechanosensation. Studies in relatively simple, genetically tractable organisms such as D. melanogaster (Jarman 2002) C. elegans, (Bianchi 2007; Chalfie 2009) and zebra fish (Nicolson et al., 1998) have identified genes required for mechanosensation which have homologues in higher organisms. Many mechanosensory components identified by genetic approaches have been functionally characterized in model systems and subsequently recognized as being involved in human mechanosensation and other processes. Members of the Deg/ENaC and TRP families of ion channels, and stomatins have been identified or characterized in the context of model organism mechanosensation and
subsequently found to play similar and expanded roles in mammals (Arnadottir et al., 2010; Bianchi et al., 2002; Wetzel et al., 2007)

The Touch Receptor Neurons (TRNs) of *C. elegans* are a particularly useful model system for the study of mechanosensation and the development of specialized neuronal subtypes. Founding members of important metazoan protein families including the DEG/ENaC sodium channels (Driscoll et al., 1991) and the LIM-Homeodomain transcription factors were identified in the TRNs (Way et al., 1988). Genetic, electrophysiological, biochemical, and gene expression profiling approaches have clarified TRN development and function furthering the understanding of mechanosensation and neuronal cell-fate specification in general. Several questions remain however. One of the prominent unresolved questions regarding TRN development and function pertains to the role of the unique 15-protofilament microtubules (MTs) found in abundance in these neurons but not in any other *C. elegans* cells. The large diameter MTs are required for mechanosensation but their role remains unclear. Chapters III and IV of this dissertation will discuss efforts to clarify the role of the large diameter MTs in the TRNs.

Higher order nervous systems have evolved a multitude of specialized neuronal subtypes giving them the functional diversity needed for the complex functions required of them. Our understanding of nervous system function and development would be greatly aided by elucidation of the mechanisms by which neuronal subtype heterogeneity is manifested. Identification of the complete set of genes necessary for the function of any well-characterized neuron is therefore an area of much research. Well-characterized cell lineages and behavioral output make the TRNs one of the more tractable models for understanding what transcripts make one specific neuronal sub-type different from another. The suite of genes that need to be expressed in order to develop a functioning TRN is still unclear.
The LIM-homeodomain transcription factor MEC-3 is needed for the expression of most TRN-specific genes and is probably the terminal transcription factor in a series of transcriptional regulators, which impart a touch neuron fate (Way et al., 1988). Genetics and gene expression profiling have revealed a handful of genes regulated by MEC-3 and a set of putatively MEC-3 regulated transcripts (Zhang et al., 2002; Zhang et al., 2002). Identifying the full set of transcripts that are needed to impart a TRN fate would provide insight into the development of specialized neurons in general and present new targets for functional characterization. Chapter II will discuss efforts to confirm putatively MEC-3 regulated transcripts and characterize their function. The rest of this chapter will present background relevant to the more specific discussions found in the following chapters.

*Caenorhabditis elegans*

The free-living soil nematode *C. elegans* has proven to be a valuable system for studying a wide variety of fundamental biological processes including mechanosensation. The animal’s small size, short life cycle, transparency, sequenced genome, ability to be frozen and stored, ease of mutation generation, and primarily asexual means of reproduction make it an ideal genetic organism. *C. elegans* has only 959 somatic nuclei and the developmental lineage of each is known (Sulston et al., 1977). Serial electron microscopy reconstructions and detailed tracings of cell lineages have illustrated structural relationships throughout the animal’s body, the source of each adult cell, and the cell-cell relationships including synaptic connectivity particularly in the nervous system (Ward et al., 1975; White et al., 1976). *C. elegans* exhibits many of the fundamental tissue types found in higher organisms including muscle, neurons, and epithelia. *C. elegans* also exhibits a wide variety of behaviors. Several *C. elegans* behaviors were identified as being degraded due to EMS induced mutations in Brenner’s original
publication regarding *C. elegans* (Brenner, 1974). By grouping mutations according to what behavior or phenotype they influenced, the foundation was laid for the establishment of relationships between genetics, behaviors, specific cells, and specific proteins.

Underlying *C. elegans* behaviors are a small and comparatively well understood nervous system consisting of 302 neurons in the adult hermaphrodite (White et al., 1986). *C. elegans* is amenable to laser ablation of individual neurons allowing specific neurons to be linked with specific behaviors. The animal’s transparency allows for easy identification of gene expression patterns based on fluorescence. The genetic basis of behavioral phenotypes can often be correlated with a handful of specific cells and the genes known to be expressed in them. *C. elegans* is simple enough to allow reconstruction of the connectivity and developmental lineage of all of the animal’s cells yet complicated enough to exhibit many characteristics of higher metazoans and behaviors involving the integration of many cellular and genetic factors. Behaviors can be dissected and have yielded some of the few examples of behaviors whose underlying basis is understood at cellular, genetic, and molecular levels. The gentle touch response was one of the first neuronal circuits underlying a specific behavior to be characterized at the level of specific cells, specific genes needed for the function of those cells, the interconnectivity of the neuronal circuit, and the behavior itself.

**TRN differentiation**

The final differentiated state of any given cell is largely a function of what genes it expresses and in what context. Cells become progressively more specialized as the organism or cell lineage matures. Tightly regulated cascades of transcription factors progressively define specific terminally differentiated cell fates, particularly in neuronal tissue where a wide variety of
different final cell fates are needed. Differences between terminally differentiated neurons are often based upon the expression of a particular neurotransmitter receptor type, a specific ion channel, biosynthetic enzymes for a given neurotransmitter or other relatively minor differences that result in major functional distinctions between otherwise similar neuronal subtypes (discussed in Hobert, 2008). There are 118 morphologically distinct neuron classes among the 302 neurons in the C. elegans nervous system (Hobert, 2005). Saturation mutagenesis identified the Mec (Mechanosensory abnormal) genes needed for touch cell function and presumably only one terminal transcription factor necessary for the adoption of a TRN fate, the LIM-Homeodomain transcription factor MEC-3 (Chalfie et al., 1981). Gene products regulated by MEC-3 make up the subset of proteins necessary for TRN terminal differentiation and function including the channel complex as well as other supporting structural and enzymatic components required for transduction. Whereas MEC-3 appears to be the final transcription factor specifying TRN fate, its activity may be fine-tuned by the aristaless homolog ALR-1 (Topalidou et al., 2010).

The other Mec mutations cause defects in the ability of the cells to transduce mechanical stimuli rather than affecting the identity of the cell itself. The other Mec mutations can be viewed as analogous to the lack of a star player on a football team resulting in sure defeat whereas mec-3 mutations cause the whole team to sleep in and miss the game. The TRNs, though apparently still present in mec-3 mutants, may be mis-localized and lack processes exhibiting the large-diameter MTs or specialized mantle characteristic of touch neurons (Chalfie et al., 1981). TRNs appear to adopt the fate of their sister BDU neurons in mec-3 mutant backgrounds (Way et al., 1988). Not all neurons expressing mec-3 adopt a touch neuron fate. The mechanosensory FLP and PVD neurons both express mec-3 though they are not involved with gentle touch perception to the body (Way et al., 1989). The adoption of a touch neuron fate is actively repressed in FLP and PVD neurons by the action of the EGL-44 and EGL-46
transcriptional regulators (Wu et al., 2001). Mutations in egl-44 and egl-46 cause the FLP and PVD neurons to express TRN genes (Wu et al., 2001). A variety of other mutations result in abnormal numbers of touch neurons either due to inhibition of cell death or alteration of cell lineages (Mitani et al., 1993). The full suite of genes that MEC-3 regulates in order to specify a TRN fate remains unknown. Mutagenesis is inadequate to identify genes with subtle mec phenotypes, hypersensitive phenotypes, redundant mec genes or genes important for mechanosensation but exhibiting pleiotropy.

The ideal solution to the question of what transcripts MEC-3 regulates in the TRNs would be to compare the entire transcript composition of the TRNs to the rest of the organism as a whole, to other specific cell types, or to other TRNs lacking MEC-3 activity. Transcripts showing differing concentrations between mRNA pools might be targets of MEC-3 regulation and serve a role in TRN function or development allowing subsequent functional characterization. By comparing the entire RNA population of mec-3 vs. wild type animals, it would in theory be possible to have a complete description of what elements of the genome are needed in the touch neurons and which are not. Whole animal mRNA comparisons failed to detect mRNA differences attributable to mec-3 though microarray assays on sorted TRNs proved more useful.

The TRNs were the first specific neuronal subtype to be subjected to cell-specific gene expression profiling in C. elegans (Zhang et al., 2002). Attempts to use gene expression profiling techniques to identify additional genes necessary for touch cell function and differentiation by comparing mRNA from wt and mec-3 whole animal extracts failed to recognize known MEC-3 regulated transcripts presumably due to the small number of TRNs present in each sample. Some of the mec genes are expressed exclusively in touch neurons making it possible to perform Fluorescence Activated Cell Sorting (FACS) on wild type and mec-3 TRNs
expressing GFP in the TRNs. Wild type animals bearing a $P_{mec-18} \text{gfp}$ construct which is expressed exclusively in the six touch neurons were compared to $\text{mec-3(e1308)}$ mutants carrying a $P_{mec-3} \text{gfp}$ construct which is expressed in the TRNs as well as the paired PVD and FLP neurons (Way et al., 1989). mRNA was isolated from sorted cells, amplified and applied to DNA chips representing 90% of the *C. elegans* genome. 96 transcripts showed differences between these two populations (Zhang et al., 2002). The presence of many of the known mec genes in this group seemed to validate the method. Efforts to confirm and characterize these putatively MEC-3 regulated genes will be the focus of chapter II.

**Mechanosensation in *C. elegans***

*C. elegans* exhibits multiple mechanosensory response behaviors mediated by different neurons (though some neurons may be involved in multiple mechanosensory modalities). The gentle nose touch response is mediated by at least three sets of neurons including the ASH, FLP, and OSH neurons (Kaplan et al., 1993). Perception of gentle touch to the body involves the TRNs. Whereas avoidance of harsher mechanical stimuli depends upon the extensively branched PVD neurons and remains intact in the absence of TRN function. Other mechanosensory behaviors such as the plate tap response may involve neurons from all of the above classes. This dissertation focuses on the gentle touch response mediated by the six touch receptor neurons.

**Gentle touch in *C. elegans***

*C. elegans* will stop and reverse direction in response to gentle touch to the body of the animal with an eyebrow hair glued to a toothpick (Sulston et al., 1975). A small subset of
neurons which had been noted for their distinct large-diameter microtubule structure (Chalfie et al., 1979) underlie this avoidance response. Laser ablation studies confirmed that the six microtubule neurons are responsible for this behavior. Whereas saturation mutagenesis identified a set of mutations selectively resulting in touch insensitivity (Chalfie et al., 1981). The TRNs include three cells in both the anterior and posterior segments of the animal; the embryonically-derived, paired ALM and PLM neurons as well as the post-embryonically derived single AVM and PVM neurons. Response to gentle touch in C. elegans thus represents an ideal model for the study of a well-characterized behavior with a known neuronal basis in a genetically amenable organism allowing the investigation of fundamental developmental and sensory questions.

Figure 2. The six TRNs with cross section showing their characteristic mantle and densely packed large-diameter MTs. (Garcia-Anoveros et al., 1997).
General characteristics of touch neurons

TRNs exhibit several distinctive features. The TRNs exhibit long processes with a resultant receptive field covering all but the most anterior aspects of the animal. The ALM and PLM neurons exhibit long anterior projecting processes (the ALM neurons exhibit one small collateral which branches off and joins the nerve ring), in close apposition to the hypodermis of the animal, which wraps around the TRN processes. This makes for a tight coupling of the animals tough exterior cuticle and the underlying sensory neurons, which detect perturbations to the cuticle. The PLM but not ALM neurons also exhibit a short posteriorly directed “tail spike”. The ALM neurons lie on either side of the animal anterior to the vulva but posterior to the pharynx. Whereas the PLM neurons are found in the tail posterior to the anus. The AVM and PVM neurons are located midway between the vulva and pharynx or anus respectively. Both AVM and PVM exhibit a process that projects ventrally for a short distance to the ventral cord where the process takes an anterior turn. One of the more striking features of the TRNs is their characteristic, cross-linked, large-diameter, acetylated microtubules (Chalfie et al., 1982). TRN microtubules tend to terminate close to the cell membrane at regular intervals. Microtubule-protofilament organization is also unique in the touch neurons with microtubule bundles consisting of 15-protofilaments rather than the usual 11 (Chalfie et al., 1982). Disruption of the large-diameter microtubules by chemical or genetic means result in a mec phenotype. Disruption of the mantle or TRN process attachment can also result in touch insensitivity.

Genetics of gentle touch

Saturation EMS mutagenesis screens revealed a group of mutations that eliminated or reduced the animal’s response to gentle touch while causing no other gross abnormalities or
impairments (Chalfie et al., 1981). These mechanosensory defective or Mec mutations specifically affect the large-diameter microtubule cells, thus yielding a system where the cells responsible for the behavior are known and mutations can be inferred to affect these cells. Experiments addressing the expression of the mec genes revealed that they were either expressed in the TRNs or in tissues nearby with their protein products interacting with the TRNs. These initial mutagenesis screens yielded many avenues for research in the realms of cell-fate determination, mechanically gated ion channel physiology, microtubule dynamics, and gene regulation.

Components of the mechanosensory transduction apparatus

Several of the mec mutations map to genes, which encode subunits of an ion channel or accessory proteins thereof. mec-4 and mec-10 encode the two pore forming subunits of an amiloride sensitive sodium channel. Some mutations in mec-4 result in a constitutively open channel that induces swelling and necroses in touch neurons. mec-4, together with deg-1, formed the basis of the Deg/ENaC gene family (Driscoll et al., 1991). A subset of sodium channels found in the epithelial cells of kidney distal tubules were soon recognized as homologues of mec-4 (Canessa et al., 1993; Lingueglia et al., 1993) and many more members of the Deg/ENaC family of sodium channels have since been identified. Ectopic expression of mec-4d (constitutively open) and mec-10 in Xenopus oocytes allows detection of amiloride-sensitive sodium current but only when co expressed with the stomatin-like protein mec-2 (Goodman et al., 2002). Mutations in the paraoxanase-like gene mec-6 suppress the degenerin phenotype induced by mec-4(d) or mec-10(d) (Huang et al., 1994). Electrophysiological work in vivo has since definitively demonstrated that the MEC-4/MEC-10 sodium channel is indeed a mechanosensory transduction channel in metazoans, that it is mechanically gated (O'Hagan et al., 2005) and that its activity is directly modulated by the products of the mec-2, mec-6, and
unc-24 genes (Chelur et al., 2002; Zhang et al., 2004; Brown et al., 2008). These data suggest that the core mechanosensory channel complex in the TRNs consists of MEC-4, MEC-10, MEC-2, MEC-6, and perhaps UNC-24.

Figure 3. A) Putative interactions between ECM components, the mechanosensory channel complex and large-diameter MTs in C. elegans TRNs. B) In Vivo recording from a single PLM neuron with stimulus presence indicated by top trace and evoked current on bottom. Modified from Chalfie, (2009).

Cytoskeletal and extracellular matrix components required for mechanosensation

As previously noted, the large diameter microtubules are a distinctive feature of TRNs. Mutations that disrupt this distinctive microtubule arrangement have been mapped to two genes (Chalfie et al., 1986; Savage et al., 1989; Fukushige et al., 1999). mec-7 encodes a β-tubulin primarily expressed in the TRNs, (Hamelin et al., 1992) whereas mec-12 encodes an α-tubulin subunit found in the TRNs (Fukushige et al., 1999). MEC-7/MEC-12 heterodimers make up the large-diameter microtubules found in the touch neurons. The mec-4d/mec-10 channel does not
require tubulin subunits for activity in reconstituted systems presumably due to the constitutively open nature of the \textit{mec-4d} mutation (Goodman et al., 2002). Disruption of the large-diameter microtubules induced by mutations in \textit{mec-7} or \textit{mec-12} or by microtubule depolymerising agents result in a Mec phenotype. Some alleles of \textit{mec-7} and \textit{mec-12} disrupt the normal punctate distribution of the channel complex on the neuronal process (Bounoutas et al., 2009) whereas others result in a widespread decrease in TRN protein levels and putatively resultant Mec phenotype (Bounoutas et al., 2010). Microtubules and mechanosensation will be the focus of chapters III and IV of this thesis and further background will be presented later in this chapter.

Components of the extracellular matrix (ECM) are necessary for proper TRN function and presumably make up part of the pronounced mantle, which surrounds TRN processes. Adult TRN processes are in close apposition to the hypodermis of the animal and disruptions of this arrangement can interfere with touch sensitivity. Mutations disrupting several genes encoding extracellular matrix components result in a Mec phenotype. ECM \textit{mec} mutations map to three genes; \textit{mec-1}, \textit{mec-5}, and \textit{mec-9} (Chalfie et al., 1981; Du et al., 1996). \textit{mec-1} encodes an EGF/Kunitz domain-containing protein known to be common in extracellular matrix. Mutations in \textit{mec-1} result in a disrupted ECM, disrupted touch neuron process attachment, and mechanosensory defects (Emtage et al., 2004). \textit{mec-9} encodes a protein with EGF/Kunitz domains as well though they are arranged differently than those in MEC-1 (Du et al., 1996). \textit{mec-5} encodes a secreted collagen protein produced in the muscle cells surrounding the touch neuron processes rather than the TRNs themselves (Du et al., 1996). Extracellular proteins seem to be important in localization of the mechanosensory channel complex (Emtage et al., 2004). Some alleles of \textit{mec-12} such as \textit{u63} effect MEC-5 localization suggesting that a touch neuron product is required for the localization of the muscle cell product MEC-5 (Emtage et al., 2004).
Other mec genes

The remaining mec genes exhibit enzymatic activity or their function is unknown. *mec-8* encodes an RNA splicing factor known to target *unc-52* in muscle cells (Lundquist et al., 1996) as well as *mec-2* in the touch neurons (Lundquist et al., 1994). *mec-15* encodes an F-box protein involved in synapse formation, transcription, and protein degradation in response to MT depolymerization (Bounoutas et al., 2010; Bounoutas et al., 2009). *mec-17* encodes an α-tubulin acetyltransferase which may acylate the MEC-12 α-tubulin (Akella et al., 2010). *mec-18* and *mec-14* remain uncharacterized. It is likely that many other genes are essential for TRN function but exhibit pleiotropic phenotypes when mutated or are redundant. Chapter III of this dissertation will address some of these genes.

Microtubules (MTs)

Cells of all types require movement and structure. Cells often must migrate to their final position or send processes over long distances. Cell division requires the movement of chromosomes and membranes. Mature post-mitotic cells must transport organelles, vesicles, and protein complexes within their cytoplasm. Cells must also establish and maintain morphologies relevant to their function. The ability to migrate, divide, transport cargos, and maintain morphology requires intracellular structural organization that is largely based on the cytoskeleton. The cytoskeleton consists of three main elements: microtubules, actin filaments and intermediate filaments. Although actin (Perrin et al., 2010; Mogensen et al., 2007) and other cytoskeletal elements may be important for mechanosensory neuron function and development, they are outside the scope of this dissertation, which will focus on the role of microtubules in mechanosensation.
Microtubules are found in all eukaryotes and their basic structure is the same throughout the domain. Monomers of α and β-tubulin form heterodimers that are polymerized end-to-end to form protofilaments. Protofilaments are arranged in tubular structures generally consisting of 10-15 protofilaments in parallel to form one microtubule (MT). Although MTs vary in length and protofilament number, the basic tubular arrangement surrounding a lumen is consistent across all MTs. Despite this similarity in basic structure, MTs can participate in a wide variety of disparate processes depending upon modifications made to the basic structural template.

Microtubules are subject to a wide variety of post-translational modifications and interact with a diverse array of proteins. MT modifications and interactions with other proteins allow the basic structure of MTs to be adapted for a wide variety of cellular needs and processes. MTs are dynamic structures that undergo polymerization and depolymerization in a tightly regulated fashion and that can be adjusted depending upon cellular needs. Motility and cytokinesis may require less stable, dynamic microtubules whereas long-range transport and morphological stability in post-mitotic cells may require more stable microtubules. The proteins associated with MTs and post-translational modifications of tubulins largely regulate MT dynamics.

Microtubule assembly and dynamics

MT polymerization and depolymerization are tightly regulated and variable depending upon cellular activities, subcellular location, and place in the cell cycle. MTs populations exhibit “dynamic instability” both in vivo (Kirschner et al., 1986) and in vitro (Mitchison et al., 1984) meaning that at any time some MTs are slowly polymerizing whereas others are rapidly depolymerizing. Cellular manipulation of this property allows them to skew the balance toward polymerization or depolymerization depending upon current cellular needs. MTs involved in mitosis show a ten-fold higher rate of instability vs. non-mitotic MTs (Mitchison 1988). MTs localized to axonal growth cones tend to be less stable than those found in non-motile locations.
Stable MTs are often acetylated or de-tyrosinated though these modifications do not appear to be required for stability and their significance is not fully understood. MTs in long-lived post-mitotic cells, such as mature neurons, tend to be particularly stable, as indicated by increased resistance to MT depolymerizing agents such as colchicine (Ferreira et al., 1989).

Newly formed MTs begin their life at a microtubule-organizing center (MTOC). MTOCs consist of a γ-tubulin ring with associated proteins (reviewed in (Murphy et al., 1996). MTOCs are found at centrosomes and basal bodies but MT nucleation can proceed acentrosomal in developing axons (Stiess et al., 2010). The γ-tubulin ring forms a template for the addition of tubulin heterodimers. The growing end of the MT is known as the plus end whereas that at the MTOC is the minus end. Polymerized tubulin heterodimers bind GTP and hydrolyze it to GDP (Weisenberg 1976). GTP bound tubulin is not susceptible to depolymerization whereas GDP bound tubulin is (Weisenberg 1976; Penningroth et al., 1978). A cap of GTP-bound tubulin is usually found at the plus end of growing MTs imparting stability upon them. When the rate of GTP hydrolysis outpaces the addition of new GTP bound dimers to the MT end, a rapid depolymerization known as “catastrophe” occurs (McIntosh, 1984). Regulation of the balance between catastrophe and the addition of new GTP bound heterodimers provides cells with a convenient mechanism to regulate the stability of their own internal scaffold depending upon their needs at any given time.
Figure 4. Dynamics of MT assembly and disassembly (Conde et al., 2009).

Microtubule associated proteins

Microtubule Associated Proteins (MAPs) bind to MTs and regulate their stability and interactions with other cellular components. MAPs fall into two general categories (reviewed in (Gard et al., 1987; MacRae 1992; Maccioni et al., 1995). The type I MAPs, including MAP1a and MAP1b, bind to MTs via charged interactions and act to mediate interactions with other proteins and the plasma membrane. Type II MAPs include MAP2, MAP4, and TAU with MAP2 and TAU being found exclusively in neurons. MAP2 is preferentially localized to dendrites
whereas TAU is largely found in axons (Caceres et al., 1992). The type II MAPs bind to MTs via their C-termini resulting in increased stability whereas their more variable N-termini interact with other proteins. MAP binding affinities are modulated by MAP phosphorylation state (Drechsel et al., 1992) and tubulin modifications. Hyperphosphorylation decreases MAP affinity for tubulin and is observed in a variety of neuropathies reviewed in (Lee et al., 2001) where free MAPs form aggregates that correlate with neuronal dysfunction and death. It is not known if free MAP aggregates are causative or a side effect of some other pathology such as β-amyloid processing dysfunction. Other MAPs including XMAP215 and MAP4 are largely involved in cell division where they are heavily phosphorylated in response to cell division signals leading to their disassociation from MTs preceding the increase in MT dynamism needed for chromosome segregation (Gard et al., 1987). Some MAPs including the C. elegans TAU homolog PTL-1 will be discussed in chapter III.

**Post Translational Modifications of tubulin**

Tubulins undergo a wide variety of PTMs primarily on their C-termini, which are exposed in polymerized MTs. Known tubulin PTMs include but are not limited to, reversible acetylation, tyrosination, glycylation, glutamylation, and phosphorylation. The significance of these modifications has proven difficult to pinpoint though recent work, including the identification of several of the enzymes responsible for tubulin PTMs, has increased the understanding of PTM significance. Disruption of tubulin PTMs results in surprisingly weak phenotypes though recent work has illustrated roles for PTMs in MT transport velocity, affinity for severing enzymes and MT assembly. Selected tubulin PTMs relevant to neurons will be briefly discussed here (reviewed in (Fukushima et al., 2009)). Acetylation in particular will be discussed in further
detail in chapter IV. MTs and mechanosensation will be discussed later in this chapter following an introduction to mechanosensation in *C. elegans*.

**Tubulin Tyrosination/de-Tyrosination**

α-tubulins but not β-tubulins are subject to tyrosination/de-tyrosination. α-tubulins contain a C-terminal tyrosine residue immediately following translation. α-tubulin C-terminal tyrosine is removed at some point after heterodimer incorporation into a growing protofilament, revealing a C-terminal glutamate residue (Webster et al., 1987). De-tyrosinated α-tubulin is also known as glutamylated tubulin (Gundersen et al., 1987). α-tubulins are re-tyrosinated following MT depolymerization unless they have been de-glutamylated (see below). De-tyrosinated α-tubulin is generally found in older, stable MTs though the significance of this is not fully understood. De-tyrosination does not confer stability on MTs but is more likely a consequence of stability. The effects of de-tyrosination on MT function are most likely mediated by changes in affinity for MAPs or motor proteins (Kreitzer et al., 1999). The enzymes responsible for tyrosination/de-tyrosination have been identified. Enzymes mediating the tubulin tyrosination cycle will be discussed in chapter III.

**Tubulin glutamylation**

Tubulin glutamylation involves the addition of 1-6 glutamate residues to the C-terminal of tubulin (Edde et al., 1990). Though tubulin glutamylation often requires preceding de-tyrosination, poly-glutamylation can occur on other tubulin residues and is independent of tyrosination/de-tyrosination. As with many tubulin PTMs, the significance of poly-glutamylation is not fully understood though some effects have been observed. The glutamylation state of MTs alters their sensitivity to the MT-severing enzyme Spastin (Lacroix et al., 2010). The binding of TAU to tubulin is regulated by the length of polyglutamate side chains in vitro (Boucher et al., 1994). Other MAPs and motor proteins bind MTs in a poly-glutamate mediated
manner (Larcher et al., 1996; Bonnet et al., 2001; Ikegami et al., 2007). Poly-glutamylation-mediated MAP and motor binding is most likely mediated by a change in tubulin’s C-terminal conformation where MAPs and motors bind, rather than direct interaction, with poly-glutamate side chains. The enzymes responsible for poly-glutamylation/de-glutamylation have been identified and will be discussed in chapter III.

**Acetylation**

α-Tubulin acetylation was first noted in the flagellar MTs of *Chlamydomonas* by elucidating the ways in which these MTs differed from those found in the rest of the cell (L'Hernault et al., 1983; L'Hernault et al., 1985). The development of monoclonal antibodies specific for acetylated α-tubulin revealed the presence of acetylated α-tubulins in other MT subpopulations, including cilia and flagella from a variety of organisms (Piperno et al., 1985), as well as axons of rat cerebellar neurons (Cambray-Deakin et al., 1987), and PC12 cells induced to differentiate by NGF administration (Drubin et al., 1985). MTs bearing acetylated α-tubulin generally share the qualities of stability and limited subcellular distribution. Acetylated MTs in *Chlamydomonas* show resistance to nocodazole and colchicine-induced depolymerization (LeDizet et al., 1986). Rat meningeal fibroblasts contain MTs showing acetylation and detyrosination which are cold resistant whereas MTs containing only detyrosinated tubulin are not (Cambray-Deakin et al., 1987). Similar observations have been made in rod photoreceptors (Sale et al., 1988). In most cases acetylated MTs are not uniformly distributed throughout cells containing them but rather appear to define specific MT subpopulations (Bulinski et al., 1988) whereas other cells contain almost exclusively acetylated α-tubulin (Black et al., 1989).

α-tubulin acetylation occurs at the Lysine 40 residue of the polypeptide. Although N-terminal acetylation is a common PTM affecting over 90% of cellular polypeptides, Lysine acetylation is limited to several hundred polypeptides (Iwabata et al., 2005; Kim et al., 2006).
The full set of lysine acetylated proteins has yet to be identified in *C. elegans* though it does appear that FOXO transcription factors (Daitoku et al., 2004) and most of the enzymes involved with energy metabolism are regulated in some way by lysine acetylation, (Wang et al., 2010; Zhao et al., 2010). As with tubulin acetylation, the nature of this regulation remains unclear.

The physiological significance of acetylated α-tubulin has remained elusive. Although acetylated-α tubulin is largely found in stable microtubules and often appears and disappears in coordination with the appearance and disappearance of stable MTs (L'Hernault et al., 1985; Lim et al., 1989) it does not appear to be absolutely necessary for MT stability as some stable MTs lack acetylated tubulin (Schulze et al., 1987). There are also examples of acetylated tubulin being found in labile MTs (Sasse et al., 1988). Overexpression of non-acetylatable α-tubulin in *Chlamydomonas* skews the MT α-tubulin content towards non-acetylated MTs yet results in no observable phenotype (Kozminski et al., 1993). Similar results were observed in *Tetrahymena thermophila* where cells completely lacking acetylatable tubulin were indistinguishable from wild-type (Gaertig et al., 1995). It may be the case that acetylation is a consequence of MT stability rather than a cause, as illustrated by acetylation following MT stabilization by MAPs (Takemura et al., 1992).

Although gross abnormalities resulting from the disruption of α-tubulin acetylation have not been observed, a variety of important cellular processes are known to be modulated by MT acetylation including MAP affinity for MTs (Saragoni et al., 2000). Formation of immune synapses (Serrador et al., 2004), the processivity of at least one molecular motor (Reed et al., 2006), and the affinity of the MT severing enzyme Katanin (Sudo et al., 2010). α-tubulin acetylation affects or is altered in several pathologies including injury-induced Wallerian degeneration of axons (Suzuki et al., 2007), and Alzheimer’s disease-related fibrilary tangles (Hempen et al., 1996) though the significance of this remains unclear.
The understanding of α-tubulin acetylation has been aided by the identification of the enzymes responsible for the addition and removal the acetyl group itself. HDAC-6 (Hubbert et al., 2002; Zhang et al., 2003), and SIR2 (North et al., 2003) directly deacetylate α-tubulins and other lysine acetylated proteins with their over/under expression proving useful in the manipulation of MT acetylation and the investigation of its effects. A deacetylase specific to α-tubulin has not been identified. The C. elegans genes mec-17 and its paralog atat-2 are the sole known direct tubulin acetyltransferases (Akella et al., 2010; Shida et al., 2010), though elements of the elongator complex may also perform this function (Solinger et al., 2010; Creppe et al., 2009). MT acetylation and mechanosensation will be discussed later in this chapter and in chapter IV.

Figure 5. Common neuronal tubulin modifications. A) Tubulin modifications, the enzymes involved, and the interactions affected. B) General location of MTs bearing particular modifications in neurons (Fukushima et al., 2009).
Microtubules and mechanosensation

The requirement of cytoskeletal components, including microtubules, for mechanosensation has been illustrated in a variety of systems. Both cartilage and bone respond to exertion of force with the cytoskeleton playing an important role in transducing compression and shear forces (Chondrocyte mechanosensation is reviewed in (Blain 2009) and osteoblast mechanosensation is discussed in (Myers et al., 2007). Specialized mechanoreceptor neurons require intact cytoskeletons in order to function properly. Given the multitude of functions that MTs perform, it is often difficult to establish whether or not the dysfunction observed in a mechanosensitive cell upon MT disruption is simply due to general cell pathology or a deficiency in the actual transduction of mechanical signals. The hypotheses and evidence regarding direct roles of MTs in mechanosensation will now be discussed.

Microtubules and mechanosensory channel gating

The speed at which mechanoreceptors respond to stimuli would appear to be most consistent with a model wherein physical force directly results in the opening of mechanically gated ion channels. Two basic models have been postulated to explain mechanosensory channel gating in metazoans (Figure 6). According to the “tethering” hypothesis of mechanosensory channel gating, perturbation of the cell membrane or sensory structures such as cilia results in shear forces between the extracellular and intracellular protein tethering elements, which interact with the channel directly pulling it open. The rigid nature of microtubules makes them well suited to act as the intercellular anchor for such a tethering model. Another possibility is that extra and intracellular proteins serve to impart stretch on the membrane near mechanosensory channels causing them to open via changes in membrane tension. Mutations in genes encoding subunits of the large-diameter microtubules in C. elegans
TRNs result in touch insensitivity (Savage et al., 1989; Fukushige et al., 1999) as do mutations in extracellular matrix components (Du et al., 1996), which could support either idea.

The tethering and membrane tension hypotheses have been studied in the context of the cochlear hair cells, which form the basis of hearing. Cochlear hair cells are one of the best-studied examples of this type of mechanosensory channel gating. Several observations make the tethering model appealing in this context including channel localization and the requirement for intact tip-links (reviewed in (Schwander et al., 2010). Microtubules do not appear to be directly required for this example of probable tether-mediated gating of a mechanosensory channel however. The stereocilia of auditory hair cells do not contain MTs. Their structure appears to be maintained by actin. One axonemal kinocilium containing MTs is present on each developing hair cell but this is reabsorbed during development. MTs also are found at the base of the cilia and may serve to anchor them but they are not present near the area of actual tethering to serve as an intracellular anchor. It may also be the case that tip links tug on the membranes of neighboring stereocilia, causing channels to open via membrane tension without any actual channel tethering.

Figure 6. Tether vs. Membrane tension models of mechanosensory channel gating as illustrated in hair cell cilia. (A and B) Tether model. Here, the transduction channel binds directly to protocadherin 15 (PCDH15) and to the tether. Green fill indicates ion flux as channels open. (C and D) Membrane-tension model. By contrast, the transduction channel is not attached to PCDH15 and instead feels lateral membrane tension. From Gillespie et al., (2009)
The tethering model fails to explain some aspects of TRN channel gating. Electrophysiological recordings derived from the TRNs of mec-7 and mec-12 mutant animals in vivo show a reduced but not eliminated mechanosensory current suggesting that at least some channel gating can occur even in the complete absence of the large-diameter microtubules (Bounoutas et al., 2009). Recordings from wild-type TRNs in vivo show a current at the onset and offset of a mechanical stimulus but not between (O’Hagan et al., 2005). If the tethering hypothesis is true, then one would expect current to be constant whereas the stimulus is present since membrane distension and resultant shear forces between tethering components would be constant. It is possible that there is some sort of fast adaptation that occurs but no such mechanism has been identified. Ultrastructural analysis reveals that the putative members of a tethering complex do not necessarily overlap (Cueva et al., 2007). Extracellular components required for mechanosensation are required for channel complex organization which may be their function rather than, or in addition to tethering (Emtage et al., 2004). Cytoskeletal and extracellular matrix components may serve to impart a baseline tension upon a small segment of membrane with rigid microtubules acting as cellular tent posts keeping a stretch of membrane taut whereas extracellular components serve the same function or act to convey force to the channel pushing it relative to the rigid membrane thus moving the channel but not the lipid bilayer resulting in some conformational change which leads to channel opening. As mentioned in the case of hair cell stereocilia, it may be the case that membrane distension alone can cause mechanosensory channels to open with cytoskeletal elements playing a secondary role. The tethering hypothesis has yet to definitively refute these considerations.

Microtubules and other cytoskeletal elements are required for appropriate ion channel activity in neurons in general and other cells as well that have no known mechanosensory function. The non-mechanosensory squid giant axon shows reduced sodium and potassium
currents as well as altered resting membrane potential upon perfusion with the MT poisoning agents vinblastine, or colchicine reviewed in (Sakai et al., 1985). Myeloid Leukemia cells show increased sodium conductance within 1-3 minutes following treatment with the actin poisoning agent cytochalasin (Negulyaev et al., 1996). Cytoskeletal integrity seems to be a general requirement for proper ion flux across cell membranes, though the MT specializations observed in mechanosensory neurons suggest that they are in some way important in a mechanosensation-specific way.

The large diameter MTs may be necessary for the appropriate localization of other elements of the mechanosensory apparatus rather than or in addition to a role in channel gating. mec-7 and mec-12 mutations resulting in the absence of large diameter microtubules correlate with defects in channel complex localization as evidenced by antibody staining targeting the mechanosensory channel component MEC-2 (Bounoutas et al., 2009). This would seem to suggest that the large-diameter microtubules play some sort of transport or complex stabilization function and, in their absence, the mechanosensory complex cannot be delivered to or maintained at its appropriate destination with mechanosensory defects being the result. Experiments with the non-specific microtubule depolymerizing agent colchicine would seem to at least partially refute this hypothesis. Animals reared on colchicine display touch insensitivity and defects in mechanosensory channel complex localization consistent with a transport role for the TRN microtubules. However, adult animals transferred to plates containing colchicine exhibit a strong mechanosensory defect within 48 hours with no concomitant defect in channel complex localization (Bounoutas et al., 2009). These data suggest that TRN microtubules are required for more than just appropriate channel localization.

Intact large-diameter microtubules may be necessary for protein homeostasis in the TRNs disruption of which may lead to cellular dysfunction and mechanosensory defects
Mutations in \textit{mec-7} and \textit{mec-12} reduce fluorescence derived from \( P_{\text{mec-18 pra}:gfp} \), \( P_{\text{unc-119} gfp} \), and \( P_{\text{mec-3 pra}:gfp} \) constructs. The GFP protein is usually quite stable. The stability of GFP makes the molecule ill-suited for assessing short-term changes in gene expression. The praja domain, when added to GFP or any other protein targets the protein for degradation by E3 ubiquitin ligases resulting in a short lived version of the fluorophore which allows for finer temporal resolution at the level of promoter activity (Poyurovsky et al., 2003). One possibility is that intact microtubules are required to sequester complexes containing transcriptional repressors. This model has been illustrated in the case of \textit{Cubitus Interruptus} (\textit{Ci}) in \textit{D. melanogaster} (Wang et al., 2004). \textit{Ci} is a component of the hedgehog-signaling pathway which, depending on its phosphorylation state, possesses both transcriptional activation and repressive properties. Though \textit{C. elegans} apparently lacks the hedgehog pathway, it may be that similar mechanisms are in place for other transcriptional repressors in the TRNs. The removal of MTs might allow such otherwise sequestered repressors to repress gene expression in such a manner that TRN function is compromised. Such a mechanism might explain the apparently general transcriptional repression observed upon MT disturbance due to mutation or treatment with de-polymerizing agents such as colchicine. Under such a model, the large-diameter MTs might only be required to maintain some sort of general cellular protein homeostasis with mechanosensory components being amongst many victims of a general transcriptional repression phenomenon upon MT depolymerization.

The large-diameter MTs may serve to facilitate electrical conductance down the TRN process. MTs conduct electrical current (Priel et al., 2006), modeled in (Freedman et al., 2010), though the physiological significance of MT electrical conductance has yet to be illustrated in vivo. Perhaps TRN MTs are large in order to more efficiently conduct current. This might explain why small-diameter MTs (which are still present in \textit{mec-7} and \textit{mec-12} mutants) are
inadequate for proper TRN function and why current recorded from stimulated TRNs in vivo is reduced but not eliminated in TRNs lacking the large diameter MTs. It may also be the case that the large amounts of free tubulin present upon MT depolymerization interfere with mitochondrial ion channels as has been reported in cancer cells (Maldonado et al., 2010) resulting in cellular dysfunction though this would probably be a transitory phenomena.

The sum of these observations is an intriguing yet cloudy picture of MT function in TRNs. Although it could be the case that they play roles in mechanosensory channel complex transport and localization, channel gating, electrical conductance, or general TRN protein homeostasis, none of these explanations are independently adequate to explain all of the mechanosensory defects observed when the MTs are disrupted. It is possible to have mechanosensory defects due to MT disruption and yet still have appropriately localized mechanosensory channels (Bounoutas et al., 2009). It is also possible to have mechanosensory current flow albeit reduced, through mechanosensory channels even in the absence of the large-diameter MTs (Bounoutas et al., 2009). These observations are inconsistent with either channel localization or tethering defects being the complete explanation for mechanosensory defects observed when the large-diameter MTs are disrupted.

Another fundamental question remains unanswered: why are TRN MTs large? What is it about mechanosensory transduction in TRNs that makes normal diameter MTs inadequate? Mutants lacking the large-diameter MTs still exhibit normal sized 11-protofilament MTs (though overall MT number is reduced), which seem inadequate to compensate for the loss of the large-diameter MTs in regards to touch sensitivity. It may be the case that there are simply too few MTs of normal size to compensate for loss of the large-diameter MTs and that, given similar numbers, the small-diameter MTs might be adequate to restore touch sensitivity. It is possible that the large diameter of TRN MTs is simply a side effect of their heavy acetylation. Perhaps
the presence of the tubulin acetyltransferases MEC-17 and ATAT-2 in the MT lumen induces steric interference resulting in a larger lumen requiring more protofilaments to compensate for the increase in MT diameter. Large-diameter MTs tend to be much longer than the small diameter MTs found in other cells (Chalfie et al., 1979). Perhaps their large diameter imparts upon them rigidity, which is more important for long MTs than short ones. Such rigidity might be necessary for transduction even in the absence of increased MT length. Many ciliary MTs appear in doublets, which seem to impart rigidity. If mechanotransduction requires more rigid MTs, why not simply invoke the doublet solution rather than reinventing the MT rigidity wheel? A large-diameter MT presents more sites for MAP interaction which may be necessary for mechanotransduction though the weak phenotype of C. elegans TAU homolog mutations seems to speak against this concept (Gordon et al., 2008). These questions remain open. Efforts to clarify the relationship between large-diameter MTs and mechanosensation in the TRNs will be discussed in chapters III and IV.

α-tubulin acetylation and C. elegans mechanosensation

The TRNs of C. elegans provide a convenient system for the study of α-tubulin acetylation. As discussed previously, the large-diameter MTs found in TRNs are the most heavily acetylated MTs seen in C. elegans (Fukushige et al., 1999). The MEC-12 α-tubulin found in the large-diameter TRNs appears to be the only lysine-40 acetylateable α-tubulin in C. elegans and is required for proper TRN function allowing the study of acetylated tubulin in a metazoan with easily assayed, MT-dependant behaviors. Acetylated MEC-12 is detectable in other C. elegans neurons including ciliated sensory neurons and D-type motor neurons though these cells show far less intense signal than the TRNs when animals are stained with the 6-11-B-1 antibody that recognizes acetylated α-tubulin (Fukushige et al., 1999). Manipulation of α-
tubulin acetylation in *C. elegans* has yielded results similar to those observed in *Chlamydomonas* and *Tetrahymena* with resulting phenotypes being subtle. The *mec-12 (e1607)* Mec phenotype can be rescued by injection of non-acetylatable *mec-12* (Fukushige et al., 1999). An integrated single copy of non-acetylatable *mec-12* also rescues the touch phenotype though not to wild-type levels (Akella et al., 2010). *atat-2* mutants display nose touch defects (mediated by non-TRN ciliated sensory neurons). Gain of function mutations in the rho-GTPase *mig-2* result in locomotion defects which are presumed to be a result of MEC-12 hyperacetylation in the D-type motor neurons (Solinger et al., 2010). Enzymatically dead MEC-17 fails to rescue the *mec-17 mec* phenotype when compared to rescue using wild-type MEC-17, though even these animals are not fully touch sensitive (Shida et al., 2010). These apparently contradictory data fail to explain what role if any, the heavy acetylation of TRN MTs plays in *C. elegans* gentle touch.

Overall the effect of MT acetylation disruption in *C. elegans* TRNs, as in other cell types studied, appears to be subtle with the exception of the *mec-17* phenotype. *mec-17* deletions result in a strong mechanosensory defect yet no significant reduction in the acetylation state of TRN MTs is detectable by antibody staining (Akella et al., 2010; Shida et al., 2010). In addition, non-acetylatable versions of MEC-12 can rescue the touch defect in *mec-12* mutants to a large degree (though not to wild-type levels) (Fukushige et al., 1999). MEC-17 does not appear to acetylate histones in vitro and as yet has no other identified substrates (Shida et al., 2010). If the *mec-17* touch defect is solely due to α-tubulin acetylation defects then why is no significant reduction in acetylation detectable by antibody staining? Perhaps slight perturbations in the α-tubulin acetylation state are sufficient to result in TRN dysfunction or perhaps other MEC-17 non-histone acetylation substrates are present in the TRNs.
MT acetylation and the MT lumen

Acetylation at Lysine-40 is the only known tubulin modification to face the MT lumen (Dasgupta et al, 1983). The significance of this and of the MT lumen itself remains unclear. All MTs contain a luminal space of varying size depending on the number of protofilaments in the MT. The presence of lumen in MTs may simply be a consequence of structural efficiency or it may play some role itself perhaps by facilitating specific transport or acting as a conductor of current. Acetylation may exert its effects by inducing conformational changes in tubulin, which effect interactions on the MT exterior surface or it may influence luminal processes. The presence of electron-dense material of unknown composition in the lumen of MTs has been noted in several cell types including the TRNs by electron microscopy. Burton described discrete spherical objects 4-5nm in diameter appearing in the lumen of frog olfactory neuron axonal MTs (Burton 1984). Might this electron dense material be acetyltransferases? If so, what might be the significance of their presence in the MT lumen? Experiments aimed at addressing the role of MT acetylation in the TRNs will be discussed in chapter IV.

The CCT chaperonin complex

Transcripts encoding subunits of the CCT chaperone complex were among the set of putatively MEC-3 regulated transcripts discussed previously. Examination of mec-3 vs. wild-type TRN microarray data revealed that all eight C. elegans genes encoding subunits of the CCT complex were present in the putatively MEC-3 regulated set of transcripts, though five of them did not meet the criteria for significance. The presence in the TRNs of a chaperone complex whose primary folding substrates are actin and tubulin is not surprising considering the densely packed MTs observed in the TRNs but its regulation by MEC-3 is unexpected given the
widespread expression of the *cct* genes. Efforts to address the role of the CCT complex in the TRNs will be addressed in chapter II though relevant background will be presented here.

The TCP-1/CCT family of molecular chaperones belongs to an essential and ubiquitous class of proteins known as molecular chaperones. Most polypeptide chains carry enough information in their amino acid sequence alone to spontaneously adopt secondary and occasionally tertiary conformation in vitro though at time scales slower than physiologically relevant processes (reviewed in (Rossmann et al., 1981). The assembly of multi-protein complexes often will not occur spontaneously even though subunits may be appropriately folded. The mechanism by which translated polypeptide chains attain their final functional state and are then assembled into large oligomeric protein complexes has been and remains an area of intense research. It has become clear that many if not most peptides require the help of molecular chaperones in vivo in order to attain functionality within physiologically reasonable time frames. The first hints of how protein folding occurs in vivo were present but not recognized as such in the 1970s and 80s. Every organism studied had shared a response to heat and other stressors consisting of a quickly expressed set of genes and accumulation of Heat Shock Proteins or Hsp (reviewed in (Ashburner et al., 1979; Neidhardt et al., 1984).

First illustrated by the observation of “puffs” on *D. melanogaster* polytene chromosomes in response to heat and other stressors, (Ritossa 1964; Ritossa 1964; Ritossa et al., 1964) the heat shock response was inferred to have some thermo-protective effect. A large body of subsequent work supported this hypothesis though many aspects of the heat shock response’s protective effect remained poorly understood as did its widespread role in non-stressed cells. In response to a wide array of stressors, all know organisms illustrated a dramatic increase in the amount of a 70 kd (Hsp70) protein throughout the cell (Lowe et al., 1983). Similar results were observed in prokaryotes where mutations in the *E. coli* GroEL and GroES genes induce defects
in bacteriophage lambda, T4, and T5 cap or tail assembly with the defects being failure in the assembly of large oligomeric protein structures (Goldenberg et al., 1981). Both GroEL and GroES are necessary for survival and are also part of the E. coli heat shock regulon.

The molecular chaperone concept was proposed in 1987 (Ellis, 1987) and confirmed in 1988 when it was recognized that GroEL was a homologue of Rubisco binding protein which is required for assembly of the large oligomeric Rubisco holoenzyme (Hemmingsen et al., 1988). Several ancient and ubiquitous gene families encode proteins whose purpose is to help other proteins achieve and maintain biologically relevant conformations or at the very least prevent harmful aggregations. Three categories of molecular chaperones were proposed: 1) Nucleoplasmin 2) Hsp-70 large chain immunoglobin binding proteins and 3) the bacterial-mitochondrial-chloroplast class (Ellis et al., 1989). The last category was given the official title ‘chaperonins’ and later Group I chaperonins. Group I chaperonins are defined as functioning to maintain proteins in their appropriate folded state and prevent inappropriate aggregation due to exposure of hydrophobic domains during and immediately following translation or transit across organelle membranes which often requires protein denaturation, thus facilitating assembly into oligomeric complexes (paraphrased from Hemmingsen et al., 1988). An additional class of chaperones was added with the discovery of the cytosolic eukaryotic chaperone complex (Rommelaere et al., 1993).

The three canonical Group I chaperonins share sequence homology as well as similar tertiary structure. In eukaryotes, their function is limited to mitochondria and chloroplasts where they are thought to prevent inappropriate aggregation of proteins that must be denatured in order to transit the organelle membranes (Ostermann, 1990). Similarities in function, amino acid sequence, and their presence in all known eukaryotes seem to suggest that the group I chaperonins have been with us since the original endosymbionts. Group I chaperonins also
serve to facilitate the assembly of large holoenzyme complexes, which function in the organelles. Although the bacterial GroEL chaperone is not membrane bound, its canonical role in bacteriophage coat assembly at the cell membrane is somewhat analogous.

**Group II chaperones**

The group II chaperonins consist of the archaebacterial chaperones (thermosome) and the eukaryotic cytosolic Chaperone Containing T-complex-1 or CCT complex. The previously identified mouse early embryonic transcript Tcp-1 (Sanchez et al., 1985) is actually a subunit of the chaperone complex (Gupta 1990; Frydman et al., 1992; Nelson et al., 1992). Genes encoding subunits of the CCT complex have been identified in all eukaryotes examined to date. The eukaryotic CCT genes and their arcahea homologs share almost 40% homology at the amino acid level. The CCT complex is composed of two eight-subunit rings. Nascent polypeptides are delivered to CCT via prefoldins (Vainberg et al., 1998) and other co-chaperones where they are folded in an ATP dependant manner in the CCT complex lumen. Each subunit is encoded by a separate gene, there being eight CCT gene homologues in eukaryotic genomes, (Kubota et al., 1995) though a ninth tissue specific ccf gene has been reported in mouse testis (Kubota et al., 1997). The amino acid sequences of each subunit protein are approximately 30% similar to each other and encode protein products of similar length and amino acid composition. Each subunit possesses ATPase domains, which probably accounts for much of the homology between different subunit genes. In contrast, each CCT subunit gene shows a high level of sequence conservation across species consistent with its ancient and essential role.
Figure 7. CCT (shown here as a 3-dimensional reconstruction from single-particle electron microscopy, (Llorca et al., 2001) with its known and putative substrates as well as the cellular processes regulated as a result of its activity. (Brackley et al., 2009).

Group II chaperonin substrates

The CCT complex is known to be essential for the appropriate folding of a variety of nascent protein substrates primarily consisting of actin and tubulin monomers. Loss of function
mutations in any of the yeast cct genes result in lethality suggesting that a lack of any one of the CCT gene products renders the entire complex ineffective (Reviewed in (Stoldt et al., 1996). Conditional mutations in any of the yeast cct genes result in global defects in cytoskeleton assembly particularly in regards to actin and tubulin. Other folding substrates of CCT continue to be identified. Appropriate accumulation of Cyclin E as well as its proper folding is CCT-dependent in yeast and human cell culture (Won et al., 1998). Other proteins identified as CCT chaperone complex targets include the VHL tumor suppressor complex (Feldman et al., 1999), the cell cycle regulatory kinase Plk1 (Liu et al., 2005), G-protein subunits (Wells et al., 2006), and Sphingosine kinase 1(Zebol et al., 2009). Aside from the approximately 30 well-elucidated examples of polypeptides requiring CCT activity for their biogenesis (Spiess et al., 2004) the picture becomes hazy. One study estimated that 9-15% of all newly translated polypeptides transit through the CCT complex and thus may require its activity to adopt a proper conformation (Thulasiraman et al., 1999) whereas another resulted in an estimate of 1% (Sternlicht et al., 1993; Grantham et al., 2006). There are no reliable motifs or amino acid sequences that identify a polypeptide as being a CCT substrate. Further complicating matters is the fact that interaction with CCT subunits does not necessarily mean that a polypeptide is a substrate for CCT activity. Several co-chaperones interact with CCT but are not folding substrates. Work towards a CCT interactome has clarified the picture somewhat confirming known substrates as well as identifying several members of the septin complex as CCT folding substrates (Dekker et al., 2008). It is clear however that the CCT complex is selective regarding which proteins it folds and that the majority of cellular proteins are not CCT folding substrates.

Individual CCT subunits may possess biologically relevant activity in vitro and in overexpression experiments. siRNA targeting each subunit transcript independently in a colon cancer cell line results in variable cell shape and recovery from actin depolymerising agent phenotypes (Brackley et al., 2009). The same study also illustrated differential localization of
CCT subunits by antibody staining in ST3 cells suggesting that monomers may be acting independently. The physiological role of monomeric CCT polypeptides remains unclear though it is likely that they may play a role in cytoskeletal integrity by a folding-independent mechanism.

Inadequate CCT function has been implicated in a variety of neurological disease states some of which may be unrelated to normal substrates of the complex. Many neurological pathologies of the aging or congenitally defective nervous system have been linked to the accumulation of inappropriate protein aggregates. CCT activity may attenuate pathology stemming from the aggregation of abnormal polyglutamate repeat containing versions of Huntingtin (Tam et al., 2006). Sensory neuropathies correlate with mutations in CCT subunit encoding genes in mice and humans (Lee et al., 2003; Hsu et al., 2004; Bouhouche et al., 2006). Mutations in tubulin-encoding genes which interfere with binding to prefoldin or CCT have been implicated in Microgyria and a suite of brain developmental disorders known as the TUBB3 disorders (Tian et al., 2010). Further discussion of CCT and disease can be found in (Lundin et al., 2010). Given the essential roles of the cytoskeleton, it is not surprising that defects in tubulin or actin folding result in a wide range of pathologies though it is interesting that they seem to largely be neurological in nature. It may be the case that neuronal-specific alterations in CCT or its substrates are the only viable alterations in higher animals with more widespread defects resulting in early lethality.

**CCT chaperonins in C. elegans**

The *C. elegans* genome contains homologues of the eight canonical CCT chaperonin subunit genes. Relatively little has been published regarding CCT in *C. elegans*. Some work has been done on a few of the subunits; the *C. elegans* homologue of Tcp-1 was cloned and
characterized (Leroux et al., 1995) illustrating the splicing pattern of the transcript and confirming the protein’s ability to bind ATP in vitro. The expression pattern of *C. elegans cct-1* has been localized to muscle and neuronal tissues (Leroux et al., 1997). *cct* transcripts have appeared in several gene expression profiling experiments confirming their expression throughout the animal’s life (Madi et al., 2003). Recent work has confirmed that CCT mediated tubulin biogenesis is essential for cell division and migration in the developing animal though the activity of prefoldin is also required (Lundin et al., 2008). Feeding RNAi targeting the various *cct* subunits results in pleiotropic, severe phenotypes. My work regarding the role of the *cct* genes in mechanosensation will be discussed in chapter II.

**Organization of this thesis**

This thesis will describe two separate though related bodies of work. Chapter II deals with the putatively MEC-3 regulated genes derived from microarray comparison of sorted TRNs derived from *mec-3* and wild type animals. Experiments designed to confirm MEC-3 regulation of transcripts and their role in mechanosensation will be described. Experiments related to the CCT chaperonin complex will be described in chapter II. Chapters III and IV will deal with microtubules and mechanosensation in *C. elegans*. Chapter III will discuss the use of TRN-specific RNAi to identify novel MT-related aspects of mechanosensation in the TRNs. Chapter IV will focus on MT acetylation in the TRNs especially in regards to the activity of the MEC-17 acetyltransferase.
Chapter II

Identification and characterization of new MEC-3-regulated transcripts in *C. elegans*

TRNs
Abstract

The LIM-Homeodomain transcription factor MEC-3 is required for TRNs to reach and maintain a fully differentiated, functional state. mec-3 TRNs develop all the features of neurons but fail to express the suite of genes necessary for mechanosensation. Saturation mutagenesis would fail to identify mutations in genes needed for TRN function that may be functionally redundant or exhibit pleiotropic, hypersensitive, or subtle phenotypes. To identify other genes regulated by MEC-3, Zhang et al. (2002) examined the differences in mRNA expression between mec-3 and wild type TRNs using DNA microarrays (Zhang et al., 2002). In this chapter I describe experiments I performed to confirm MEC-3 regulation of candidate transcripts and characterize positive candidates. I confirmed MEC-3 regulation of several novel TRN genes and examined their significance in TRN development and function. I also identified a previously unrecognized role for the CCT chaperonin complex in the TRNs.

Introduction

LIM-HD transcription factors

The LIM (LIN-1, ISL-1, and MEC-3)-homeodomain (LIM-HD) protein family is a highly conserved set of eukaryotic developmental regulators (Curtiss et al., 1998; Hobert et al., 2000). Named for their homeodomain DNA-interaction motif and LIM protein-protein interaction domains, LIM-homeodomain factors have been implicated as transcriptional regulators involved with a wide variety of developmental events in eukaryotes from early patterning events to later terminal differentiation of specific sub-populations of cells (Sanchez-Garcia et al., 1993; Lumsden 1995; Varela-Echavarria et al., 1996). Despite their roles in diverse tissue types, a general function of LIM-HD transcriptional regulation appears to be the establishment and
maintenance of terminally differentiated cells particularly in the nervous system (reviewed in (Hunter et al., 2005). Mutations in genes encoding LIM-HD transcription factors often result in the failure of specific cell subpopulations to reach a terminally differentiated state. The situation becomes more complex in higher metazoans where LIM-HD transcription factors (TFs) often appear to act in a combinatorial fashion (Sharma et al., 1998). Phenotypes observed as a result of later acting LIM-HD protein dysfunction are often due to small groups of cells not performing their specialized functions as opposed to more global cell loss or dysfunction.

Of particular interest is the role that LIM-HD TF’s play in neuronal sub-type specification. MEC-3 was the first LIM–HD TFs shown to have a role in specifying a very specific subset of neurons, acting as perhaps the final transcriptional regulator in a long network of TFs specifying TRN cell fate (Chalfie et al., 1981). Similar LIM-HD dependant specification of terminal neuronal identity has been illustrated in bipotential neurons of the basal forebrain (Fragkouli et al., 2009), telencephalic progenitor cells and the Emx-1 lineage (Chou et al., 2009).

The full mechanism by which MEC-3 and other LIM-HD TF’s specify cell fate through transcriptional regulation is unclear. Both activation and repression of transcription contribute to the sequential cell-fate selection events, which give rise to specific, terminally differentiated cells. Transcriptional activation has been illustrated for a wide variety of LIM-HD TFs. Lhx2 deficient mice fail to express BMP-4, and 7 mRNA in the developing optic cup (Yun et al., 2009). Lmx1b knockout mice fail to express the slit diaphragm protein encoding genes Nphs2, Col4a3, and Col4a4 in podocytes (Miner, et al., 2002; Rohr et al., 2002) though this result has not been observed in patients suffering from Nails-Patella syndrome (correlated with mutations in lmx1b) (Heidet et al., 2003). ISL-1 activity induces the expression of several genes in pancreatic Islet cells possibly including insulin (Zhang et al., 2009). Although the ability of LIM-HD transcription
factors to activate transcription has been well-documented, there are fewer examples of LIM-HD mediated repression of transcription though some exist (Avraham et al., 2009).

MEC-3, UNC-86, and TRN specification

Terminal differentiation of the six TRNs requires the activity of the LIM-HD transcription factor MEC-3 and its DNA binding partner, UNC-86 (Way et al., 1988; Xue et al., 1993). unc-86 encodes a POU (Pit-Oct-Unc)-domain containing transcription factor expressed in 57 hermaphrodite neurons (Finney et al., 1990). UNC-86 partners with other transcription factors in subsets of these 57 neurons to impart different terminal cell fates depending upon the transcription factor UNC-86 interacts with. In TRNs, MEC-3 and UNC-86 bind some of the same DNA regions and their cooperative activity is required for appropriate expression of genes encoding components of the mechanosensory apparatus (Xue et al., 1992; Xue et al., 1993). UNC-86 can initially drive expression of mec-3 in mec-3 mutants but later expression of mec-3 requires MEC-3 itself (Xue et al., 1992). Mutations in mec-3 result in touch insensitivity due to the lack of expression of the other known mec genes. TRNs are still present but they fail to generate the components of the mechanosensory apparatus. In contrast, the FLP and PVD neurons express mec-3 but fail to express the genes necessary for a TRN cell fate due to the active repression of MEC-3 activity by EGL-44 and EGL-46 (Wu et al., 2001). MEC-3 is the most terminal known transcription factor in a cascade of transcriptional regulators that serve to refine a base-line neuronal fate imparted by UNC-86 into a very specific subtype with the decision as to whether or not to adopt a TRN fate depending upon MEC-3 activity.

Most of the known mec genes require MEC-3 activity for their transcription in TRNs (Zhang et al., 2002). The majority of mec genes were originally identified by mutagenesis however mutagenesis is inadequate to identify genes that may be involved in mechanosensation but present subtle, pleiotropic, or redundant effects upon mutation.
Mutagenesis will also detect genes not regulated by MEC-3 in the TRNs that might be important for mechanosensation. To identify genes regulated by MEC-3 that might be important for mechanosensation, gene expression profiling techniques are necessary. Microarray comparisons of mRNA populations derived from FACS sorted populations enriched for mec-3 or wild-type TRNs yielded a set of 96 transcripts differentially expressed in the two mRNA populations. 71 transcripts showed enrichment in wild type vs. mec-3 derived mRNA whereas 25 exhibited enrichment in mRNA derived from mec-3 TRNs vs. wild-type TRNs. The 71 transcripts represent genes likely expressed in a MEC-3 dependant manner and include several of the known mec genes (Zhang et al., 2002). These putatively MEC-3-regulated transcripts could encode proteins necessary for TRN development or function that were not identified by mutagenesis.

The set of 25 transcripts showing enrichment in mec-3 TRNs represent two possibilities: FLP expression or MEC-3 repression. mRNA used for the mec-3 microarray experiments were derived from populations of cells enriched for TRNs by FACS. The \( P_{mec-3\_gfp} \) construct used to label wild-type TRNs selectively enriched for wild type TRNs as mec-18 is only expressed in these six cells. The fact that the known TRN-specific genes are regulated by MEC-3 complicated the task of obtaining a population of cells enriched for the TRNs by FACS in a mec-3 mutant background. The best marker for TRNs that could be used in the mec-3 mutant background was a \( P_{mec-3\_gfp} \) construct which is expressed in TRNs as well as the paired FLP neurons which are involved in the perception of touch to the animal’s nose (Kaplan et al., 1993). Early mec-3 expression can be driven by UNC-86 though maintenance of expression past early development requires MEC-3 itself (Way et al., 1989). Thus the population of cells enriched for mec-3 mutant TRNs was also enriched for FLP neurons. The 25 transcripts showing increased expression levels in the mRNA derived from mec-3 TRNs could be transcripts that are normally repressed by the MEC-3 or they could simply be genes expressed in the FLP neurons.
This chapter will discuss the experiments I performed on the list of transcripts showing differential TRN expression in a putatively MEC-3-dependent manner. I set out to confirm MEC-3 regulation of the transcripts showing differential expression by microarray and to establish whether or not MEC-3 has a previously unreported transcriptional repression function in the TRNs. I also sought to characterize the role of newly identified MEC-3-dependent genes in TRN development and function.

**Results**

**Confirmation of MEC-3 regulation**

Promoter GFP constructs for all 96 genes (with the exception of *twk-28*, the last five of the putatively MEC-3 repressed transcripts, transcripts confirmed in (Zhang et al., 2002), and the known *mec* genes) on the list were constructed by traditional cloning, PCR fusion (Hobert, 2002), or by high-throughput techniques (Dupuy et al., 2007). I injected promoter GFP constructs into *mec-3* mutant animals and then crossed hermaphrodites from resulting stable lines with N2 males. Nearly all promoter GFP transgenic lines were bright enough to follow using a fluorescence dissection microscope. I examined homozygous stable lines in both N2 and *mec-3* backgrounds using fluorescence microscopy, comparing TRN expression or the absence thereof in both backgrounds. The presence of three subunits of the eight subunit CCT chaperonin complex on the microarray list prompted me to test the other CCT subunit-encoding genes for MEC-3 regulation as well.

I confirmed several genes as being MEC-3 regulated in the TRNs (table 1) whereas most transcripts examined appear to be false positives or genes requiring regulatory elements outside of the region used in my promoter gfp constructs (table 2). Newly identified MEC-3
regulated genes include the acid phosphatase F14E5.4/acp-2 (Figure 2), F-actin capping complex subunit cap-1 (Figure 4), arsenide inducible protein aip-1 (Figure 7), GABA receptor subunit ZC482.5, the ribosomal protein-encoding rpl-20 (Figure 3), unc-24 (appendix 1), and three genes of unknown function; F10A3.11 (Figure 6), T04B2.3 (Figure 5), F42A9.9 (Figure 1). Additionally, C03A3.3, T07D1.3, cct-2, and cct-4 had been confirmed as being MEC-3 regulated in our previously published work (Zhang et al., 2002). Aside from the CCT subunits, the new MEC-3 regulated genes do not appear to fall into any recognizable functional class. The enrichment of two of the eight CCT subunit transcripts in the wild-type TRN mRNA versus mec-3 TRN mRNA led me to design promoter GFP constructs for the remaining C. elegans CCT subunit-encoding genes in order to determine if these might be MEC-3 regulated in the TRNs as well. I confirmed MEC-3 regulation of three additional genes encoding subunits of the CCT/tcp-1 chaperonin complex in the TRNs. No evidence of MEC-3-mediated transcriptional repression was observed and the putatively MEC-3 repressed transcripts most likely represent genes expressed in the FLP neurons, genes whose regulatory elements were not included in my promoter constructs, or false positives.

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Description (Wormbase.org)</th>
<th>Fold difference*</th>
<th>N2 expression</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>F42A9.9</td>
<td>Unknown</td>
<td>4.52</td>
<td>Unknown head and tail neurons</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>ZC482.5</td>
<td>GABA receptor</td>
<td>4.24</td>
<td>See Topalidou et al., forthcoming</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>acp-2</td>
<td>Acid Phosphatase</td>
<td>3.91</td>
<td>TRNs, FLP? Vulval muscle</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>unc-24</td>
<td>Stomatin/lipid transfer Protein-like</td>
<td>3.58</td>
<td>(Zhang et al., 2004)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>rpl-20</td>
<td>Ribosomal protein</td>
<td>2.49</td>
<td>Many tissues</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>cap-1</td>
<td>F-actin capping complex subunit</td>
<td>2.21</td>
<td>Widespread neuronal expression</td>
<td>-</td>
<td>√</td>
</tr>
<tr>
<td>Transcript</td>
<td>Description (Wormbase.org)</td>
<td>Fold difference*</td>
<td>N2 expression</td>
<td>S1</td>
<td>S2</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------</td>
<td>------------------</td>
<td>---------------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>T04B2.3</td>
<td>Unknown</td>
<td>2.16</td>
<td>Body and pharyngeal muscle, neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>aip-1</td>
<td>Putative chaperone</td>
<td>2.06</td>
<td>Many tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F10A3.11</td>
<td>Unknown</td>
<td>2.06</td>
<td>Intestine FLP and other head neurons</td>
<td>√</td>
<td>√</td>
</tr>
</tbody>
</table>

Table 1. Newly identified MEC-3 regulated TRN transcripts (not including cct subunit genes or those published in (Zhang et al., 2002)). **“** fold difference mec-3 vs. N2 TRN mRNA. S1: √=MEC-3::UNC-86 binding site present in 5' UTR. S2: √=Overrepresented heptanucleotide present in 5' UTR (Zhang et al., 2002).
### Table 2. Summary of expression patterns of transcripts derived from microarray data which fail to show MEC-3 regulation in TRNs. **=* fold difference mec-3 vs. N2 TRN mRNA. S1: √=MEC-3::UNC-86 binding site present in 5’ UTR. S2: √=Overrepresented heptanucleotide present in 5’ UTR (Zhang et al., 2002).

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Description (Wormbase.org)</th>
<th>Fold difference</th>
<th>N2 expression</th>
<th>S1</th>
<th>S2</th>
</tr>
</thead>
<tbody>
<tr>
<td>bag-1</td>
<td>Homolog of human BAG chaperone</td>
<td>2.15</td>
<td>Intestine, neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F46E10.2</td>
<td>Unknown</td>
<td>2.15</td>
<td>Some neurons, intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>His-58</td>
<td>H2B histone</td>
<td>2.15</td>
<td>Most tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sra-6</td>
<td>Serpine receptor, class A</td>
<td>2.14</td>
<td>Head and tail neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K07C11.4</td>
<td>Carboxylesterase</td>
<td>2.13</td>
<td>Intestine, gonad, pharynx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F19B2.5</td>
<td>Helicase-like transcription factor</td>
<td>2.12</td>
<td>Many neurons, hypodermis, intestine, body wall muscle</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>C02F5.3</td>
<td>GT-binding protein DRG2</td>
<td>2.10</td>
<td>Many neurons, intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R05D7.5</td>
<td>Unknown</td>
<td>2.09</td>
<td>Most tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>calu-1</td>
<td>Calumenin (calcium-binding protein) homolog</td>
<td>2.09</td>
<td>Pharynx, intestine, hypodermis, tail neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C27B7.6</td>
<td>Serine/threonine specific protein phosphatase PP1, catalytic subunit</td>
<td>2.09</td>
<td>Muscle, intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B0336.11</td>
<td>Unknown</td>
<td>2.08</td>
<td>Muscle, hypodermis, head neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mtx-1</td>
<td>Translocaose of outer mitochondrial membrane complex, subunit TOM37/Metaxin 1</td>
<td>2.08</td>
<td>Most tissues</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mxl-3</td>
<td>Upstream transcription factor 2/L-myc-2 protein</td>
<td>2.06</td>
<td>Head muscle, head neurons, intestine, hypodermis</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>srp-1</td>
<td>Serpin, serine protease inhibitor</td>
<td>2.06</td>
<td>Body wall muscle</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>F42H10.3</td>
<td>Nebulin repeat protein</td>
<td>2.05</td>
<td>Muscle, many neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C55A1.6</td>
<td>Unknown</td>
<td>2.03</td>
<td>Intestine, head neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ttr-2</td>
<td>Trans thyretin-related family domain</td>
<td>2.03</td>
<td>Intestine, head neurons</td>
<td>√</td>
<td>√</td>
</tr>
<tr>
<td>pam-1</td>
<td>Puromycin-sensitive aminopeptidase</td>
<td>2.02</td>
<td>Intestine, head and tail neurons</td>
<td></td>
<td></td>
</tr>
<tr>
<td>art-3</td>
<td>ADP-ribosylation factor related</td>
<td>2.01</td>
<td>Many neurons, muscle, intestine</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>acr-13</td>
<td>Acetylcholine receptor</td>
<td>2.01</td>
<td>Body wall muscle, many neurons</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**MEC-3::UNC-86 binding sites, overrepresented putative regulatory sequences, and expression of candidate transcripts**

Analysis of 5’ sequence was performed on the putatively MEC-3 regulated transcripts in order to identify MEC-3::UNC-86 binding sites or other conserved regulatory sequences which...
might be involved in the regulation of MEC-3-dependant genes in TRNs (Zhang et al., 2002). A 5’ Heptanucleotide sequence was found to be overrepresented among the top twenty differentially expressed transcripts (mec-3 vs. wt TRN mRNA). Of the newly identified MEC-3-regulated TRN transcripts (including the three cct subunits and the two previously confirmed transcripts present in the microarray data), 4 of 14 transcripts contain the MEC-3::UNC-86 consensus binding sequence and 6 of 14 contain the overrepresented Heptanucleotide 5’ to their coding region. Of the 47 apparent false-positives whose expression I confirmed using promoter GFP constructs, 11 of 47 contain the MEC-3::UNC-86 binding site and 13 of 47 contain the overrepresented heptanucleotide 5’ to their coding region. These data suggest that neither the MEC-3::UNC-86 binding site nor the overrepresented heptanucleotide are essential for MEC-3 regulation of the differentially expressed transcripts identified by microarray comparison of mRNAs from sorted TRNs. Transcripts not expressed in TRNs also contain these putative regulatory elements in sequence immediately 5’ to their coding region and transcripts showing MEC-3 regulation in TRNs are no more likely to contain these regulatory elements than transcripts not exhibiting MEC-3 regulation.

**Characterization of newly identified MEC-3 regulated transcripts**

I employed a combination of expression analysis, genetics, and RNAi to ascertain what role, if any, the newly identified MEC-3 regulated transcripts might have in TRN development or function. Each transcript is discussed below.

T07D1.3 and C03A3.3

T07D1.3 and C03A3.3 appear fifth and seventh on the list of transcripts putatively regulated by MEC-3 in TRNs exhibiting 8.75 and 8.13-fold enrichment respectively in mRNA.
derived from wild type TRNs vs. mec-3 TRNs (Zhang et al., 2002). T07D1.3 and C03A3.3 transcriptional regulation by MEC-3 was previously confirmed (Zhang et al., 2002). I performed feeding RNAi targeting T07D1.3 and C03A3.3 transcripts in RNAi-permissive backgrounds resulting in no discernible Mec phenotype. Full knockouts were also indistinguishable from wild type by blind observation. T07D1.3 encodes a 144 amino acid protein with similarity to aldehyde dehydrogenases whereas C30A3.3 encodes a 287 amino acid protein with similarity to metallo-β-lactamases.

F42A9.9

mRNA transcribed from the F42A9.9 gene shows 4.52-fold enrichment in mRNA pools taken from wild type TRNs vs. mec-3 TRNs (Zhang et al., 2002). F42A9.9 encodes a protein of unknown function with no recognized homologs. The expression of P_{F42A9.9}gfp does not appear to be completely absent in mec-3 TRNs and this transcript may be an example of incomplete regulation by MEC-3 (Figure 1). Faint expression is observed in what appear to be ALM and PLM neurons in the mec-3 background though these cells appear to exhibit morphological defects, which might confound their identification. F42A9.9 is expressed in cells who’s location is consistent with that of the ALM sister BDU neurons. TRNs are clearly identifiable in the wild type background as are the presumed BDU neurons. No mechanosensory defect was observed upon F42A9.9 feeding RNAi.
Figure 1. Expression pattern of $P_{F42A9.9gfp}$ in mec-3 and N2 backgrounds. (A and B) Schematic representation of TRNs (Syntichaki et al., 2004) and BDU respectively (Wormatlas.org, 2011). (C and D) Apparent $P_{F42A9.9gfp}$ expression in ALM (white triangles) and BDU (blue triangle) in mec-3 animals. (E and F) $P_{F42A9.9gfp}$ expressed in apparent PLM (F shows distal PLM morphology defect). (H-J) $P_{F42A9.9gfp}$ expression in TRNs and BDU of N2 animals (white triangle in J shows an ALM process running past the BDU cell body indicated by a blue triangle).
F14E5.4/\textit{acp-2}

\textit{acp-2} encodes an acid phosphatase of the lysosomal and prostatic acid phosphatase family and shows 3.91-fold mRNA enrichment in wild type vs. \textit{mec-3} TRNs (Zhang et al., 2002). The expression of \( p_{\text{acp-2gfp}} \) is restricted to a few neurons including apparent FLPs, TRNs, an unidentified cell posterior to the pharynx, and unidentified posterior cells just anterior to the anus (Figure 2). TRN expression is not observed in \textit{mec-3} animals. Vulval muscle expression is also observed in adults. \textit{acp-2} deletion mutants exhibit no discernible mechanosensory defect.

\textbf{Figure 2.} \( P_{\text{acp-2gfp}} \) expression in \textit{mec-3} and N2 backgrounds. (A and B) Schematic representation of TRNs (Syntichaki et al., 2004) and FLP(Wormatlas.org, 2011) respectively. (D and E) \( P_{\text{acp-2gfp}} \) expression in \textit{mec-3} animals showing FLP and an unidentified posterior neuron. (F-H) \( P_{\text{acp-2gfp}} \) expression in N2 animals showing TRN and FLP expression.
The unc-24 mutation was recognized in Sidney Brenner’s original EMS screen with mutants exhibiting an uncoordinated phenotype manifesting as a defect in forward locomotion (Brenner, 1974). unc-24 appears fourteenth on the list of transcripts putatively regulated by MEC-3 in TRNs exhibiting a 3.58-fold mRNA enrichment in wild-type TRNs vs. TRNs isolated from mec-3 animals (Zhang et al., 2002). unc-24 mutants can reverse proficiently but move as if their nose were against an invisible barrier when prodded on the tail. unc-24 has been cloned and encodes a bipartite protein exhibiting two distinct domains both of which are found independently in other proteins (Barnes et al., 1996). UNC-24 contains a Stomatin domain similar to the characterized mec-2 gene, which influences the activity of the MEC-4/MEC-10 mechanosensory channel complex (Goodman et al., 2002). This similarity caused me to investigate whether or not unc-24 mutants exhibited a touch-insensitive phenotype. unc-24 mutants exhibited a slight defect in response to gentle touch to the anterior of the animal’s body but no defect in response to harsh touch. The unc-24 Mec phenotype was weak enough to have been easily overlooked in screens for touch insensitive mutants. Temperature sensitive mutants of mec-4 and mec-6 enhance the Mec phenotype of subtle Mec mutations at permissive temperature. I created unc-24; mec-4(u45) and mec-6(u247); unc-24 strains and assayed them for touch sensitivity. These strains exhibited a significant Mec phenotype at permissive temperatures. A similar defect in harsh touch was not observed suggesting that the observed Mec phenotype was not the result of a non-specific defect in locomotion. This result prompted us to mate an existing unc-24gfp line with mec-3 animals to assess the dependence of unc-24 expression on MEC-3 in the TRNs. We observed that unc-24 is indeed regulated by MEC-3 in the touch neurons. The details of unc-24’s involvement in mechanosensation can be found in Appendix 1.
rpl-20/ribosomal large subunit

rpl-20 encodes a component of the large ribosomal subunit and shows 2.49-fold enrichment in wild type vs. TRN mRNA populations (Zhang et al., 2002). P_{rpl-20}gfp shows widespread expression in mec-3 and N2 backgrounds with TRNs present in N2 and possibly mec-3 as well though PLM was not identifiable (Figure 3). Mutations in rpl-20 are lethal and this gene was not examined further due to the ubiquitous importance of ribosomes. rpl-20 may be an example of a TRN transcript not regulated by MEC-3 but showing differential expression due to MT loss in mec-3 animals (see discussion).

Figure 3. P_{rpl-20}gfp expression in mec-3 and N2 backgrounds. (A and B) Schematic representations of TRNs (Syntichaki et al., 2004) and BDU (Wormatlas.org, 2011) neurons. (C-E) P_{rpl-20}gfp expression in mec-3 animals showing BDU, and possibly ALM neurons. (F and G) P_{rpl-20}gfp in N2 animals showing BDU, ALM, and PLM neurons.
cap-1 (F-actin capping complex subunit)

The F-actin capping complex subunit cap-1 transcript displays 2.21-fold enrichment in N2 TRNs vs. mec-3 TRNs (Zhang et al., 2002). mec-3 animals may show expression of \( P_{\text{cap-1gfp}} \) in the PVM and the PLM but no expression is discernible in the anterior TRNs. N2 animals show \( P_{\text{cap-1gfp}} \) expression in all six TRNs (Figure 4). Organism-wide feeding RNAi against cap-1 results in severe pleiotropic defects whereas TRN-specific RNAi yielded no discernible mechanosensory defect.

Figure 4. \( P_{\text{cap-1gfp}} \) expression in mec-3 and N2 backgrounds. (A) Schematic representation of TRNs (Syntichaki et al., 2004). (B-E) \( P_{\text{cap-1gfp}} \) expression in mec-3 animals showing empty anterior (C), apparent PVM (D) and possible PLM (white triangle in E). (F-H) \( P_{\text{cap-1gfp}} \) expression in N2 animals showing TRNs (white triangles
T04B2.3

T04B2.3 transcripts show 2.16-fold enrichment in wild type vs. mec-3 TRNs (Zhang et al., 2002). \( P_{T04B2.3} \text{gfp} \) shows widespread expression with TRNs apparently visible in both mec-3 and N2 backgrounds (Figure 5). T04B2.3 encodes two isoforms of a protein of unknown function with homologs found only in other closely related nematode species. RNAi targeting T04B2.3 failed to yield any discernible phenotypes.

Figure 5. \( P_{T04B2.3} \text{gfp} \) expression in mec-3 and N2 backgrounds. (A and B) Schematic representations of TRNs (Syntichaki et al., 2004) and BDU (Wormatlas.org, 2011) neurons respectively. (D and E) \( P_{T04B2.3} \text{gfp} \) in mec-3 animals showing apparent expression in TRNs and BDU though these cells appear to lack processes or show morphology defects confounding their identification. (F and G) \( P_{T04B2.3} \text{gfp} \) expression in N2 animals showing TRNs.
**F10A3.11**

F10A3.11 mRNA shows 2.02-fold enrichment in wild-type TRNs vs. mec-3 TRNs (Zhang et al., 2002). \( P_{F10A3.11}\text{gfp} \) expression is limited to intestine and a handful of neurons including cells consistent with FLP, an unidentified cell in the tail anterior to the anus, cells near the vulva and TRNs in N2 animals (Figure 6). TRN expression is not observed in mec-3 mutants. F10A3.11 encodes a 283 amino acid protein of unknown function containing a PAN protein/protein, protein/carbohydrate interaction domain. Deletion mutants of F10A3.11 show no mechanosensory defect discernible by blind comparison with N2 animals. F10A3.11 affects lipid accumulation in \( daf-2/\text{insulin receptor} \) mutants by RNAi (Ashrafi et al., 2003).

![Figure 6](image_url)

Figure 6. \( P_{F10A3.11}\text{gfp} \) expression in mec-3 and N2 animals. (A-E) \( P_{F10A3.11}\text{gfp} \) expression in mec-3 animals showing possible FLP (B), unidentified posterior neuron (C) and cells straddling the vulva (D). (E-G) \( P_{F10A3.11}\text{gfp} \) expression in N2 animals showing TRNs and possible FLP neurons.
Transcripts of the Arsenide Inducible Protein (aip-1) show a 2.06-fold difference between wild type and mec-3 TRNs (Zhang et al., 2002). $P_{aip-1gfp}$ shows widespread expression in many head and tail neurons as well as the intestine and hypodermis. TRN expression is present in N2 animals but cannot be ruled out in mec-3 animals due to the many cells observed in the tail, which confound identification of PLM (Figure 7). ALM was not observed in the mec-3 background. RNAi vs. aip-1 does not induce a noticeable mechanosensory defect (chapter III). aip-1 is expressed in response to arsenide toxicity and other stressors (Ferguson et al., 2010). AIP-1 may be involved in ubiquitin-mediated proteolysis and has also been shown to have a protective effect in C. elegans Alzheimer’s models by attenuating the toxicity of ß-amyloid overexpression (Hassan et al., 2009). AIP-1 may act as a chaperone in response to stress. The need for such a function would not have been identified by my RNAi assays since the animals were presumably not stressed.
The CCT chaperonin complex

As mentioned previously, transcripts for three of the eight *C. elegans* genes encoding subunits of the CCT chaperonin complex were enriched in mRNA derived from wild type TRNs versus mRNA derived from *mec-3* TRNs as shown by microarray data (Zhang et al., 2002). I created promoter GFP constructs to ascertain the expression pattern of the remaining CCT subunit genes and determine whether or not they are also regulated by MEC-3 in TRNs. I found

Figure 7. *P*_{aip-1}gfp expression in *mec-3* and N2 animals. (A) Schematic representation of TRNs (Syntichaki et al., 2004). (B-D) *P*_{aip-1}gfp expression in *mec-3* animals showing no apparent TRNs though PLM expression cannot be ruled out in *mec-3* animals due to the presence of many fluorescent cells in the tail. No ALM expression is observed in *mec-3* animals. N2 animals show ALM and PLM *P*_{aip-1}gfp expression.
that cct-2, cct-6, and cct-8 are MEC-3 regulated in the TRNs. Expression patterns for cct-3, cct-5, and cct-7 were obtained in mec-3 animals but have yet to be crossed into N2. *C. elegans* CCT subunit genes showed widespread expression in a variety of tissues including many neurons in the head and tail including a neuron consistent with the position of the CAN pair of neurons which are required for viability (Figure 8). Five of eight subunits of the CCT complex showed MEC-3 dependant expression in TRNs suggesting that the CCT chaperonin complex is important for some aspect of TRN development or function. It is expected that the remaining three CCT subunit genes are also MEC-3 regulated in the TRNs.

Figure 8. Expression of *P*cct-2gfp, *P*cct-6gfp, and *P*cct-8gfp in mec-3 and N2 animals. (A and B) Schematic representations of TRN (Syntichaki et al., 2004) and CAN (Wormatlas.org, 2011) neurons respectively. (C and D) *P*cct-2gfp in mec-3 and N2 animals showing TRNs. (E-H) *P*cct-6gfp in mec-3 and N2 animals showing ALM and PLM in N2. (I-M) *P*cct-8gfp showing expression in TRNs (N2) and CAN (both).
The expression of five of the eight CCT chaperonin complex subunits in TRNs is not surprising. Actin and tubulin appear to be the major substrates for CCT mediated folding in eukaryotes (Kubota et al., 1995) and the TRNs are especially tubulin rich due to their tightly packed 15-protofilament MTs. Less is known about actin in the touch neurons. No Mec mutations have been shown to map to genes encoding actin subunits, which can probably be attributed to the severe pleiotropic effects caused by actin mutations. What is surprising is the fact that the subunits of a ubiquitous protein folding apparatus would be under the regulation of a transcription factor whose purpose seems to be one of applying “finishing touches” to a very specific subset of cells.

The ubiquitous and essential nature of the CCT complex complicated attempts to address its role in mechanosensation. Knockouts of any of the cct genes result in lethality in all systems studied thus far. Conditional cct mutants exist in S. cerevisiae (Stoldt et al., 1996) but none are known in C. elegans or other metazoans. I attempted feeding RNAi against the cct genes at different developmental stages in an effort to bypass the severe defects observed in cct balanced lethal strains where homozygotes arrest and die shortly after L1. Animals hatched on plates seeded with RNAi bacteria targeting any of the cct genes exhibited an arrest at L2 followed by a quick death. Animals placed on RNAi bacteria at later larval stages however did not exhibit such severe effects and could be assayed for touch sensitivity. L4 animals moved from OP50 plates onto cct RNAi plates exhibited a convincing Mec phenotype when assayed the next day. These animals exhibit normal harsh touch response and overall locomotor behavior.

*TRN-specific or pan-neuronal RNAi targeting cct subunits results in mechanosensory defects*

I employed TRN-specific and pan-neuronal RNAi to target cct genes in the TRNs or pan-neuronaly in order to avoid the pleiotropy associated with cct mutations or organism-wide RNAi.
C. elegans neurons are normally refractory to feeding RNAi due to lack of the SID-1 RNAi transporter which is expressed in most non-neuronal tissues (Winston et al., 2002; Feinberg et al., 2003). Tissue specific rescue of sid-1 mutants in TRNs or pan-neuronal yielded strains which could only undergo RNAi in tissues where the RNA transporter is expressed. Six of the eight C. elegans cct subunit homologs are present in the C. elegans feeding RNAi library. TRN-specific and pan-neuronal RNAi animals were grown at 15° on bacteria carrying RNAi constructs targeting cct-1, cct-2, cct-4, cct-5, cct-6, and cct-7. I assayed adults for anterior touch sensitivity. cct RNAi in the pan-neuronal permissive background resulted in arrest around the L2 larval stage. Animals persisted in this arrested state for at least two weeks before dying. Although it is possible that this arrest was a result of a purely neuronal cct RNAi phenotype due to cct expression in the CAN neuron, the observation that unc-119 (the unc-119 promoter drives pan-neuronal sid-1 rescue) expression is observed in early hypodermal precursors (Calixto et al., 2010) probably accounts for this arrest. Pleiotropic effects were not observed in the TRN-specific RNAi background. TRN-specific RNAi results were similar for all six cct subunits tested with five of six transcripts targeted by RNAi yielding statistically significant anterior mechanosensory defects as compared to RNAi targeting gfp (Figure 9).
cct-1 mutants show a Mec phenotype, disrupted TRN MTs but not mechanosensory channel complex localization defects

Although almost all cct mutants are lethal in C. elegans, a transposon-mediated insertion in the coding region of cct-1 yields animals that are sickly, and slow growing but can be assayed for mechanosensation. cct-1 mutants display mechanosensory defects (Figure 10). I stained cct-1 animals with the 6-11b-1 antibody, which recognizes acetylated α-tubulin and the MEC-2 antibody in order to assess MT integrity and MEC-4/MEC-10 channel complex localization respectively. MT integrity or channel complex localization might be disrupted in animals carrying cct mutations since tubulin is one of the CCT complex’s primary folding substrates. cct-1 animals show apparently disrupted, fragmentary 6-11b-1 staining along TRN processes. MEC-2 antibody staining recapitulates the wild-type pattern in cct-1 mutants. These data
suggest that the cct-1 mechanosensory defect is due to some disruption of TRN MT integrity though there appear to be enough intact MTs to allow correct channel complex localization.

Figure 10. 6-11b-1 and MEC-2 antibody staining in cct-1 and N2 animals. (A) Schematic representation of TRNs (Syntichaki et al., 2004). (B) Comparison of N2 and cct-1 young adult mechanosensory phenotypes (30 animals per strain). (C–G) 6-11b-1 antibody staining in cct-1 and N2 animals showing fragmented staining in cct-1 animals vs. N2. (H–K) MEC-2 antibody staining in cct-1 and N2 animals showing similar patterns.
cct rescue experiments

In order to conclusively illustrate a role for the CCT complex in mechanosensation, I attempted to rescue the cct lethal phenotype in non-TRN tissues in the hope of isolating the TRN effect of cct mutations. The sto-4 promoter is expressed in most neurons but not the TRNs (J. Kratz personal communication). I designed constructs bearing the genomic coding sequence of cct-2 and cct-6 driven by the sto-4 promoter and transformed balanced lethal cct-2, and cct-6 mutant strains with these constructs. Both $P_{sto-4}^\text{::CCT}$-2 and $P_{sto-4}^\text{::CCT}$-6 constructs failed to yield viable transformants in cct-2 and cct-6 balanced lethal backgrounds. The sid-1 promoter is expressed in the majority of non-neuronal C. elegans tissues. I generated constructs carrying genomic cct-2, and cct-6 sequences driven by the sid-1 promoter and transformed balanced lethal cct-2 and cct-6 mutant lines with these constructs. Both $P_{sid-1}^\text{::CCT}$ constructs failed to yield viable transformants. $P_{sid-1}^\text{::CCT}$-6 transformation yielded transformants but failed to rescue the lethality of the cct-6 mutation. I also attempted mosaic rescue analysis using genomic cct-2 and cct-6 driven by their own promoters as well as a fosmid carrying containing 40KB of genomic DNA including cct-6. Mosaic analysis relies upon the inconsistent inheritance of extrachromosomal arrays during cell division in C. elegans. By transforming cct mutants with genomic cct constructs along with a TRN GFP marker, it might be feasible to identify rescued animals expressing the extrachromosomal array in either the anterior or posterior TRNs only thus allowing comparisons to be made between rescued or non-rescued anterior and posterior mechanosensory phenotypes. These experiments failed to yield viable transformants. Tissue-specific and mosaic rescue experiments in cct mutant backgrounds to date have failed.
Discussion

I used promoter GFP constructs to assess MEC-3 regulation of 78 transcripts showing differential representation in mRNA populations derived from FACS wild type and mec-3 TRNs (Zhang et al., 2002). These 78 transcripts included 20 of the 25 putatively MEC-3 repressed genes as well as the putatively MEC-3 upregulated genes (with the exception of the known mec genes). Nine transcripts were confirmed as being wholly or partially regulated by MEC-3 in TRNs. I also found MEC-3 regulation of three additional subunits of the CCT chaperonin complex in TRNs. Expression patterns for the final three CCT subunit-encoding genes were obtained in mec-3 animals but have yet to be crossed into N2 animals. The other transcripts tested did not show MEC-3 regulation in the TRNs and may represent false positives or genes requiring regulatory elements outside of the 5′ region I used in my promoter GFP constructs. It is difficult to estimate the likelihood of false positives in microarray data since many of the resulting gene lists are not confirmed by in vivo expression analysis. Expression data for many C. elegans transcripts exist and it might be possible to come up with baseline false positive rates for C. elegans gene expression profiling experiments by comparing known expression patterns to published gene lists. The majority of the putatively MEC-3 regulated transcripts appear to represent false positives or possibly FLP-expressed genes. The promoter GFP approach may have failed to identify MEC-3 regulated transcripts whose regulatory regions lie outside of the 5′ region used in my constructs though I doubt that this difficulty would explain all of the apparent false positives. The apparently high false positive rate may be a manifestation of the immaturity of the technology at the time of the original microarray experiments or it may be attributable to issues with the analysis or probe design as discussed in (Hariharan, 2003). Comparison with other microarray data sets might help clarify what transcripts are false positives.
The newly identified MEC-3 regulated genes do not fall into any easily recognizable categories. \textit{acp-2} encodes an acid phosphatase, which could be important in the regulation of a variety of processes. The human homolog of \textit{acp-2} attenuates Epidermal Growth Factor signaling in prostate tissue by de-phosphorylating its receptor (Lin et al., 1988). EGF signaling partially acts via the Retinoblastoma pathway in \textit{C. elegans} (Thomas et al., 1999). The \textit{C. elegans} RB homolog \textit{lin-35} facilitates RNAi when mutated (Lehner et al., 2006). \textit{acp-2} knockout strains showed no mechanosensory defect though a slight defect was observed upon \textit{acp-2} RNAi in \textit{lin-35} animals. There may be a relationship between \textit{acp-2} and \textit{lin-35} in TRNs though this has not been conclusively established. \textit{cap-1} encodes a subunit of the F-actin capping complex which is involved in regulating actin branching dynamics (Waddle et al., 1993). Although proper actin branching may be important for TRNs no mechanosensory defect was illustrated by \textit{cap-1} RNAi. \textit{aip-1} possesses putative chaperone activity and attenuates \(\beta\)-amyloid toxicity in neurons (Hassan et al., 2009; Ferguson et al., 2010). No mechanosensory phenotype was observed by \textit{aip-1} RNAi though \textit{aip-1} may be involved in a neuronal stress response, which was not addressed by my assays. F10A3.11 encodes a protein, which may be involved in lipid metabolism. No mechanosensory defect was observed in F10A3.11 mutants though its limited neuronal expression may suggest TRN significance in a redundant or environment-specific manner such as during dauer or starvation. The other newly identified MEC-3 regulated transcripts encode proteins of unknown function and illustrate no mechanosensory defects.

Some of the MEC-3 regulated transcripts may be examples of genes that are expressed in the TRNs but are not under MEC-3 regulatory control. \textit{mec-7} and \textit{mec-12} encode beta and alpha-tubulins respectively that comprise the TRN large-diameter microtubules. Mutations in \textit{mec-7} and \textit{mec-12} appear to cause a general transcriptional malaise in TRNs which affects both MEC-3 and non MEC-3 regulated transcripts though the extent of this is unclear as only
five genes have been studied for this effect (Bounoutas et al., 2010). *mec-7* and *mec-12* are themselves under MEC-3 transcriptional regulation. Some of the apparently MEC-3 regulated genes may actually represent non-MEC-3 regulated transcripts, which show differential TRN expression in *mec-3* mutants due to transcriptional repression caused by the lack of *mec-7* and *mec-12* expression in *mec-3* animals. *rpl-20, cap-1, F42A9.9, and T04B2.3* may be examples of genes expressed in TRNs that are not under MEC-3 regulation despite showing decreased expression in *mec-3* animals. This decrease may be attributable to general TRN transcriptional depression since apparent TRNs are faintly visible in *mec-3* animals expressing these constructs. *F10A3.11, acp-2, T07D1.3, and C03A3.3* are more likely to represent genuine targets of MEC-3 transcriptional regulation since no hint of TRN expression was observed in *mec-3* animals carrying promoter GFP constructs for these transcripts.

**The CCT chaperonin complex and TRNs**

Five of the eight *C. elegans* genes encoding subunits of the CCT chaperonin complex show MEC-3 transcriptional regulation in TRNs. The expression patterns of the remaining three *C. elegans cct* genes remain uncharacterized. The wide neuronal expression of *cct* subunits may have confounded efforts to identify *Pcct:Gfp* expression in MEC-3 TRNs though I saw no evidence of such expression. I illustrated mechanosensory phenotypes for *cct* genes by TRN-specific RNAi, late-stage organism-wide RNAi, and analysis of the viable *cct-1* mutant line. *cct-1* animals display defects in acetylated α-tubulin antibody staining but apparently intact mechanosensory channel complex localization. These data suggest that *cct* mechanosensory defects are due to TRN MT disruption caused by the lack of CCT’s tubulin chaperone role. Defects in tubulin folding could result in TRNs lacking appropriately assembled MTs though some degree of tubulin folding is apparently still present in *cct-1* mutants since stretches of acetylated α-tubulin staining are still present in *cct-1* TRNs. *cct-1* mechanosensory defects are
most likely due to MT disruption though it is unclear if this leads to defects in channel gating, transcriptional repression or both. The observation that L4 animals placed on cct RNAi bacteria exhibit mechanosensory defects within 24 hours suggests that newly synthesized and folded tubulin is necessary in TRNs throughout the animals life or that the CCT complex performs some other vital function independent of tubulin folding even in late developmental stages.

I performed a variety of cct rescue experiments aimed at conclusively identifying a TRN role for the CCT complex. Although MEC-3 regulation and mechanosensory defects suggest that CCT activity is needed in TRNs these data would be bolstered by non-TRN rescue of the cct phenotype. I was unable to rescue the cct lethality phenotype with genomic cct constructs, a fosmid bearing the cct coding region or tissue-specifically. My rescue experiments may have failed for a variety of reasons. Overexpression of one subunit of a multi-subunit complex may disrupt assembly of the functional complex by disrupting the subunit stoichiometric balance. I transformed cct mutant lines with varying concentrations of rescuing construct though I may not have found the right one. Further rescue experiments employing different concentrations might result in successful rescue.

The apparent expression of P_{cct}gfp constructs in the CAN pair of neurons further complicated rescue experiments. The CAN neurons and the M4 pharyngeal neuron are the only C. elegans neurons known to be required for viability. M4 is required for feeding whereas CAN neuron function is unknown though it may act to regulate body homeostasis. CCT dysfunction in the CAN neuron could result in lethality if it compromised CAN function. The P_{sto-4}CCT rescue constructs I designed do not express in the CAN neurons, which might have compromised their ability to rescue cct lethality. P_{sid-1}CCT rescue constructs would also fail to rescue cct mutants should cct lethality in any way be based upon CAN neuron dysfunction since the sid-1 promoter is not expressed in neurons.
Materials and Methods

Expression constructs

I employed three different methods for generating promoter GFP fusions. All constructs for the putatively repressed list and the initial part of the upregulated list were created using standard techniques: Primers were designed to amplify the region upstream of each gene’s coding region by PCR with hopes that the gene’s regulatory regions would be included. 2000 base pairs or the coding region of the next gene upstream were used as cutoffs for construct size. The 3’ primer incorporated a restriction enzyme recognition site to facilitate cloning into the p95.75 GFP expression vector (Fire et al., 1990). Promoter regions were amplified by PCR; gel purified, digested, and cloned using standard bacterial cloning techniques. A PCR fusion approach (Hobert 2002) was also used to create promoter GFP constructs. Two sets of primers were designed for the promoter region and the coding region of gfp. The initial set would amplify the desired stretch of DNA normally but the 3’ primer would include an overhang complimentary to the gfp sequence. The second set of primers consisted of a 5’ primer slightly nested to the first 5’ primer of the promoter region whereas the 3’ primer would be slightly nested to the initial 3’ gfp primer. PCR products from the first round were mixed together in equal ratio GFP PCR product/promoter region PCR product. The nested primers described above were used for a second round of PCR and the resulting fused promoter GFP product was then directly injected. PCR products used in these experiments were not purified in any way as purification had been shown to interfere with the second PCR reaction. Sequencing was not possible for these constructs. Denis Dupuy created the third group of promoter GFP constructs using high-throughput methods in the lab of Marc Vidal. This group included some transcripts not included in the published list of 71 due to failure to meet the two-fold difference cut-off.

Creation of transgenic lines
Expression constructs were injected using previously described microinjection techniques (Mello et al., 1991; Mello et al., 1995) into young adult mec-3(e1338) hermaphrodites. Transformants were identified amongst the progeny of injected animals by the presence of a transformation marker and individually plated. I used several different markers during the course of this work including rescue plasmids for dpy-20, lin-15, unc-119, pceh-22gfp, and $P_{myo-3}mCherry$. Multiple stable lines were analyzed for expression patterns.

Matings and genetics

N2 x mec-3(e1338); promoter GFP: N2 males were crossed into mec-3(e1338) animals carrying promoter gfp constructs. Touch sensitive hermaphrodite offspring from these matings were picked and allowed to self-fertilize. Matings producing no touch sensitive F1 progeny were considered to be failures and repeated. Individual touch sensitive F2s displaying fluorescence were picked and allowed to self. Plates exhibiting only touch sensitive F3 progeny were considered homozygous wild type in regards to mec-3. I rarely if ever observed gfp stable lines that could not be picked out under a fluorescent dissecting microscope. The creation of the unc-24(e138); mec-4(u45), and mec-6(u247); unc-24(e138) lines are described in Appendix 1.

RNA interference

RNA interference assays were conducted as described by Fraser et al. (2000). HT115 (DE3), an RNase III-deficient E. coli strain with IPTG-inducible T7 polymerase activity carrying the L4440 T7 driven RNAi insert of interest were picked from frozen glycerol stocks and streaked onto LB amp, tetracycline plates and grown overnight at 37°. LB ampicillin (50 $\mu$g/ml) liquid cultures were then inoculated and grown at 37° for approximately eight hours. RNAi induction plates (standard NGM agar plates plus 25 $\mu$g/ml carbinicillin, 1mM IPTG) were then
seeded with a thin lawn of bacteria and allowed to air dry under a flame. Following a day of bacterial dsRNA induction at room temperature, gravid sid-1, lin-15b, him-4, P\textsubscript{unc-119}sid-1, P\textsubscript{myo-2}mCherry, mec-18::gfp or sid-1, lin-15b, him-4, p\textsubscript{mec-18}::SID-1, p\textsubscript{myo-2}::mCherry animals were washed off of several plates with M9 buffer and bleached (100 µl bleach, 50 µl 5M NaOH per 350 µl worm solution) to isolate eggs. Eggs were washed several times with M9 buffer and placed in equal amounts on the induced RNAi plates. Animals were cultured at 15° for six days prior to analysis. Multiple plates were tested blindly for each RNAi condition with 30 animals being assayed per plate. Each plate was counted as a single experiment. Error bars represent SEM. Statistical significance was determined using Student’s T test, P<.05.

Gentle touch assays

Young adult hermaphrodites were assayed for touch sensitivity using an eyebrow hair (mine) glued to a toothpick under a standard dissection scope. Previous results have suggested that cells of the posterior touch circuit are somewhat refractory to feeding RNAi induced effects in the strains used (Calixto et al., 2010). I therefore analyzed only anterior mechanosensory phenotypes caused by RNAi. Animals on food were stroked longitudinally across the anterior portion of their body immediately posterior to the pharynx. A cessation of forward motion or a reversal were both counted as a positive touch response. Animals were allowed to resume forward locomotion following a positive response prior to being touched again. Each animal was touched five times with thirty animals being touched per plate. Except in the case of contamination or starvation, thirty animals were tested on three plates in each experiment. Mutant strains were assayed for both anterior and posterior mechanosensation in the manner described above with ten touches total per animal. All assays were performed blindly.

Microscopy
Promoter GFP expression and antibody staining were examined using an axiophot 2 (Zeiss) microscope. RNAi was performed as described previously. Six day old young adult hermaphrodites were picked from RNAi plates and placed in M9 buffer on a 4% plus Nicodazole pad affixed to a standard glass slide which was then cover slipped. Animals were allowed to reach a paralyzed state prior to analysis. Slides were scored blindly.

**Antibody staining**

Antibody staining was performed using the formaldehyde fixation protocol as previously described (Savage et al., 1994) MEC-2 N-terminus antibody (Zhang et al., 2004) staining was performed at 1:500 dilution primary antibody stained overnight at room temperature. Secondary goat anti-rabbit rhodamine or Alexafluor antibodies were used at 1:1000 dilution overnight at room temperature. 6-11-b1 (Sigma) antibody staining was performed at 1:500 dilution overnight at room temperature with secondary goat anti-mouse antibodies used at 1:1000 dilution overnight at room temperature.

**Strains and reagents**

mec-3(e1338), mec-4(u45), mec-6(u247), and N2 (Bristol isolate) were extant in our laboratory. cct-1(NL708), cct-2(ok3438), and cct-6(ok2904) were obtained from the CGC. acp-2(ok2129), F10A3.11KO, T07D1.3(ok2272), and C03A3.3(ok2834) were obtained directly from the C. elegans knock out consortium. TRN and pan-neuronal RNAi strains are described in (Calixto et al., 2010).
Chapter III

TRN-specific RNAi screen for microtubule-related proteins involved in mechanosensation
Abstract

The characteristic 15-protofilament microtubules (MTs) found in the six touch receptor neurons (TRNs) of *C. elegans* are required for the perception of gentle touch to the animal’s body. Disruption of the large diameter MTs in the TRNs by MT depolymerizing drugs or mutations in the tubulin-encoding genes *mec-7* and *mec-12* result in touch insensitivity. The basis of the MT-related mechanosensory phenotype is not fully understood. MTs in general perform a wide variety of functions and interact with many other proteins leading to several possible explanations for TRN dysfunction in their absence. I set out to clarify the importance of large diameter MTs in the TRNs by examining proteins known to interact with or regulate MTs. Many such proteins had been previously unexamined in this context due to lethality of mutations therein. A TRN-specific RNAi approach was employed to overcome this obstacle. Several genes were identified as being necessary for mechanosensation, which have not been previously implicated in the process including regulators of TAU phosphorylation, Katanin, and a tubulin deglutamylase.

Introduction

Saturation mutagenesis in *C. elegans* revealed multiple mutations in 15 genes specifically effecting the perception of gentle touch to the body (Chalfie et al., 1981). *mec-12* and *mec-7* encode the alpha and beta tubulin subunits, which make up the characteristic large diameter, heavily acetylated, MTs found in the TRNs. Disruption of the large-diameter MTs by mutations in *mec-7* or *mec-12* or by chemical means result in mechanosensory defects though the mechanisms of this defect remain unclear. Given the wide array of MAPs, MT modifying enzymes, chaperones, regulators, and other proteins, which regulate, or interact with MTs it is
The failure of mutagenesis to recognize other MT-related genes suggests several possibilities. MAPs, molecular motors, MT-modifying enzymes, and other proteins involved with MTs may be so important that mutations in them result in severe pleiotropic effects confounding identification of mechanosensory defects. The majority of proteins involved with MTs may exhibit a high degree of redundancy, subtle Mec phenotypes when mutated, or simply not be important for mechanosensory functions in the TRNs. Two C. elegans MAPs may have roles in mechanosensation but phenotypes are weak (Gordon et al., 2008) or suspect (Hueston et al., 2008) and these genes most likely would not have been identified by mutagenesis screens for touch insensitive mutants. I sought to clarify the importance of TRN MTs in mechanosensation by studying mutants of known MT-related proteins including MAPs, chaperones, motor proteins, and modifying enzymes. Many of these candidate genes lack mutants suitable for testing touch sensitivity in C. elegans due to lethality or pleiotropic effects. I employed a TRN-specific RNAi approach as well as mutant analysis where possible in an attempt to identify new MT-related proteins required for mechanosensation.

**TRN-specific RNAi**

RNA interference (RNAi) by feeding has proven to be a powerful tool for large-scale analysis of gene function in C. elegans (Timmons et al., 2001). Libraries of bacteria containing constructs targeting most of the C. elegans genome exist and animals are simply grown on plates seeded with bacteria bearing a plasmid construct targeting the transcript of interest for degradation by the dicer pathway (Knight et al., 2001). Phenotypes believed to be attributable to solely neuronal dysfunction were difficult to obtain by RNAi. The basis of this neuronal RNAi
refractivity has since been shown to be due to the failure of neurons to express the dsRNA transporter encoded by the sid-1 (systemic interference deficient) gene (Winston et al., 2002). C. elegans neurons are largely hampered in their ability to uptake the interfering RNA molecules in contrast to the rest of the animal’s tissues, which express the sid-1 gene with few known exceptions. We overcame this neuronal RNAi refractivity by generating sid-1 mutant strains carrying integrated, pan-neuronal or TRN-specific sid-1 rescue constructs (Calixto et al., 2010). Since these strains only express sid-1 in the TRNs (P_{mec-18}sid-1) or pan-neuronaly (P_{unc-119}sid-1), other tissues should not experience an RNAi effect allowing pan-neuronal or TRN-specific phenotypes to be examined whereas avoiding pleiotropy attributable to RNAi-induced phenotypes in other tissues. This approach has been used with success to screen genes with known lethal phenotypes for roles in mechanosensation (Calixto et al., 2010).

Results

I assayed a total of 48 genes of possible relevance to the large diameter TRN MTs by feeding RNAi. Anterior touch was tested (the PLM neurons are less susceptible to feeding RNAi in this strain for unknown reasons) and target genes showing fewer than 3 out of 5 positive anterior responses upon RNAi were considered for further study.

Tubulin chaperones

RNAi targeting mRNA encoding subunits of the CCT chaperonin complex result in mechanosensory defects (chapter II). I assayed other known or putative tubulin chaperones and co-chaperones. The six-subunit Prefoldin complex acts in conjunction with CCT bringing nascent polypeptides to the CCT complex for processing (Vainberg et al., 1998). I also tested other known or putative tubulin chaperones including nud-1 (Faircloth et al., 2009) and tor-2
(Granata et al., 2009). F16D3.4 encodes a putative β-tubulin folding cofactor D whereas K07H8.1 encodes α-tubulin folding cofactor E (Tarailo et al., 2007). Arsenide-inducible protein aip-1 does not have known chaperone activity but its expression in response to stress in consistent with chaperone-like behavior and it is also of interest due to its regulation by MEC-3 in the TRNs (chapter II). Mechanosensory phenotypes elicited by RNAi versus mRNA encoding prefoldin subunits and putative tubulin chaperones did not differ significantly from gfp RNAi phenotypes and were not studied further (Figure 1).

Figure 1. Feeding RNAi versus prefoldins and other chaperones/putative chaperones does not result in significant mechanosensory defects. N=experiments.
**Tubulin modifiers**

Tubulins are subject to a wide variety of post-translational modifications (PTMs) including severing, phosphorylation, acetylation, tyrosination, glutamylation as well as the reverse of these modifications. Such modifications are a crucial determinant of the particular cellular role a given MT or portion thereof plays at any given time (Neuronal tubulin PTMs reviewed in (Fukushima et al., 2009)). I sought to determine what tubulin PTMs were important for TRN function by down-regulating the transcripts of known microtubule modifying enzymes (Figure 2). Experiments related to tubulin acetylation will be discussed in the following chapter.

I targeted genes encoding known or putative modifiers of tubulin and MT severing enzymes by feeding RNAi and anterior mechanosensory phenotypes were scored. CCPP-1, and CCPP-6 de-glutamylate tubulins (Kimura et al., 2010) whereas the *C. elegans* genome contains six putative Tubulin Tyrosine Ligases (ttll) some of which may glutamylate tubulin (Janke et al., 2005). Three of the six *C. elegans* *ttll* genes were present in the whole genome RNAi library. I also targeted transcripts encoding *C. elegans* homologs of the putative MT modifier LIS-1, and Tau Tubulin Kinase 2 (ttbk-2) by RNAi. Statistically significant, RNAi-induced, mechanosensory defects as compared to *gfp* were observed when the *C. elegans* homologs of TAU tubulin kinase (*ttbk-2*), the MT-severing enzymes Spastin (*spas-1*) and Katanin (*mei-1*), the Cytosolic CarboxyPePtidases 1 (*ccpp-1*) and 6 (*ccpp-6*), Lisencephaly 1 (*lis-1*), and the Tubulin Tyrosine Ligases *ttll*-4 and *ttll*-15 were targeted. RNAi targeting Histone Deacetylase six (*hdac-6*), and *ttll*-5 did not result in statistically significant mechanosensory defects relative to RNAi targeting *gfp*. Although RNAi against *ccpp-6* resulted in a statistically significant Mec phenotype, touch tests performed on the knockout strain resulted in no mechanosensory defect (Figure 8). Though eight of ten candidate genes exhibited statistically significant Mec phenotypes as compared to RNAi vs. *gfp*, I only considered the three genes exhibiting the strongest phenotypes for further study (*mei-1*, *ttbk-2*, and *ccpp-1*).
Kinases, phosphatases, and associated proteins

Multiple indirect phosphorylation events contribute to the regulation of MT dynamics via MAPs (Drewes et al., 1998). MAPs themselves are a heavily phosphorylated class of proteins with their phosphorylation state affecting their affinity for MTs and thus MT dynamics. I performed RNAi against several known regulators of phosphorylation involved with MT dynamics or that directly phosphorylate tubulin or MAPs (Figure 3). *ttbk*-2 could also fall into this category though it was included with the MT modifiers previously discussed. PAR-1/MARK is a serine-threonine kinase required for appropriate cytoplasmic partitioning, neuronal polarity as well as elements of metabolism (Biernat et al., 2002). PAR-1 has also been shown to
destabilize MTs by phosphorylating MAPs (Doerflinger et al., 2003). SPAT-1/BORA is involved in regulating cellular polarity (Noatynska et al., 2010). *rac*-2 encodes a small RHO family GTPase that has cytoskeletal elements among its targets (Struckhoff et al., 2003). GSK-3 (glycogen synthase kinase 3) and PAA-1 (structural subunit of Protein Phosphatase 2A) often act antagonistically regulating many processes including MAP phosphorylation (Qian et al., 2010). RNAi targeting *paa*-1 and *gsk*-3 transcripts resulted in statistically significant anterior Mec phenotypes as compared to RNAi targeting *gfp*.

Figure 3. TRN-specific feeding RNAi targeting regulators of phosphorylation. N=experiments.

*Structural microtubule associated proteins*

Microtubule associated proteins serve to stabilize microtubules, regulate their dynamics, and mediate their interactions with other cellular components. I used TRN-specific RNAi to
target several MAPs and other proteins with structural roles related to MTs (Figure 4). I assayed the *C. elegans* TAU homolog *ptl-1* (Goedert et al., 1996), and the Echinoderm Like Protein MAP (*elp-1*) which have been previously implicated in mechanosensation (Gordon et al., 2008; Hueston et al., 2008) and observed no significant defect. I assayed the *C. elegans* homologs of Dystrophin (*dys-1*), which is mutated in Muscular Dystrophy and Dystrobrevin (*dyb-1*), which associates with Dystrophin, due to reported *dys-1* interactions with *elp-1* (Hueston et al., 2009). *dnc-1* encodes the *C. elegans* homolog of *D. melanogaster* GLUED which helps organize the mitotic spindle (Skop et al., 1998). *ebp-2* encodes a *C. elegans* MT end-binding protein (Kozlowski et al., 2007). *nab-1* encodes the sole *C. elegans* homolog of mammalian Neurabin/Spinophilin proteins which regulate MT-actin interactions (Hung et al., 2007) and *zyg-12* mediates MT-membrane interactions (Malone et al., 2003). *apl-1* encodes the *C. elegans* amyloid-like precursor protein. RNAi targeting *elp-1*, *dys-1*, *apl-1*, and *zyg-12* resulted in statistically significant anterior Mec phenotypes as compared to RNAi targeting *gfp*. These Mec phenotypes were relatively weak however and I did not study any of these genes further with the exception of *elp-1*. I tested a strain carrying an *elp-1* deletion for touch sensitivity and was unable to differentiate it from wild-type (Figure 8).
Figure 4. TRN-specific RNAi versus MAPs and other structural MT-related proteins. N=experiments.

Tubulins

In addition to mec-7 and mec-12 the C. elegans genome encodes 8 α-tubulins and 4 β-tubulins (wormbase.org). Some of these tubulins are expressed in the TRNs and probably comprise the few small-diameter MTs observed in these cells, which may fulfill some of the residual transport capacities present in mec-12 null TRNs. It is unknown if these other tubulins influence mechanosensation. C. elegans tubulins exhibit a high degree of sequence conservation often resulting in secondary RNAi effects vs. non-targeted tubulin transcripts. I targeted the tubulins available in the RNAi construct library excluding those with overlapping secondary targets (Figure 5). RNAi vs. all tubulins assayed by RNAi resulted in statistically
significant anterior Mec phenotypes as compared to gfp. tba-8 is the only candidate tested by RNAi which does not have annotated secondary effects affecting mec-7 or mec-12 transcripts (Wormbase.org). No tba-8 mutant lines exist and these genes were not studied further.

Figure 5. TRN-specific feeding RNAi versus tubulin-encoding genes results in anterior Mec phenotypes. N=experiments.

**Kinesin-like proteins**

The kinesin family of molecular motors transport cargo along MTs and also facilitate chromosome segregation. Mechanosensory defects observed upon disruption of the large-diameter MTs may be attributable to disruption of kinesin-dependant transport of mechansensory transduction components. The *C. elegans* genome contains 20 known genes
encoding kinesin-like proteins. I tested the klp genes present in the *C. elegans* RNAi library for mechanosensory defects. RNAi for eight kinesin-like protein-encoding genes yielded some statistically significant Mec phenotypes as compared to control (Figure 6) but these phenotypes were relatively weak and I did not examine the *klp* genes further.

![RNAi results: Kinesin-like proteins](image)

**Figure 6.** TRN-specific feeding RNAi versus kinesin-like proteins. *N*=experiments.

*RNAi screen summary*

I employed TRN-specific RNAi to assess the role of 48 MT-related proteins in *C. elegans* mechanosensation. I chose a cut-off of less than three out of five positive responses to anterior touch induced by RNAi against a candidate transcript as my criteria for further study of
candidate genes. Although RNAi elicited statistically significant anterior Mec phenotypes for several other candidates, these differences were relatively small compared to control and were not studied further. Nine candidates showing fewer than three out of five positive responses to anterior touch: ttbk-2, gsk-3, tba-8, paa-1, the ccpp-1, mei-1, tba-1, tba-4, and tbb-2. Of these, three encode tubulins with annotated secondary RNAi effects against the known mec genes mec-7 and mec-12. tba-4 and tbb-2 were excluded from further analysis. The α-tubulin encoding gene tba-8 illustrated a touch defect by RNAi but has no annotated secondary RNAi effects. elp-1 and ptl-1 were included in further experiments due to their previously illustrated roles in mechanosensation as were the three C. elegans members of the Phocein protein family due to published effects on MTs and neuronal morphology (Schulte et al., 2010).

Characterization of candidates

I obtained mutant strains for candidate genes where possible and assayed them for touch sensitivity. I obtained strains carrying balanced lethal deletions in the mei-1, gsk-3, and paa-1 coding sequences as well as strains carrying non-lethal mutations in the ptl-1, elp-1, ttbk-2, ccpp-1, and two of three C. elegans phoceins; F09A5.4, and C30A3.3 from the Caenorhabditis Genome Center (CGC). No strains carrying mutations in tba-8 are present in the CGC database. The mei-1 knockout results in sterility rather than lethality with homozygotes growing to apparently normal adulthood without the ability to generate viable eggs, which still allows for the assay of mechanosensory phenotypes in these animals. Though RNAi vs. elp-1 and ptl-1 did not induce mechanosensory defects matching my criteria for further study, elp-1 and ptl-1 were included for further analysis based on their published putative roles in mechanosensation (Hsu et al., 1992; Hueston et al., 2008). I used antibody staining, and genetic enhancements in order to elucidate the basis of candidate mechanosensory defects.

RNAi versus candidate genes does not result in gross morphological defects or cell death
I performed feeding RNAi against candidate genes in a pan-neuronal RNAi permissive strain: carrying a TRN-specific GFP marker (attempts to cross this $P_{mec-18}gfp$ into the TRN-specific RNAi strain resulted in a strain exhibiting mechanosensory defects presumably due to the presence of two $P_{mec-18}$ arrays in the same cells). No significant reduction in the number of GFP positive ALM or PLM cells was observed upon feeding RNAi versus candidate genes except in the $gfp$ and $mec-12$ feeding RNAi conditions (Figure 7). The reduction in the number of GFP positive ALMs in the $mec-12$ feeding RNAi condition may be a reflection of the general transcriptional repression observed in $mec-7$ and $mec-12$ mutants as discussed previously. The incomplete elimination of GFP signal in the $gfp$ RNAi condition is consistent with the generally incomplete penetrance of feeding RNAi or perdurance of GFP protein translated prior to the ingestion of sufficient RNAi bacteria. There did appear to be a global reduction in AVM GFP signal though this is probably insufficient to cause mechanosensory defects since laser ablation of AVM alone in insufficient to result in mechanosensory defects (Chalfie et al., 1985).
Figure 7. Feeding RNAi against candidates was performed in a pan-neuronal RNAi permissive strain carrying a TRN-specific gfp marker and the numbers of GFP positive cells were counted. AVM was universally reduced in all conditions including controls. ALM gfp signal was reduced in the mec-12 RNAi condition.

Characterization of candidate mutants and enhancement of mechanosensory defects in sensitized strains

I observed statistically significant mechanosensory defects in strains carrying mutations in the ccp-1, F09A5.4, C30A3.3, and mei-1 genes at the standard C. elegans cultivation temperature of 20°C (Figure 8). elp-1, pti-1 and ttbk-2 mutants were essentially wild type at this temperature. I was unable to assay the paa-1 and gsk-3 strains for mechanosensory defects due to early lethality or, in the case of gsk-3, a balancer that results in Unc animals who cannot
be reliably assayed for touch sensitivity. \textit{paa-1} mutants exist in a balanced lethal state and a lack of a fluorescent marker and undetermined timing of death or arrest in this strain precluded assessing any mechanosensitive phenotype.

Temperature sensitive (ts) mutations in known \textit{mec} genes show no phenotype at 15° but manifest strong mechanosensory defects at 25°. These “sensitized” backgrounds enhance Mec phenotypes at 15° when crossed into mutant lines exhibiting subtle or weak mechanosensory defects (Zhang et al., 2002; Zhang et al., 2004). I crossed \textit{elp-1}, and \textit{ptl-1} mutants into the \textit{mec-4(u45) ts} line. Both \textit{elp-1; mec-4(u45)} and \textit{ptl-1; mec-4(u45)} showed mechanosensory defects at permissive temperatures. \textit{elp-1} and \textit{ptl-1} encode MAPs, which bind to and stabilize MTs. Mutations in neither gene result in mechanosensory defects but the ts enhancements suggested a possibly subtle or redundant role in mechanosensation. I created a \textit{ptl-1; elp-1} double mutant in order to address this possibility. \textit{elp-1; ptl-1} double mutants show no discernible mechanosensory defects (Figure 8).
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Figure 8. Mechanosensory phenotypes of mutations in genes identified by RNAi or from literature. Animals were touched ten times in an alternating anterior/posterior manner. 30 young adults from each strain were tested blindly. mec-4(u45) strains were grown at 15°C. Other strains were grown at 20°C. Error bars represent SEM.

**TRN MT integrity**

Mec phenotypes attributable to candidate gene dysfunction might be due to disruption of the TRN MTs themselves. I addressed this possibility by 6-11b-1 antibody staining against acetylated α-tubulin, which brightly stains TRNs by specifically recognizing acetylated MEC-12 α- tubulin. The two Phocean homologs F09A5.4 (Figure 9), and C30A5.3 (Figure 11) as well as cccp-1 (Figure 10) show a fragmented, discontinuous pattern of TRN MT staining as compared to N2 and dumpy controls.
Mechanosensory channel complex localization

Mechanosensory defects in candidate mutants might be attributable to MT-related transport defects. In order to test this possibility, I assayed the distribution of the MEC-4/MEC-10 mechanosensory channel complex by antibody staining for the channel complex accessory protein MEC-2. Wild type, touch sensitive animals illustrate a regularly-spaced punctate distribution of MEC-2 staining whereas disruption of TRN microtubules often results in a less regular distribution of puncta or their complete absence. No apparent MEC-2 puncta defect was observed in the strains assayed suggesting that observed mechanosensory defects were not due to channel complex mislocalization.
Figure 9. The Phocein homolog F09A5.4 shows both WT and disrupted 6-11b-1 and MEC-2 antibody staining. (A) dpy-20 control animal showing WT 6-11B-1 antibody staining. (B) N2 ALM showing characteristic punctate MEC-2 antibody staining. (C and D) grossly WT 6-11b-1 staining in F09A5.4 anterior and posterior. (E) F09A5.4 merged 6-11b-1 and MEC-2 antibody images showing lack of 6-11b-1 and MEC-2 antibody overlap. (F and G) Individual antibody images from merge. White triangles indicate areas of disrupted staining. The lower of the two PLM processes lacks MEC-2 staining and discontinuous staining by 6-11b-1. PVM shows an apparent lack of 6-11b-1 staining though MEC-2 staining is intact.
Figure 10. *ccpp-1* antibody staining. (A) *dpy-20* animal showing wt 6-11b-1 antibody staining. (B) N2 ALM showing WT MEC-2 antibody staining. (C-E) Merged and single images from *ccpp-1* animal anterior showing disrupted 6-11b-1 and WT MEC-2 staining in ALM. (F-H) Merged and single images from *ccpp-1* animal posterior showing disrupted 6-11b-1 and WT MEC-2 staining. White triangles indicate a PLM process with no 6-11b-1 and WT MEC-2 staining.
Figure 11. 6-11b-1 antibody staining in C30A3.3 animals. (A and B) N2 ALM and PLM 6-11b-1 antibody staining. (C-E) Fragmented TRN 6-11b-1 antibody staining in C30A3.3 animals.

Discussion

The hallmark large-diameter MTs of *C. elegans* TRNs are necessary for mechanosensation though the exact reasons remain unclear. I employed a TRN-specific feeding RNAi approach to disrupt 48 known MT-related genes in order to clarify the role of the TRN MTs in mechanosensation. I identified a handful of MT-related genes whose function had not previously been associated with TRN mechanosensation. Newly identified genes included
three regulators of tubulin or MAP phosphorylation, a tubulin deglutamylase, another α-tubulin, and two members of the phocein family. Some of the newly identified genes show disruption of acetylated α-tubulin or MEC-2 antibody staining in the TRNs. Several classes of genes notably showed no mechanosensory defect upon feeding RNAi including known MAPs, and kinesin-like proteins.

The lack of mechanosensory defects for the majority of genes assayed by RNAi does not rule out a role in mechanosensation. Although the TRN-specific feeding RNAi approach has the advantage of allowing the testing of lethal and pleiotropic genes it is ill-suited for the study of genes whose products are required in TRNs only in early developmental stages prior to the animal ingesting any bacteria carrying the RNAi construct. Proteins required later in TRN development or function might exhibit high rates of perdurance allowing them to function adequately even after their transcription has ceased. Feeding RNAi based approaches are also hampered by variable penetrance, which appears to differ from gene to gene. Animals fed gfp RNAi still illustrate a weak GFP signal in many cases and RNAi targeting mec-4 does not fully phenocopy known mutants. In other cases, feeding RNAi penetrance can approach 100% as in the case of unc-22 or subunits of the CCT complex (see chapter II). This variability may be based upon the strength of the target gene’s promoter or protein perdurance. My criteria for a significant phenotype may also have eliminated some genes that have subtle roles in mechanosensation from further study.

*RNAi versus prefoldin and other chaperones does not result in strong Mec phenotypes*

RNAi vs. the Prefoldin complex and other known or putative tubulin chaperones resulted in little effect suggesting that their role is at least partially dispensable in the TRNs or that RNAi is ineffective for some reason. Although the Prefoldin complex is known to work in concert with the CCT complex, it would appear that its role is not absolutely necessary in the TRNs though
organism-wide RNAi against *pfd-1* results in embryonic lethality (Lundin et al., 2008). RNAi knockdown of Prefoldin and other co-chaperone transcripts does not take effect until the animals are mature enough to ingest significant amounts of the bacteria and it may be the case that Prefoldin and other co-chaperones are essential at earlier developmental stages but lose their importance as the animal matures. This hypothesis may be supported by the observation that RNAi against *pfd-1* results in F1 but not P0 lethality (Lundin et al., 2008). There may be adequate levels of correctly folded tubulin by the time significant RNAi bacteria are ingested to make tubulin co-chaperone roles dispensable in the TRNs. This does not appear to be the case for the CCT complex however (chapter II) suggesting different requirements for Prefoldin-CCT cooperation at different developmental stages in the TRNs or differences in PFD and CCT subunit protein perdurance. *aip-1, nud-1,* and *tor-2* are not necessarily chaperones but they have been identified as having putative chaperone roles as in the case of *nud-1* or whose expression is consistent with a chaperone-like role as in the case of *aip-1* which is MEC-3 regulated. RNAi against these transcripts resulted in no significant mechanosensory defect nor did RNAi against the two tubulin co-chaperones tested (F16D3.4 and K07H8.1). It may be the case that tubulin chaperone activities are more important in developing TRNs than in later developmental stages.

**RNAi targeting kinesin-like proteins does not result in strong Mec phenotypes**

The Kinesin family of molecular motors performs a variety of tasks including anterograde axonal transport along neuronal MTs and facilitation of mitosis and meiosis. RNAi against the eight kinesin-like proteins present in the *C. elegans* RNAi library resulted in no mechanosensory defects meeting my criteria for significance. No phenotype was reported for *klp-4, klp-6,* and *klp-12* in previous RNAi experiments (www.wormbase.org). *klp-7, klp-13,* and *klp-16* have documented roles in mitosis (Robin et al., 2005; Srayko et al., 2005) and may not be important
for later TRN developmental events. RNAi most likely would not accumulate early enough to affect these early developmental processes. The expression pattern of klp-12 is known and does not include TRNs (www.wormbase.org). klp-10 mutants show no phenotype though RNAi targeting klp-10 causes severe defects which may be attributable to knockdown of klp-18, which shares high, sequence homology with klp-10 and is involved in mitosis (www.wormbase.org).

Proper localization of the MEC-4/MEC-10 mechanosensory channel complex appears to be necessary for mechanosensation. Disruption of the normal punctate distribution of the channel complex along TRN processes correlates with mechanosensory defects. The mechanism by which channel localization is established is unknown but it’s likely that it depends upon MT-dependant anterograde transport. None of the motor proteins I targeted by TRN-specific RNAi yielded a mechanosensory defect meeting my criteria for significance. This may be due to adequate kinesin function prior to ingestion of significant RNAi bacteria, KLP protein perdurance or channel complex protein perdurance. It may also be the case that I simply targeted kinesins involved in mitosis/meiosis but not anterograde transport or that the kinesins I targeted are involved in anterograde transport in a redundant fashion.

*RNAi against MAPS and other MT-associated structural proteins fail to induce strong Mec phenotypes*

The same caveats discussed for other protein families may apply to the MAPs and other structural proteins targeted by TRN-specific RNAi none of which resulted in strong mechanosensory defects. The proteins encoded by these genes may be perdurant, redundant, important only in early development before RNAi takes effect, or simply not involved in mechanosensation. I was unable to substantiate published reports that PTL-1/TAU and ELP-1 are needed for mechanosensation though my observation that they both show mechanosensory defects at permissive temperatures in the mec-4(u45) background may suggest that they have
subtle roles in TRN function. The lack of a mechanosensory defect in the *elp-1; ptl-1* strain suggest that these proteins do not function redundantly.

*Positive candidates*

What of the positive candidates identified? The three regulators of phosphorylation are interesting but proved difficult to study due to the lack of suitable mutant strains in the case of *paa-1* and *gsk-3* and the lack of a mechanosensory phenotype in the *ttbk-2* deletion strain. Of particular interest is the relationship between *ttbk-2, paa-1, gsk-3* and *ptl-1*. The proteins encoded by these genes have been widely studied in the context of Alzheimer’s disease. *ptl-1* encodes the sole *C. elegans* homolog of the TAU MAP, which binds to and stabilizes MTs particularly in axons. Hyperphosphorylated TAU loses its affinity for MTs and forms aggregates which are a hallmark of Alzheimer’s disease though it is unknown if this is causative of the pathology. GSK-3 phosphorylates TAU and co-localizes with it in the pathological aggregates (Chatterjee et al., 2009). TTBK-2 also phosphorylates TAU. *paa-1* encodes a subunit of the Protein Phosphatase 2A complex which de-phosphorylates TAU among other substrates (Qian et al., 2010). Although *gsk-3* and *paa-1* in particular have multiple targets, the common TAU target is intriguing.

How might these phosphorylation relationships fit in with a mechanosensory defect? One obvious possibility is that *gsk-3, paa-1, and ttbk-2* are required for a wide variety of cellular processes and observed mechanosensory defects are simply a reflection of a general TRN pathology rather than anything specific to their mechanosensory functions. The lethality observed upon mutations in either *gsk-3* or *paa-1* may support this hypothesis. Mutations in *ttbk-2* are not lethal however but the mutant shows no mechanosensory defect. If it is not the case that the RNAi induced mechanosensory defects observed are solely due to general TRN dysfunction, how else might these observations be explained? One possibility is that of a
disruption in TAU phosphorylation homeostasis since both positive and negative regulators of TAU phosphorylation show mechanosensory defects by RNAi. This could explain why *ptl-1* knockouts show no mechanosensory defects. The absence of the protein itself may not cause a defect but perhaps disassociation of TAU from TRN MTs due to disruption of its normal phosphorylation state could result in either a general TRN pathology akin to the aggregates observed in Alzheimer’s patients or some interference with specific elements of the mechanosensory apparatus. This might also explain the observation that both *ptl-1* and *elp-1*, which encode proteins that stabilize MTs, enhance *mec-4(u45) ts* mutations but not each other suggesting that the mechanosensory enhancement these mutations exhibit are not due to a decrease in MT stability.

*MT severing enzymes*

What might explain the Mec phenotype observed in *mei-1* mutants and by RNAi targeting *mei-1?* *mei-1* was originally identified by defects in meiosis where it serves to sever MTs allowing mitosis to progress (Lu et al., 2004). *mei-1* encodes the *C. elegans* homolog of the Katanin MT severing complex that along with Spastin, severs MTs at centrosomes and along neuronal MTs. Both complexes perform roles in mature neurons severing MTs depending upon type and degree of post-translational modification the MTs exhibit. Katanin but not Spastin preferentially targets acetylated MTs in mature dendrites but not axons presumably because axons exhibit higher levels of TAU than dendrites (Sudo et al., 2010) whereas Spastin MT affinity is affected by MT glutamylation state (Lacroix et al., 2010). Overexpression of TAU can suppress Katanin mediated MT severing as can deacetylation of MTs (Sudo et al., 2010). Katanin has also been shown to act at the plus end of MTs along the leading edge of growing cells or processes (Baas et al., 2011) RNAi against Spastin showed a statistically significant yet weak phenotype. How could loss of MEI-1 function in TRNs result in mechanosensory defects?
TRN MTs are heavily acetylated and a baseline rate of MT severing may be important for maintaining appropriate levels of MT dynamism in TRNs. It may be the case that a lack of TRN MEI-1 function results in TRN MTs that are too stable or perhaps too numerous resulting in interference with TRN function, appropriate process growth, or synapse formation. No gross TRN abnormalities were observed upon staining with the MEC-2 antibody in mei-1 mutants however.

The most promising candidate for further study would appear to be F56H1.5 (since renamed CCPP-1). I observed a mechanosensory defect inccpp-1 mutants and a disruption of α-tubulin antibody staining. ccpp-1 has confirmed de-glutamylase activity which is most likely specific to poly-glutamate chains found on tubulin (Kimura et al., 2010). The significance of tubulin polyglutamylation is poorly understood though it occurs at the tubulin C-terminus near the sites of MAP and molecular motor binding and may affect their affinity for tubulin (Bonnet et al., 2001). The mammalian tubulin glutamylases TTLL4 and TTLL6 add polyglutamate side chains of different lengths to tubulin with side chain length distinguishable by antibody staining (Kimura et al., 2010). HeLa cells overexpression TTLL-6, which adds long polyglutamate side chains to tubulin show a 70% reduction in total MT mass after 24 hrs whereas those over expressing TTLL4 which adds shorter glutamate side chains showed no reduction in overall MT mass (Regnard et al., 1999). Overexpression of TTLL-11, which also adds long chains, showed results similar to those observed with TTLL-6 over-expression. The loss of MT mass was greatly reduced when TTLL-6 or 11 were overexpressed in the presence of siRNA targeting the MT severing enzyme Spastin suggesting that Spastin targets MTs bearing long glutamate side chains. Mutations in an enzyme, which shortens polyglutamate side chains of TRN MTs, might impede some baseline level of normal Spastin activity resulting in abnormally stable MTs or perhaps too many MTs in general resulting in mechanosensory defects by an unknown mechanism.
Phoceins

The question of general TRN pathology versus mechanosensory importance arises when considering the Mec phenotypes observed upon mutations in the phoceins. The phocein family of proteins regulates cell polarity, cytoskeletal integrity, and dendritic vesicular trafficking (Schulte et al., 2010; Bailly et al., 2007). I showed MT disruption and mechanosensory defects in two C. elegans phocein homologs. Phoceins serve to couple MT integrity with vesicular cycling, and synapse formation in neurons. The basis of mechanosensory defects observed in phocein mutants are most likely due to MT disruption though it may be the case that the TRN MTs are intact and only their acetylation is disrupted.

Possible future experiments regarding the positive candidates presented in this chapter will be discussed in chapter V.

Materials and Methods

RNA interference

RNA interference assays were conducted as described (Fraser et al., 2000). HT115 (DE3), an RNase III-deficient E. coli strain with IPTG-inducible T7 polymerase activity carrying the L4440 T7 driven RNAi insert of interest were picked from frozen glycerol stocks and streaked onto LB amp, tetracycline plates and grown overnight at 37°. LB ampicillin (50 μg/ml) liquid cultures were then inoculated and grown at 37° for approximately eight hours. RNAi induction plates (standard NGM agar plates plus 25 μg/ml carbinicillin, 1mM IPTG) were then seeded with a thin lawn of bacteria and allowed to air dry under a flame. Following a day of bacterial dsRNA induction at room temperature, gravid sid-1, lin-15b, him-4, p_{unc-119}\text{-}::\text{SID-1}, p_{myo-2}\text{-}cherry, MEC-18\text{-}::\text{gfp or sid-1, lin-15b, him-4, p}_{\text{mec-18}}\text{-}::\text{SID-1}, p_{\text{myo-2}}\text{cherry animals were washed}
off of several plates with M9 buffer and bleached (100μl bleach, 50μl 5M NaOH per 350 μl worm solution) to isolate eggs. Eggs were washed several times with M9 buffer and placed in equal amounts on the induced RNAi plates. Animals were cultured at 15° for six days prior to analysis. Multiple plates were tested for each RNAi condition with 30 animals being assayed per plate. Each plate was counted as a single experiment. Error bars indicate SEM and “*” denote statistically significant differences vs. gfp control (or klp-4 in the case of the Kinesin-Like Proteins). Statistical significance was determined using Student’s T-test, P<.05.

Gentle touch assays

Young adult hermaphrodites were assayed for touch sensitivity using an eyebrow hair (mine) glued to a toothpick under a standard dissection scope. Previous results have suggested that cells of the posterior touch circuit are somewhat refractory to feeding RNAi-induced effects in the strains used. I therefore analyzed only anterior mechanosensory phenotypes caused by RNAi. Animals on food were stroked longitudinally across the anterior portion of their body immediately posterior to the pharynx. A cessation of forward motion or a reversal were both counted as a positive touch response. Animals were allowed to resume forward locomotion following a positive response prior to being touched again. Each animal was touched five times with thirty animals being touched per plate. Except in the case of contamination or starvation, thirty animals were tested on three plates in each experiment. Mutant strains were assayed for both anterior and posterior mechanosensation in the manner described above with ten touches total per animal. All assays were performed blind.

Microscopy

Effects of RNAi on MEC-18GFP levels and antibody staining analysis were assayed at 630x using an axiophot two Zeiss Fluorescent microscope. RNAi was performed as described
previously. Six day old young adult hermaphrodites were picked from RNAi plates and placed in M9 buffer on a 4% plus Nlcodazole pad affixed to a standard glass slide which was then cover slipped. Animals were allowed to reach a paralyzed state prior to analysis. Slides were scored blindly.

**Antibody staining**

Antibody staining was performed using the formaldehyde fixation protocol as described by Savage et al., (1994). MEC-2 N-terminus antibody staining was at 1:500 dilution primary antibody stained overnight at room temperature. Secondary goat anti-rabbit rhodamine or Alexafluor antibodies were used at 1:1000 dilution overnight at room temperature. 6-11-b1 (Sigma) antibody staining was performed at 1:500 dilution overnight at room temperature with secondary goat anti-mouse antibodies used at 1:1000 dilution overnight at room temperature.

**Genetics**

Mechanosensory phenotype enhancements by *mec-4(u45)* ts matings were carried out at fifteen degrees. *mec-4(u45)* males were crossed into mutant lines at 15°. Mating efficacy was determined by the presence of multiple F1 males. F1 hermaphrodites were picked and moved to 25°. Mec F2 animals were PCR genotyped for the presence of the *elp-1* or *ptl-1* deletion then moved to 15° for multiple generations prior to touch testing. The *elp-1; ptl-1* double mutant strain was created using PCR genotyping.

**Strains**

TRN-specific and pan-neuronal RNAi strains are described in (Calixto et al., 2010). C30A5.3(ok2702), F09A5.4(ok3273), *ptl-1(ok621)*, *elp-1(ok347)*, *ccpp-1(ok1821)*, *ccpp-
6(ok382), +/-mT1 II; paa-1(ok1539)/mT1[dpy-10(e128)], unc-101(sy216) gsk-3(nr2047)/hln1[unc-54(h1040)], ccpp-6(ok382), and mei-1(ok2000) l/hT2[bli-4(e937) let-?(q782) qIs48](I,III) were obtained from the CGC. N2 Bristol, mec-4(u45), and mec-6(u247) were extant in our laboratory. Animals were raised under standard cultivation conditions.
Chapter IV

α-tubulin acetylation and mechanosensation.
Abstract

The acetylation of α-tubulin on its luminal-facing lysine 40 residue is a common tubulin post-translational modification particularly in neurons. The significance of α-tubulin acetylation is poorly understood though it correlates with stable MTs. *mec-12* is required for mechanosensation and encodes the only *C. elegans* α-tubulin bearing an acetylateable lysine 40 residue. *mec-12* is expressed in the TRNs and a few other neurons but only the TRNs display a robust signal when stained with an antibody specific to acetylated tubulin. The significance of the heavy α-tubulin acetylation observed in the TRNs remains unclear. I explored the relationship between MT acetylation in the TRNs, the MT acetyltransferase-encoding genes *mec-17*; and *atat-2*, the MT lumen, and mechanosensation. I found morphological and ultrastructural TRN defects attributable to defects in MEC-17 activity including ectopic sprouting, dramatic reduction in TRN MT number, elimination of characteristic TRN MT luminal electron density, and the presence of a pronounced posterior ALM process, which is not observed in wild type animals.

Introduction

The touch receptor neuron (TRN) microtubules (MTs) are unique amongst *C. elegans* MTs for their heavy lysine 40 α-tubulin acetylation (Fukushige et al., 1999) and their large diameter (Chalfie et al., 1982). The monoclonal antibody 6-11b-1 specifically recognizes acetylated α-tubulin (Piperno et al., 1985). Animals stained with 6-11b-1 show a robust TRN signal with much fainter staining observed in ciliated sensory neurons and motor neurons (Fukushige et al., 1999; Sollinger et al., 2010). MEC-12 is the only known α-tubulin isoform subject to lysine 40 acetylation in *C. elegans* and is responsible for the bright TRN 6-11b-1
antibody signal as well as the faint staining seen in the other neuronal subtypes (Fukushige et al., 1999). The functional significance of α-tubulin acetylation in C. elegans and in the TRNs in particular remains unclear.

The high level of TRN acetylation and its uniqueness would seem to suggest some sort of physiological significance though the bright 6-11b-1 TRN signal could be attributable to the high number of MTs in the TRNs. Non-acetylateable MEC-12 rescues the Mec phenotype of the mec-12(1607) mutation which is a presumed null (Fukushige et al., 1999) suggesting that α-tubulin acetylation is dispensable for TRN function. Rescue experiments such as that performed with non-acetylateable MEC-12 involve the injection and integration into the genome of multi-copy extra-chromosomal arrays making it impossible to determine exactly how many copies of the rescuing gene have been incorporated into the rescued animal’s chromosomal DNA. It is possible that MEC-12 acetylation is necessary for TRN function but this requirement can be overcome by non-acetylateable MEC-12 in excess. Single-copy insertion using a mos-1 mediated damage repair mechanism is a more physiologically relevant rescue approach since only one copy of the gene of interest is inserted (Frokjaer-Jensen et al., 2008). The mos-1 single copy insertion of wild-type mec-12 into a mec-12(e1607) mutant background results in an approximately 75% positive touch response, non-acetylateable versions of mec-12 result in 50-60% positive touch response vs. 25% positive response in the mec-12(e1607) background with no transgene (Akella et al., 2010). These data do not conclusively establish a requirement for α-tubulin acetylation in the TRNs since non-acetylateable versions of MEC-12 can rescue the touch defects of mec-12 mutants to a large degree and the question of TRN MEC-12 acetylation remains unresolved.

The difference in mechanosensory defect rescue efficacy between wild type and non-acetylateable MEC-12 is relatively small. Non-acetylateable versions of MEC-12 still exhibit a
near 100% increase in touch sensitivity vs. the \textit{mec-12(e1607)} mutant phenotype (Akella et al., 2010). The rescue efficacy of single-copy, non-acetylateable \textit{mec-12} would seem to suggest that excess of non-acetylateable MEC-12 is not required to exert a significant degree of rescue with a single copy of the gene being sufficient to affect rescue. \(\alpha\)-tubulin acetylation may be somewhat dispensable for touch neuron function with reduction but not elimination of touch sensitivity resulting from loss of TRN \(\alpha\)-tubulin acetylation. A simple loss of TRN \(\alpha\)-tubulin acetylation may result in a phenotype too subtle to have been identified by mutagenesis screens (alleles of \textit{mec-12} effecting acetylation were not identified by saturation mutagenesis) or one that results in pleiotropy confounding identification of EMS-induced \textit{mec-12} acetylation mutants. Pleiotropic defects due to \textit{mec-12} mutations seem unlikely since the presumed null \textit{mec-12(e1607)} mutation does not result in an uncoordinated phenotype (which is the only obvious pleiotropic phenotype one might expect to be caused by \textit{mec-12} mutations due to its expression in the D-type motor neurons). The sum of these results would seem to suggest a subtle yet detectable decrease in touch sensitivity when TRN \(\alpha\)-tubulin acetylation is disrupted though the mechanism of this effect remains unclear. Insight into the significance of TRN \(\alpha\)-tubulin acetylation in mechanosensation has also been examined by study of the enzymes responsible for the modification.

\textit{Regulators of \(\alpha\)-tubulin acetylation: Tubulin acetyltransferases}

The enzymes responsible for \(\alpha\)-tubulin acetylation have only recently been identified (Akella et al., 2010; Shida et al., 2010). The \textit{mec-17} gene of \textit{C. elegans} was first identified by mutagenesis screens for its effect on mechanosensation and is exclusively expressed in the TRNs (Zhang et al., 2002). The function of MEC-17 remained unclear until a weak homology with \textit{gcn5} (general control nonderepressible 5) histone acetylases was recognized. Histone N-terminal tail acetylation is a global transcriptional regulatory mechanism that serves to “loosen”
the DNA helix from histones allowing transcriptional machinery access to DNA (reviewed in (Nagy et al., 2010). Gcn5 also acetylates select transcription factors (Nagy et al., 2010). mec-17 mutations alone result in little or no disruption of TRN MT acetylation as measured by 6-11b-1 antibody staining and MEC-17 does not acetylate histones in vitro (Akella et al., 2010; Shida et al., 2010). MEC-17 may have as yet unidentified substrates other than \( \alpha \)-tubulin or histones though none have been identified. The *C. elegans* genome contains a mec-17 paralog dubbed atat-2 (Zhang et al., 2002). atat-2 mutants display a subtle Mec phenotype and no apparent reduction in TRN 6-11b-1-antibody staining (Akella et al., 2010). Double mutants of mec-17 and atat-2 however result in the complete elimination of acetylated tubulin staining in the TRNs and severe mechanosensory defects suggesting functional redundancy in the TRNs (Akella et al., 2010; Shida et al., 2010).

Shida et al., (2010) performed rescue experiments with wild type and enzymatically inactive mec-17 constructs in mec-17(ok2109) animals. In their hands, mec-17(ok2109) animals respond to slightly less than five out of ten gentle touches to the body. The same strain responds to slightly more than five out of ten gentle touches to the body when transformed with multiple copies of wild-type mec-17 and approximately 2.5 out of ten touches when transformed with a construct encoding an enzymatically inactive version of MEC-17. The authors claim rescue of the touch phenotype with wild type mec-17 but not enzymatically inactive mec-17. The lack of any meaningful rescue of touch sensitivity by transformation of mec-17 (ok2109) with wild-type mec-17 (Shida et al., 2010) may leave room for doubt. Rather than simply failing to rescue in these experiments, transformation of mec-17(ok2109) with an enzymatically inactive MEC-17 construct actually enhances the touch insensitivity phenotype of mec-17(ok2109) animals by approximately 100%. Overexpression of the mec-17 promoter itself may complicate the interpretation of these rescue results due to sequestration of transcription factors. The uls31 construct which encodes amino acids 1-220 of the 262 amino acids in MEC-17 (followed
by GFP) fails to rescue mec-17(ok2109) touch defects (this thesis) even though the amino acid crucial for acetylation is located at position 157 (Shida et al., 2010). Questions regarding the enzymatic activity of MEC-17 and its significance remain.

It is unclear why mec-17 and atat-2 single mutants display mechanosensory defects. If mec-17 and atat-2 are responsible for acetylating α-tubulin and acetylation is important for TRN function, then why is little to no decrease in TRN MT acetylation observed in single mutant backgrounds? If the two paralogs are redundant than why does disruption of one result in any defect at all? MEC-17 and ATAT-2 both acetylate α-tubulin at lysine 40 though it is unknown if they are functionally equivalent in terms of concentration and processivity. Slight perturbations in α-tubulin acetylation not detectable by 6-11b-1 antibody staining may be enough to cause a significant mechanosensory defect. 6-11b-1 TRN staining signal may be saturated at tubulin concentration levels lower than those present in wild type TRNs and some degree of reduction in tubulin acetylation may occur before signal strength fades. Free, acetylated tubulin heterodimers may contribute to the observed 6-11b-1 signal as well. Another possibility is that MEC-17 regulates some as yet unidentified protein by acetylation with resultant touch defects upon MEC-17 disruption.

The Elongator complex, which helps extend nascent RNA transcripts, may also be involved α-tubulin acetylation. TRN MT acetylation is reduced in elpc-3 (which encodes a subunit of the Elongator complex) mutants (Solinger et al., 2010). Elongator-mediated acetylation of α-tubulin modulates cortical neuron migration and differentiation (Creppe et al., 2009). Another subunit of the Elongator complex has recently been implicated in tubulin acetylation (Cheishvili et al., 2010). The Elongator complex has been proposed to have both nuclear and cytosolic functions with histone H3 being its nuclear target and α-tubulin the cytosolic target (Creppe et al., 2011). In contrast to these data, Akella et al., saw no decrease in
TRN MT acetylation in *elpc*-3 mutants (Akella et al., 2010). What role, if any, the Elongator complex may play in TRN MT acetylation is unresolved.

A lack of TRN α-tubulin acetylation might result in mechanosensory defects by several different mechanisms. The MT motor protein Kinesin-1’s affinity for MTs and its processivity are promoted by α-tubulin acetylation (Reed et al., 2006; Bulinski 2007). Mechanosensory defects caused by TRN α-tubulin defects could be a result of axonal transport defects which might result in mis-localization of the MEC-4/MEC-10 mechanosensory channel complex or other essential components though MT-related mechanosensory defects don’t always correlate with channel complex mislocalization (Bounoutas et al., 2009). α-tubulin acetylation affects the MT-severing enzyme Katanin’s affinity for MTs (Sudo et al., 2010). A disruption of MT dynamics induced by changes in Katanin activity could form the basis of TRN dysfunction. The affinity of some MAPs for tubulin can be affected by acetylation (Saragoni et al., 2000). α-tubulin acetylation defects may result in mechanosensory defects as a result of disrupted tubulin/MAP interactions though complete loss of MAPs such as ELP-1, PTL-1, show little to no loss of touch sensitivity (see previous chapter).

Regulators of α-tubulin acetylation and mechanosensation: α-tubulin deacetylases

Histone deacetylase 6 (HDAC-6) and Silent Information Regulator 2 (Sir-2) are known deacetylases which remove acetyl groups from α-tubulin (North et al., 2003; Zhang et al., 2003) as well as other substrates. Gain of function mutations in the rho GTPase mig-2 result in α-tubulin hyperacetylation leading to an Unc phenotype presumably due to hyperacetylation of MEC-12 in the motor neurons (Solinger et al., 2010). The mechanism of MIG-2 α-tubulin acetylation is unclear though it is probably indirect given the role of rho GTPases in a variety of signaling pathways. The *mig-2*(gm38) gain of function Unc phenotype can be suppressed by overexpression of HDAC-6 in motor neurons (Solinger et al., 2010) suggesting that HDAC-6 can
deacetylate MEC-12. Mammalian Sir2 modulates resistance to Wallerian degeneration following axonal injury in a MT-acetylation dependant manner with axons rich in acetylated MTs showing delayed degeneration (Suzuki et al., 2007). HDAC-6 and SIR-2 may act to maintain α-tubulin acetylation homeostasis in the TRNs though mutations in either gene do not appear to result in touch insensitivity.

TRN MT acetylation seems to have a subtle and as of yet, poorly understood influence on TRN function. Non-acetylateable MEC-12 α-tubulin can largely rescue mechanosensory defects caused by the near null mec-12(e1607) mutation suggesting that TRN MT acetylation is not essential for mechanosensation. The rescue of mec-12 mutations by non-acetylateable MEC-12 is less effective than that of wild-type MEC-12 however, suggesting that TRN MT acetylation is at least involved in proper TRN function. Further complicating the picture are the observations regarding the enzymes involved with the regulation of TRN MT acetylation. Mutations in mec-17 and, to a lesser extent, atat-2 result in noticeable mechanosensory defects yet no apparent reduction in TRN α-tubulin acetylation (or only slight reduction) by immunofluorescence. The Elongator complex appears to acetylate α-tubulin in several experiments yet contradictory results are reported for TRN MTs. The significance of the location of acetylation is also unknown. Acetylation is the only known MT PTM to occur on the luminal face of protofilaments. As with other systems studied, the role of TRN α-tubulin acetylation appears to be subtle and complicated.

Results

*Mutations in putative TRN MT α-tubulin acetylation regulators show a range of mechanosensory phenotypes*
I tested the touch sensitivity of strains carrying mutations in genes known or believed to effect α-tubulin acetylation in *C. elegans*. Mutations in the deacetylase-encoding genes *hdac-6* and *sir-2* did not differ significantly from N2 animals in touch sensitivity. *mig-2*(gm38) gain of function mutants displayed a subtle mechanosensory defect as did *atat-2* animals (Figure 1). Mutations in *mec-17* resulted in touch insensitivity with the knock out showing a more severe phenotype than the EMS induced point mutation. Mutants lacking the ELPC-3 Elongator complex subunit showed no mechanosensory defect. A *mec-17*(ok2109); *mig-2*(gm38) gain of function double mutant was created to test whether or not *mig-2*(gm38) induced hyperacetylation could make up for the loss of α-tubulin acetylation attributable to MEC-17 activity. No suppression of the *mec-17* touch phenotype was observed.

![Figure 1. Mechanosensory phenotypes of acetyltransferase mutants. 30 young adults per strain were blindly assayed.](image)
**α-tubulin acetylation is intact in acetyltransferase single mutants**

The 6-11B-1 anti-acetylated α-tubulin antibody was used to assess TRN levels of acetylated α-tubulin in acetyltransferase mutant backgrounds (Figure 2). My results were consistent with previously published assays of TRN α-tubulin acetylation in mec-17 and atat-2 mutant backgrounds (Akella et al., 2010). I was unable to distinguish between mec-17(ok2109), mec-17 (u265), N2, and atat-2(ok2415) mutants stained with the 6-11b-1 antibody by blind observation.
α-tubulin mutants display TRN morphology defects

I examined TRN morphology in *uls31* (partial MEC-17::GFP) and *p_mec-3_Mcherry* reporter strains as well as by MEC-18 antibody staining (Figure 3). All three conditions revealed changes in TRN morphology in the *mec-17(ok2109)* strain with the most notable change being found in the ALM neurons, which exhibited dramatic extension of a normally small or non-existent posterior process. *mec-17(u265)* mutants exhibited similar morphology defects when stained with the MEC-18 antibody but not by the *uls31* reporter construct. The *mec-17(ok2109)* long posterior ALM process is easily visible by 24 hours post-hatching and continues to elongate as the animal develops often proceeding past the vulva in mature animals (Figure 5). Cell body placement relative to the pharynx is unchanged in *mec-17(ok2109)* animals vs. controls. *atat-2(ok2415)* mutants displayed more subtle morphological phenotypes largely affecting the PLM neurons. A notable truncation of the PLM tail spike was observed in *atat-2(ok2415)* mutants. Analysis of the *atat-2(ok2415)* ALM posterior spike was confounded by the delayed maturation of this mutant strain. Some degree of ALM posterior growth was observed by 72 hours post hatching (which is when this strain reaches maturity) but similar phenotypes are observed in reporter strains alone and may be a manifestation of age-related issues. *elpc-3(ok2452)* animals displayed no apparent TRN morphology defects by MEC-18 antibody staining nor did they significantly enhance those observed in the *mec-17* knockout strain (Figure 4).
Figure 3. Representative examples of ALM Tail spike morphology in $\alpha$-tubulin acetyltransferase mutant backgrounds. Young adults were stained with the MEC-18 antibody, which exclusively labels the TRNs. White triangles=ALM cell body.
Figure 4. ALM posterior process length in cell body equivalents. MEC-18 antibody staining of young adults. Error bars represent SEM and “n” is number of ALM cells.
Figure 5. ALM posterior process elongation during development. Acetyltransferase mutants carrying uls31 or p_mec-3_cherry reporters were examined at twelve-hour intervals after hatching and the ALM posterior spike was measured. Error bars represent SEM and “n” represents total ALMs examined. Note: mec-17(ok2109); atat-2; uls31 animals await genotype confirmation.

Mechanosensory channel complex localization and α-tubulin acetylation

The processivity of some molecular motors as well as the overall speed of axonal transport can be affected by changes in α-tubulin acetylation (Reed et al., 2006; Bulinski 2007). The mec-17 mechanosensory defect might be due to disruptions in the transport or localization of the MEC-4/MEC-10 mechanosensory channel complex. I examined channel complex
localization using an antibody, which recognizes the channel complex accessory protein MEC-2. MEC-2 is normally co-localized with the MEC-4/MEC-10 channel complex along TRN axons and exhibits a punctate distribution. No differences in MEC-2 localization were discernible between wild type, *atat-2(ok2415)*, and *mec-17(ok2109)*, and animals by blind observation (Figure 6). *mec-17(u265)* exhibits diffuse MEC-2AB staining in the TRNs. The ectopic ALM posterior process was also observed by MEC-2 staining in the *mec-17(ok2109)*, and *mec-17(u265)* animals.

![Figure 6. MEC-2 antibody staining, ALM anterior processes. Red triangles indicate AVM processes showing puncta whereas white triangles indicate non-punctate ALM processes.](image)

I observed a variety of heterogeneous TRN morphology defects in *mec-17(ok2109)* and *mec-17(u265)* mutants including sprouting, ectopic processes, apparent attachment defects,
and corona-like loops extending from TRN processes (Figure 7). ALM and PLM were both affected though ALM defects are more penetrant with the vast majority of adults illustrating morphologically aberrant ALM anterior processes (in addition to the ectopic posterior process).

Figure 7. TRN morphology defects in acetyltransferase mutants. (A) Two uls31 animals showing WT ALMs. (B) Two animals. PLM showing a distal process attachment defect is indicated by white triangle. (C) ALM showing sprouting. (D) ALM with ectopic processes. (E) ALM showing loops (red triangles) and an ectopic nerve ring process in addition to the one normally present (white triangles). (F) ALM showing posterior bifurcation. (G) ALM sprouting (red triangle) and bifurcation. (H) Truncated PLM posterior processes (white triangles indicate cell bodies).
MEC-7 β-tubulin levels are unchanged in tubulin-acetyltransferase mutants

The TRN large diameter MTs are composed of MEC-12 α-tubulin and MEC-7 β-tubulin heterodimers. Acetylated α-tubulin staining is intact in mec-17 and atat-2 mutants but eliminated in mec-17(ok2109); atat-2 double mutants (Akella et al., 2010; Shida et al., 2010). It is unclear if TRN MTs are still present and unacetylated or are simply absent in double mutants. It may also be the case that changes in TRN α-tubulin acetylation result in altered MT dynamics.
possibly affecting MEC-7 β-tubulin levels. I assayed overall TRN MT integrity and MEC-7 levels using an antibody to the MEC-7 β-tubulin in vivo. No discernible difference was observed in mec-17(ok2109), mec-17(u265), or atat-2 versus wild-type TRNs with all strains showing robust staining of the TRN processes (Figure 8). MEC-7 antibody staining was observed in the ectopic ALM process at levels comparable to the rest of the cell.

*Ultrastructural analysis of acetyltransferase mutants*

I re-examined electron micrographs first used to describe the large diameter microtubules in wild-type animals (Chalfie et al., 1979). In any given serial section, some TRN microtubules appear to be completely or partially occluded by electron dense material whereas others appear clear. Serial reconstruction revealed that any given TRN microtubule oscillated between clear and occluded states along its length with no such phenomena observed in non-TRN MTs. Although non-TRN MTs were occasionally slightly occluded, they were rarely blocked (Figure 9).
We hypothesized that the electron dense material observed in TRN MT lumen might be related to MEC-17 in some way since α-tubulin acetylation occurs on the luminal face of the protofilaments. In addition, mec-17 is regulated by one of the most highly transcribed TRN promoters yet identified (Zhang et al., 2002) suggesting that it might perform more than a simple enzymatic function and might play some structural role in the large diameter MTs. We performed transmission electron microscopy on sectioned mec-17 and atat-2 mutants in order

Figure 9. Serial reconstruction of ALM MTs from wild type animals. (A) Ten serial sections showing luminal density fluctuations in the indicated MT. (B) Graphic reconstruction of MT luminal density in 100 serial sections. Each line represents a serially reconstructed MT and its luminal occlusion state. MTs above the red line are from ALM whereas those below it are from non-TRN partner ALN. Control MTs were much shorter and often appear and disappear between sections in the same place. (C) Comparison of total number of occluded, partially occluded and clear MT sections from ALM, Control (ALN) and non-TRN Ventral Cord neurons.
to examine the TRN MTs. We found greatly reduced numbers of TRN MTs in *mec-17(ok2109)* and animals. No TRN MT luminal density was observed in *mec-17(ok2109)* sections. Evidence of morphological defects were also observed at the ultrastructural level with ALM processes appearing to sprout, bifurcate, and oscillate between narrow and wider diameters. The AVM and PVM neurons are not recognizable in *mec-17(ok2109)* animals due to a lack of large, filled MTs (Figure 12). The location of AVM and PVM in the Ventral Nerve Cord (VNC) where they are surrounded by other neuronal process complicates their identification by other means though these cells are present in the mutant strains and show no gross morphological defects by antibody staining.

The relationship between ALM and its partner ALN neuron also seems disrupted in *mec-17(ok2109)* animals. The ALM and ALN processes are normally immediately adjacent to each other since growing ALM axons use the ALN process as a guidance cue. Although some variations in the diameter of ALM and ALN processes are observed in wild type neurons, the severe constriction of ALM process diameter and apparent separation between the two cells observed in *mec-17(ok2109)* mutants does not.
Figure 10. Non-serial images of ALM neurons showing reduction in the number of MTs and morphological abnormalities. Low magnification images are to the left with area of detail outlined in red. Images to the right are higher magnification images of the same cell. N2 sections show the dense bundle of dark ALM MTs (ALN membrane is slightly out of plane). mec-17(ok2109) sections show diameter fluctuations between ALM and ALN neurons (same ALM cell in all three panels from left to right with two ALMs from different animals depicted). It is possible that the apparent ALM/ALN separation is an example of a bifurcated ALM process.
Figure 11. MT number is reduced in *mec-17(ok2109)* ALM neurons. Representative images to the right. MTs were counted from ten random sections for each cell.
Figure 12. Ventral nerve cord TRNs (AVM and PVM) are not recognizable in mec-17(ok2109) animals (A, C, and E) Low magnification image of the ventral cord. N2 TRNs are in the red box and are recognizable by their dark, dense MT bundle whereas mutant strain red boxes indicate where the TRNs would be expected. No characteristic MTs can be discerned in two different mec-17(ok2109) animals though the VNC TRNs are present in this strain.
Figure 13. Serial reconstruction of ALM sprouting event in a mec-17(ok2109) animal. (A) ALM and ALN. ALM “bud” indicated by red box. MTs can be seen in the bud. The ectopic bud drifts away from ALM and ALN in panels B-D and disappears shortly thereafter. By panel I the hypodermal cell has re-envoloped ALM and ALN. Fluctuations in ALN diameter can also be seen in panels C-I. This ectopic budding event may be an example of the loops and sprouts observed by antibody staining in this strain.
Discussion

Summary

I examined the role of α-tubulin acetylation in C. elegans TRNs and its influence on mechanosensation by investigating the α-tubulin acetyltransferases elpc-3, mec-17, atat-2, as well as the presumably indirect α-tubulin hyperacetylation mutant mig-2(gm38). Strains carrying mutations in genes encoding the tubulin deacetylases hdac-6, and sir-2 were also assayed for touch sensitivity though their lack of a phenotype precluded further investigation. Mutations in mec-17 or atat-2 result in touch insensitivity but little to no apparent reduction in TRN MT acetylation was detectable by immunofluorescence. mec-17 mutants display TRN morphology...
defects and a substantial reduction in TRN MTs with only a handful of MTs present in contrast to the densely packed MT bundle present in wild type TRNs. The mec-17(u265) mutation results in MEC-2 channel complex localization defects whereas the complete mec-17 knockout does not. atat-2 mutants display no apparent channel complex localization defects and less severe TRN morphological changes than those observed in mec-17 animals. The loops and sprouting observed on TRN processes in mec-17 mutants are not observed in atat-2 animals. Knockout of the elongator complex subunit elpc-3 failed to result in a Mec phenotype or enhancement of the mec-17(ok2109) phenotype. mig-2(gm38) gain of function mutations, which result in α-tubulin hyperacetylation (Solinger et al., 2010) failed to rescue mec-17 phenotypes though mig-2(gm38) animals show a slight touch defect.

Ultrastructural analysis of sections from mec-17(ok2109) animals show greatly reduced MT numbers. mec-17(ok2109) animals show grossly normal acetylated α-tubulin antibody staining and normal β-tubulin staining. It would appear that the small number of MTs present in mec-17(ok2109) TRNs is adequate to produce a near wild type fluorescent signal upon antibody staining with the 6-11b-1 or MEC-7 antibodies. The drastic reduction in TRN MTs illustrated by electron microscopy was not detectable by antibody staining alone. It would seem that antibody staining is inadequate to detect incomplete MT TRN loss possibly due to signal saturation by the few MTs present. Another possibility is that free tubulin heterodimers are contributing to the observed antibody staining signals. Electron microscopy clarifies the observation that mec-17 mutants show normal acetylated MT staining but exhibit mechanosensory defects. The reduced number of MTs observed in mec-17(ok2109) mutants are sufficient to produce a near wild type signal upon antibody staining with 6-11B-1 or MEC-7 antibodies but evidently insufficient to allow normal TRN function suggesting that signal derived from immunohistochemistry is saturated around 20% normal TRN MT content or that sufficient free, stained tubulin is present to make up for the loss of polymerized MT signal.
mec-17(u265) carries two missense mutations resulting in amino acid changes at positions 46 and 72. Neither mutation results in a stop codon and mutant MEC-17 protein is presumably still translated. mec-17(u265) animals exhibit a less severe mechanosensory defect than mec-17(ok2109) animals but illustrate the same morphological defects in adults. In contrast to the mec-17(ok2109) mutants, mec-17(u265) animals show diffuse MEC-2 antibody staining in the ALM and PLM neurons suggesting a mislocalization of the mechanosensory channel complex.  uly31, which encodes all but the last 40 amino acids of MEC-17 fused to GFP, rescues the ALM morphology defect in mec-17(u265) animals but not the mechanosensory defect suggesting that these two phenotypes are separable.  uly31 does not rescue the mec-17(ok2109) morphology or touch defects.

*Interpretation*

The initial observation that mec-17 mutants are touch insensitive yet display normal TRN MT α-tubulin acetylation was confusing. Sequence homology and in-vitro assays strongly suggest that MEC-17 is a α-tubulin acetyltransferase yet no defect in α-tubulin acetylation is observed in mec-17 mutants by antibody staining. The activity of ATAT-2 may be sufficient to compensate for the absence of MEC-17 activity in TRNs at the level of MT acetylation but this fails to explain the mec-17 mechanosensory defect. If the mec-17 mechanosensory defect is attributable to a reduction in TRN MT acetylation and ATAT-2 performs the same function as MEC-17 in a redundant manner then why are mec-17 animals touch insensitive? Ultrastructural analysis appears to have resolved this issue. Despite their grossly normal TRN acetylated α-tubulin antibody staining, mec-17(ok2109) TRNs contain many fewer MTs than wild type TRNs. The remaining MTs also lack the luminal density observed in WT TRN MTs. These data suggest that mec-17 animals are touch insensitive as a result of a drastic reduction in the number of TRN MTs.
How could a loss of MEC-17 activity result in such a dramatic decrease in the number of TRN MTs? \( \alpha \)-tubulin acetylation may be required for TRN MT stability with MTs being more susceptible to depolymerization in the absence of MEC-17 activity. This possibility seems unlikely since the MTs that are present in \( \text{mec-17(ok2109)} \) TRNs appear to be acetylated by ATAT-2 as evidenced by antibody staining. It may be the case that ATAT-2 is a less processive \( \alpha \)-tubulin acetyltransferase, is expressed at different developmental stages, or is expressed at levels lower than \( \text{mec-17} \) and is thus incapable of fully compensating for the loss of MEC-17 function allowing it to stabilize a smaller number of MTs. If \( \alpha \)-tubulin acetylation is required for TRN MT stability and ATAT-2 only partially compensates for the loss of MEC-17, it would be expected to see even fewer TRN MTs in the \( \text{mec-17(ok2109); atat-2} \) double mutant. MT \( \alpha \)-tubulin acetylation is eliminated in \( \text{mec-17(ok2109); atat-2} \) animals (Akella et al., 2010; Shida et al., 2010) though it is unknown how severe TRN MT loss is in these animals.

The complete absence of MT TRN luminal electron density in \( \text{mec-17(ok2109)} \) animals may explain the decrease in overall TRN MT number. As mentioned previously, \( \text{mec-17} \) is transcribed at very high levels (Zhang et al., 2002), which may be inconsistent with a simple enzymatic function for the protein. In vitro experiments indicate that MEC-17 prefers polymerized tubulin as a substrate for its acetyltransferase activity (Akella et al., 2010) suggesting that MEC-17 most likely performs enzymatic function in the MT lumen. The absence of TRN MT luminal electron density in \( \text{mec-17(ok2109)} \) mutants raises the possibility that said electron density is composed of MEC-17 protein. In vitro observations also indicate that MEC-17 can acetylate free tubulin heterodimers but with much slower kinetics (Akella et al., 2010). It may be the case that slow kinetics keeps MEC-17 attached to free tubulin heterodimers as they are assembled into protofilaments and that this is somehow required for assembly or maintenance of the large diameter TRN MTs with MEC-17 serving a structural role. As
mentioned previously, enzymatically inactive MEC-17 rescue experiments have yielded cloudy results. A clearer hypothesis in this regard will have to await experiments aimed at determining the protofilament number and diameter of TRN MTs in mec-17 mutant EM sections.

Disruption of the regulation of some unknown protein may account for the decrease in mec-17(ok2109) TRN MTs. Several hundred proteins have been shown to be targets of lysine acetylation in other systems including many components of metabolic pathways (Zhao et al., 2010). A prominent example is the FOXO family of transcription factors whose DNA binding and thus transcriptional activity is partially regulated by acetylation (Daitoku et al., 2004; Motta et al., 2004). MEC-17 may have substrates other than α-tubulin. The C. elegans FOXO transcription factor DAF-16 is regulated by the deacetylase SIR-2 (Tissenbaum et al., 2001; Daitoku et al., 2004) but the acetyltransferase has not been identified. A daf-16 null mutation initially seemed to suppress the mec-17(ok2109) morphology defect but not the mechanosensory defect though this experiment has thus far failed to be repeatable (data not shown and experiments ongoing) but this does not exclude the possibility that MEC-17 may be involved in a similar regulation of as yet unknown proteins. Antibodies against all lysine 40 acetylated proteins yielded bright green animals with widespread staining precluding TRN identification (though a co-stain with a TRN-specific antibody was not performed). The possibility of other MEC-17 targets has yet to be adequately addressed.

How might a lack of MEC-17 activity result in TRN morphological abnormalities? A simple reduction in TRN MT number is probably insufficient to result in the extensive sprouting and ectopic process growth observed in mec-17 TRNs though it is not inconceivable. mec-12(e1607) TRNs lack the large diameter MTs but are not known to exhibit morphology defects similar to those observed in mec-17(ok2109) animals. It may be that the lack of α-tubulin acetylation by MEC-17 leads to sprouting, ectopic process growth, and other morphological
abnormalities. The evidence regarding α-tubulin acetylation and neuronal morphology seems to contradict this hypothesis. The Elongator complex appears to enhance migration and sprouting of cortical neurons via α-tubulin acetylation with migration and sprouting reduced when Elongator function is impaired (Creppe et al., 2009). The N-terminal acetyltransferase ARD1-NAT1 (which may also target interior lysines) is required for dendritic extension in cultured neurons (Ohkawa et al., 2008). In addition, the hypothesis that decreased MT acetylation due to a loss of MEC-17 activity results in ectopic sprouting is complicated by the fact that ATAT-2 seems to effectively acetylate the few MTs remaining in mec-17(ok2109) TRNs as evidenced by antibody staining though there are still many fewer TRN MTs overall. Finally, the AVM and PVM neurons, which arise later in development than the ALM and PLM neurons, do not exhibit morphology defects in mec-17(ok2109) animals though they do exhibit an apparent loss of the large diameter MTs. Although ectopic sprouting induced by a lack of acetylated MTs cannot be ruled out, a more likely possibility is that MEC-17 has other substrates whose deregulation results in TRN morphology changes. A lack of MTs in mec-17(ok2109) TRNs may be the basis of morphological abnormalities though this would have to be a TRN-specific phenomena as low MTs are observed in most C. elegans neurons and does not appear to correlate with degree of sprouting.

My observations regarding the mec-17(u265) may provide insight into MEC-17 function. mec-17(u265) animals exhibit a less severe mechanosensory defect than that observed in the mec-17(ok2109) strain. The decreased phenotypic severity of the mec-17(u265) mutations suggests that MEC-17 mutant protein is made and is at least somewhat functional despite the fact that both u265 missense point mutations lie within the GCN5 acetyltransferase domain of the protein though it is difficult to estimate what effect they might have on the function of the GCN5 domain. The mec-17(u265) morphology defect but not the mechanosensory defect can be rescued by the uls31 construct which includes all but the last 40 amino acids of MEC-17
suggesting that the morphology defect and the mechanosensory defect are separable phenotypes. The last 40 amino acids of MEC-17 lie outside of the GCN5 domain and do not share homology with any known proteins or motifs. MEC-17 encoded by the (u265) gene presumably includes the 40 terminal amino acids. It may be the case that the 40 terminal amino acids of MEC-17 are required for appropriate sub-cellular localization or interaction with other proteins allowing the uls31 construct to rescue the mec-17(u265) morphology defect but not the mechanosensory phenotype. uls31 does not rescue any aspect of the mec-17(ok2109) phenotype suggesting that the last 40 amino acids are needed for MEC-17 function. The mec-17(ok2109) and mec-17(u265) mechanosensory phenotypes may be caused by different mechanisms with mechanosensory channel localization disrupted in mec-17(u265) mutants but not mec-17(ok2109) animals. Further experiments are required to clarify what insight mec-17(u265) and uls31 rescue may provide into MEC-17 function. These will be discussed in chapter V.

Deacetylase mutants and indirect hyperacetylation mutations appear to have little effect on TRN function. mig-2(gm38) gain of function mutations fail to rescue the mec-17(ok2109) mechanosensory defect despite the apparent MEC-12 hyperacetylation observed in motor neurons of this mutant (Solinger et al., 2010). mig-2 gain of function-induced hyperacetylation of α-tubulin must not act via MEC-17 since mec-17 is not expressed in the motor neurons. The failure of the mig-2(gm38) mutation to rescue mec-17(ok2109) may suggest that mec-17 phenotypes are not solely due to decreases in α-tubulin acetylation or they may indicate that mig-2(gm38) does not induce hyperacetylation in TRNs or at least not at levels adequate to compensate for the loss of MEC-17 function. Mutations in the α-tubulin deacetylase-encoding genes hdac-6 and sir-2 failed to effect mechanosensation. This may suggest that α-tubulin acetylation saturation does not interfere with TRN function.
Conclusions

Mutations in the acetyltransferase-encoding genes *mec-17* and *atat-2* result in touch insensitivity and TRN morphology defects whereas disruption of the putative α-tubulin acetyltransferase activity of the Elongator complex do not result in similar defects. The basis of the mechanosensory phenotype is most likely a drastic reduction in the number of MTs present in the TRNs, which may be due to loss of a putative structural role for MEC-17 or due to a lack of MEC-17 acetyltransferase activity that ATAT-2 cannot compensate for. This absence of TRN MTs may change the physical properties of the TRN process resulting in less effective distribution of physical perturbances to the cell membrane leading to the opening of fewer mechanosensory channels rendering most stimuli sub-threshold. Some precedent for this idea may be observed in cochlear pillar cells which support hair cells and contain extensively cross-linked, bundled, 15-protofilament MTs. Disturbance of the cross-links results in a decrease in cell "stiffness" (Tolomeo et al., 1997). The decreased number of TRN MTs may have a similar effect yielding a smaller non-bundled MT composition which is unable to effectively transmit force to the mechanosensory channels yet is adequate to maintain axonal transport.

The basis of acetyltransferase mutation-induced TRN morphology changes remains unclear. A lack of MT acetylation may induce ectopic sprouting and process growth though this seems contrary to reported relationships between α-tubulin acetylation and neuronal process growth. It may be the case that loss of MEC-17 activity results in deregulation of some as yet unidentified protein leading to ectopic growth though such a putative factor would appear to not be present in the AVM and PVM neurons. The reduced TRN MT number observed in *mec-17(ok2109)* animals might account for the constriction of ALM process diameter by a simple decrease in TRN cytoskeletal rigidity rendering the cell less resistant to constriction by the
surrounding hypodermal cell. Discussion of relevant future experiments will be presented in chapter V.

Materials and Methods

Strains and cultivation

*C. elegans* strains used in these experiments were cultivated under standard conditions as described in (Brenner, 1974) All experiments and matings were performed at 20° unless otherwise noted. The Caenorhabditis Genetics Center (CGC) provided *mec-17(ok2109), elpc-3 (ok2452), atat-2(ok2415), hdac-6(tm3436), sir-2, and mig-2(gm38)* mutant lines. N2, *mec-17 (u265), uls31, and p_mec-3::Mcherry*, were extant in our laboratory. *mec-17(ok2109); uls31, atat-2(ok2415); uls31, and mec-17(ok2109); elpc-3(ok2452)* strains were constructed by following Mec phenotypes for *mec-17*, fluorescence for *uls31* and *p_mec-3::Mcherry*, whereas PCR was used to confirm the presence of the *elpc-3* and *atat-2* deletions. *P_mec-17(ok2109); mig-2(gm 38), and mec-17(ok2109); mig-2(gm 38); uls31* strains were constructed by following Mec, Unc, and fluorescent phenotypes.

Touch assays

Gentle touch assays were performed as described in (Chalfie et al., 1981). Ten alternating touches were administered to the animals anterior (immediately behind the pharynx) and posterior. A failure of the animal to change direction, stop, or slow down in response to the touch was considered a negative response. Tests were administered blindly.

Morphology time-courses
Morphology time course analysis of the ALM posterior tail spike was performed as follows: Well-fed gravid adults were synchronized by bleaching onto standard worm growth plates lacking food. Newly hatched animals were picked approximately 2-4 hours later onto standard plates containing food and scored every 12 hours (microscopy described below) for 48 hours *atat-2(ok2415)* animals exhibit slowed growth and these animals were scored for an additional 24 hours.

**Immunohistochemistry**

Immunohistochemistry was performed as described by Finney and Ruvkun (1990). Rabbit MEC-2-N (Zhang et al., 2004), MEC-18, MEC-7, and mouse 6-11B-1 primary antibodies were incubated at 1:200, 1:1000, 1:500, and 1:500 dilutions respectively from 3 hours to overnight at room temperature. Rabbit and mouse secondary antibodies conjugated to rhodamine detected primary antibodies or Alexafluor488 incubated at room temperature for three hours to overnight at 1:1000 dilution.

**Microscopy and morphology defect determination**

Reporter strains and fixed, stained animals were blindly scored using an Axioskop 2 microscope (Zeiss) with a SPOT digital camera (Diagnostic Instruments, Inc.). Fixed animals were mounted on 4% agarose pads whereas live animals were mounted on 4% agarose pads containing 1% 2,3-butanedione monoxime, and 0.1M HEPES (pH 6.9). ALM posterior process length was determined by assigning a standard length to ALM cell bodies. The Axioscope eyepiece scale bar is approximately 10 ALM cell bodies long at 630X magnification. The same measurement was applied to all strains at all ages. 1000x magnification was used to illustrate finer structures such as branching and looping of ALM processes. Images were processed to enhance features of interest or morphology.
Electron Microscopy

High pressure and standard fixation techniques were used to prepare animals for electron microscopy. Chemical immersion fixation was performed in the lab of Dave Hall as described in (Hall 1995). High pressure freeze fixation was performed as described in (Hall 2011). All electron microscopy images were taken in the lab of Dave Hall using a Phillips CM10 Transmission Electron Microscope. Images in this thesis were taken at 64,000x or 92,000x for high magnification images and 34,000x for lower magnification images.
Chapter V

Discussion and future directions:
In this thesis I present work related to two main aspects of TRN function and development. Chapter II discusses work aimed at clarifying the suite of genes regulated by the TRN fate-specifying LIM-HD transcription factor MEC-3. Chapters III and IV discuss experiments aimed at clarifying the role of the large-diameter, heavily-acetylated MTs in the TRNs with effort focused on identifying new MT-related proteins involved in mechanosensation and clarifying the role of α-tubulin acetylation in the TRNs. Although some discussion is presented in the relevant chapters, I will now elaborate upon each set of work with emphasis on future directions.

**MEC-3 regulated genes (chapter II)**

The LIM-HD transcription factor MEC-3 is required for the establishment of TRN cell fate. MEC-3 is required for transcription of the genes which serve to make the TRNs functional mechanoreceptors including the components of the mechanosensory channel complex, the large diameter MT subunits, crucial ECM components and other genes, which support the mechanosensory functions of the TRNs. In the absence of MEC-3 activity, TRNs adopt a neuronal cell fate but fail to express the other mec genes suggesting that MEC-3 is needed for the final step of TRN differentiation. In order to gain a fuller understanding of the suite of proteins needed to make a functional TRN, mRNA from mec-3 and wild type TRNs was amplified and subjected to microarray comparison. Two sets of putatively MEC-3 regulated transcripts were revealed with one set appearing to be putatively repressed by MEC-3 or expressed in the FLP neurons and the other appearing to be upregulated by MEC-3 in wild type TRNs. Characterization of these putatively MEC-3 regulated candidates could yield new genes needed for TRN function, genes whose activity must be repressed for TRN function, or genes expressed in the FLP neurons. I performed experiments aimed at clarifying the mechanism of
MEC-3’s role in TRN fate determination and the function of any newly identified MEC-3 dependant genes.

I found no evidence for MEC-3-mediated transcriptional repression nor was I able to unambiguously identify FLP expression of transcripts in the absence of TRN expression. It is possible that the 5’ sequences used for each promoter construct did not include all of the necessary regulatory elements required to recapitulate endogenous expression though it is doubtful that this would be the case for all 20 genes whose expression patterns were determined. All of the transcripts enriched in the mec-3 TRN population showed less than two fold differences in expression vs. mRNA derived from wild-type TRNs and it is likely that these transcripts represent false positives or FLP transcripts that I was unable to unambiguously confirm by expression pattern analysis.

I confirmed several transcripts identified by microarray analysis whose TRN expression requires MEC-3 activity. The requirement for MEC-3 activity is based on the observation that promoter GFP constructs for a given gene are not expressed in mec-3 TRNs but are expressed in N2 TRNs. Several of the transcripts I investigated appear to show what may be incomplete regulation by MEC-3. F42A9.9, cap-1, rpl-20, and T04B2.3 may be faintly expressed in mec-3 TRNs. Morphological defects associated with a lack of MEC-3 activity and widespread neuronal fluorescence (for some promoters) complicate TRN identification in mec-3 animals but the ALM lie in a part of the body with few neurons and are relatively easy to identify. Transcripts partially regulated by MEC-3 might be regulated by other transcription factors such as UNC-86 as well. UNC-86 is sufficient for early expression of mec-3 itself and might be responsible for partial regulation of the transcripts showing expression in the mec-3 background.

Transcripts showing apparently partial MEC-3 regulation may not be directly regulated by MEC-3 at all. It may be the case that F42A9.9, cap-1, rpl-20, and T04B2.3 represent victims
or a microtubule defect-induced transcriptional malaise. Mutations in mec-7 and mec-12 result in decreased TRN transcription that affects both TRN-specific and general transcripts (though only five genes have been examined in this context) (Bounoutas et al., 2010). MEC-3 regulates mec-7 and mec-12 expression thus mec-3 mutations might phenocopy the transcriptional depression seen in mec-7 and mec-12 mutants. The extent of this transcriptional depression in mec-3 TRNs varies from gene to gene with some fluorescent reporters showing no TRN signal by 48 hrs post hatching in the presence of colchicine. MT depolymerization or absence might allow normally MT-sequestered transcriptional repressors to act in the TRNs. Such a putative repressor might have specific targets or it might generally inhibit transcription. Transcripts expressed in TRNs but not regulated by MEC-3 might still show differential expression in wild-type vs. mec-3 TRNs simply because of MT-dependant transcriptional repression though such an effect would have to occur early in development to affect the mRNA populations used for microarray analysis.

The possibility of partial MEC-3 regulation of the above-mentioned transcripts could be addressed by crossing N2 strains carrying promoter GFP constructs for F42A9.9, cap-1, rpl-20, and T04B2.3 into unc-86 animals to see if their TRN expression is eliminated. MEC-3 requires UNC-86 for transcriptional activation (Duggan et al., 1998) whereas UNC-86 is able to drive transcription in the absence of MEC-3 (though this may be limited to early larval stages in TRNs). If F42A9.9, cap-1, rpl-20, and T04B2.3 promoter GFP constructs show no TRN expression in unc-86 animals, it would be possible to infer that these transcripts are at least partially regulated by TFs other than MEC-3 and not simply victims of MT disruption-induced transcriptional repression. This approach may be confounded by the observation that UNC-86 can apparently only drive mec-3 transcription early in development. The MT disruption-induced transcriptional repression effect begins to take effect 24 hrs post hatching at a time when UNC-86 may no longer be independently driving transcription in the TRNs. Another possibility is to
examine F42A9.9, cap-1, rpl-20, and T04B2.3 promoter GFP constructs in a mec-3; dlk-1 background. Mutations in dlk-1 which encodes a MAPKKK suppress the transcriptional repression effect caused by MT disruption (Bounoutas et al., 2010). If F42A9.9, cap-1, rpl-20, and T04B2.3 expression are not regulated by MEC-3, then these promoter GFP constructs should be equally bright in mec-3; dlk-1 and N2 animals eliminating the possibility that their differential expression is due to MT disruption.

F10A3.11, F14E5.4/acp-2, aip-1 and the cct subunits are more likely to be directly regulated by MEC-3 since promoter constructs for these transcripts are not expressed in mec-3 TRNs. RNAi and mutant analysis showed no mechanosensory defect associated with these transcripts (with the exception of the cct genes) but this does not necessarily rule out a role for these genes in TRNs especially in the case of F10A3.11 and acp-2, which show very limited neuronal expression including only 8-10 neurons. RNAi targeting F10A3.11 affects lipid accumulation in the intestine of daf-2 mutants (Ashrafi et al., 2003). F10A3.11 may be important for regulating some aspect of lipid accumulation/composition that is necessary for TRN function. Membranes of mechanosensory neurons may require distinct lipid compositions to facilitate channel gating. F10A3.11 mutants show no mechanosensory defects though F10A3.11’s role may be specific to periods of starvation perhaps acting to maintain TRN membrane qualities during metabolic stress. Analysis of daf-2; F10A3.11 or starved/dauer F10A3.11 animals might reveal a Mec phenotype. aip-1 may also be important for TRN function under stressful conditions consistent with its putative chaperone and arsenide response activities ((Hassan et al., 2009; Ferguson et al., 2010). acp-2 may act to regulate the activity of the C. elegans EGF receptor LET-23 in a manner similar to its human homolog (Sharma et al., 2005). Combinatorial TRN-specific RNAi vs. let-23 and acp-2 or genetics could be employed to address this hypothesis.
The MEC-3 regulation of CCT complex subunits is somewhat confusing. It makes sense that cells packed with tubulin might have special regulatory mechanisms to deal with the suite of proteins needed to process high levels of tubulin. However, if high levels of tubulin require overexpression of cct subunits, one might expect MEC-3 to enhance a baseline level of cct expression present in most tissues rather than be the exclusive regulator of TRN cct transcription. No evidence of TRN cct expression is observed in mec-3 mutants suggesting that MEC-3 is required for cct gene transcription in TRNs. The regulation of cct transcripts by MEC-3 in the TRNs suggests that MEC-3 is not only needed for the expression of genes specific to mechanosensation but that it also regulates the expression of genes whose function are not necessarily tied to mechanosensation in general. The cct genes would appear to be the first example of mec genes, which are also essential for viability and have become regulated by the “niche” transcription factor MEC-3 in a specific cell-type context.

It is notable that no other genes encoding MT-related proteins appear in the microarray data (with the exception of mec-17) perhaps suggesting that the densely packed TRN MTs do not require increased expression of MAPs or other MT-related proteins or that such transcripts are not under MEC-3 regulation. It is also notable that transcription of Prefoldin complex subunits is not MEC-3 dependant, which might be expected since the subunits of its partner CCT complex are. Overall, it would appear that, with the exception of the cct transcripts, MEC-3 does not regulate any factors essential for general TRN function that have not been previously identified and that newly identified MEC-3 regulated genes may be important in specific contexts only or that their function is redundant.
Microtubules and Mechanosensation in *C. elegans* (Chapters III and IV)

**Background**

*C. elegans* TRN MTs are unique for their large diameter and heavy α-tubulin acetylation. The significance of both the large diameter and heavy acetylation has remained obscure. The products of the *mec-12* and *mec-7* genes encode α and β-tubulins, respectively, which comprise the subunits of the large-diameter MTs. Mutations in either of these genes result in an absence of the large diameter MTs and a mec phenotype by several possible mechanisms including disrupted channel gating, channel localization defects, TRN transcriptional repression or changes in TRN physical properties such as membrane tension or axonal rigidity. Few proteins known to regulate MT dynamics or to associate with them in important ways have been implicated in mechanosensory defects (aside from the weak effects of PTL-1 and EPL-1 which I was unable to confirm) and the mechanism of MT-mediated mechanosensory defects remains unclear.

The significance of the heavy α-tubulin acetylation of TRN MTs is unclear. Mutations in α-tubulin acetyltransferases result in mechanosensory defects yet non-acetylable MEC-12 is able to rescue the mec phenotype in *mec-12* mutants to varying degrees (Akella et al., 2010; Fukushige et al., 1999). α-tubulin acetyltransferase mutants display mechanosensory defects yet antibody staining of acetylated α-tubulin appears normal or only slightly reduced (Akella et al., 2010; Shida et al., 2010). Transformation of *mec-17* null (*ok2109*) animals with wild-type or enzymatically inactive *mec-17* multi-copy arrays yield minimal to no rescue and enhancement of the *mec-17(ok2109)* touch defect respectively (though excessive copies of the *mec-17* promoter may confound these results) (Shida et al., 2010). The elongator complex has been implicated in α-tubulin acetylation though there are conflicting reports with elongator complex mutants showing reduced TRN MT acetylation (Solinger et al., 2010) or not (Akella et al., 2010).
Different groups report varying severity of mechanosensory phenotypes upon disruption of acetyltransferase genes with mec-17(ok2109) animals showing an 80% reduction in touch sensitivity (C. Keller, this thesis) vs. 50-60% reduction in other work. The mec-17(ok2109); atat-2 double mutant has also been reported as being severely mec (this thesis and (Akella et al., 2010)) or as indistinguishable from the mec-17(ok2109) phenotype (Shida et al., 2010). As with other systems examined, the significance of α-tubulin acetylation in TRNs is unclear.

The work presented in this thesis provides several new insights into the role of the unique TRN MTs and their heavy α-tubulin acetylation in mechanosensation. I used TRN-specific RNAi to identify previously unrecognized MT-associated proteins involved in mechanosensation. I also studied the role of the acetyltransferase MEC-17 which is needed for proper TRN function and development. I have shown that mutations in mec-17 result in gross TRN morphology defects as well as ultrastructural defects with MT number reduced, MT luminal density eliminated, and mechanosensory channel complex localization disrupted in one case (mec-17(u265)). Comparison of phenotypes derived from mec-17(ok2109) (deletion) and mec-17(u265) (missense) animals illustrates that mechanosensory, channel localization, and morphology defects attributable to mec-17 are separable, differ between the two mutations, and may depend upon different parts of the protein. Through RNAi screens and characterization of newly identified MEC-3 regulated genes I have revealed previously unrecognized components related to MTs and necessary for TRN function including the CCT chaperone complex, the MT severing enzyme Katanin, the cytosolic carboxy peptidase ccpp-1, phoceins, and regulators of TAU/tubulin phosphorylation. These observations present several routes for further study to be discussed below.
Newly identified MT-related mechanosensory components

Chapter III of this thesis presents the results of TRN-specific RNAi and mutant analysis targeting genes encoding MT-related proteins and their possible roles in TRN development or function. I identified several genes encoding proteins not previously recognized as being involved in TRN development or function including the protein phosphatase 2a subunit PAA-1, GSK-3 kinase, the tubulin deglutamylase CCPP-1, α-tubulin 8, MEI-1/Katanin and two C. elegans phocelin homologs. I identified the basis of the mechanosensory defects observed in ccpp-1, C30A3.3, and F09A5.4 animals whereas I was unable to clarify the basis of mechanosensory defects induced by RNAi vs. paa-1, gsk-3, ttbk-2, tba-8, or mei-1.

Putative regulators of phosphorylation

PAA-1, GSK-3, and TTBK-2 share TAU as a target for regulation by phosphorylation and the phenotypes observed upon RNAi against the genes encoding these proteins may be due to deregulation of TAU phosphorylation state homeostasis. TAU binds to and stabilizes MTs in a phosphorylation-dependant manner with hyperphosphorylated TAU losing its affinity for MTs with MT instability being the result. It may be the case that over/under phosphorylation of TAU results in a shift in TRN MT stability dynamics away from an optimal state resulting in mechanosensory defects due to unstable or hyper-stable MTs. Hyperphosphorylated TAU forms aggregates containing GSK-3 and are correlated with Alzheimer's disease pathology. A perturbation of TAU phosphorylation state homeostasis may result in analogous aggregate formation leading to TRN dysfunction. Experiments designed to address the TAU phosphorylation deregulation hypothesis are confounded by the severe, lethal phenotypes observed in gsk-3 and paa-1 mutant strains, which complicate genetic approaches. paa-1 and gsk-3 balanced lethal strains exist though homozygotes arrest and die at unknown
developmental time points. The RNAi phenotype induced by RNAi against \textit{ttbk-2} may be a false positive as the deletion strain shows only a very slight mechanosensory defect.

Despite the above-mentioned difficulties, several experiments could be performed to address the significance of TAU phosphorylation regulators in the TRNs. Overexpression of the \textit{C. elegans} TAU homolog \textit{ptl-1} in the TRN-specific RNAi strain followed by RNAi targeting \textit{gsk-3}, \textit{paa-1}, or \textit{ttbk-2} could be performed to address the TAU phosphorylation deregulation hypothesis. If TRN MT instability caused by loss of TAU affinity for MTs is the basis of the mechanosensory defect observed in \textit{gsk-3} or \textit{ttbk-2} RNAi conditions, it could be expected that excess PTL-1 might suppress this phenotype. Alternatively, excess PTL-1 could enhance mechanosensory defects under these conditions if it is the case that MT hyper-stability or PTL-1 aggregate formation are the basis of observed mechanosensory defects. Another possibility is to over express \textit{gsk-3}, \textit{paa-1}, or \textit{ttbk-2} in a TRN specific manner. Such overexpression would presumably perturb PTL-1 phosphorylation state homeostasis and result in mechanosensory defects if the hypothesis is accurate. I examined \textit{ptl-1} mutants and found that they exhibit no mechanosensory defect though the \textit{ptl-1} deletion does not eliminate the entire coding sequence and some truncated version of PTL-1 may still be produced. \textit{paa-1}, \textit{gsk-3}, and \textit{ttbk-2} are components of complicated signaling pathways and examination of viable mutants encoding other members of the signaling network might provide insight into the basis of the observed mechanosensory defects. It is also possible that the mechanosensory phenotypes observed are simply a manifestation of some general cell pathology unrelated to mechanosensation though examination of GFP-labeled TRNs under \textit{gsk-3}, \textit{paa-1}, or \textit{ttbk-2} pan-neuronal RNAi conditions revealed no apparent TRN cell-death or morphology defects.
Katanin (mei-1)

The MT severing enzymes Spastin and Katanin sever MTs at the centrosome and at other locations along the length of MTs allowing them to move about the cytoplasm. Katanin and Spastin target MTs at their more stable points though they exhibit different MT affinities depending upon the tubulin PTMs present. Katanin MT severing activity has also recently been identified at the more dynamic plus end of MTs near the leading edge of growing axons (Baas et al., 2011). Why would an absence of MEI-1 activity in TRNs result in mechanosensory defects? Katanin preferentially targets neuronal MTs at sites of heavy acetylation though this effect is attenuated in areas with high levels of TAU protein, which is more prevalent in axons than dendrites (Sudo et al., 2010). Dendritic MT severing by Katanin can be suppressed by TAU overexpression (Sudo et al., 2010). TRN MTs are heavily acetylated and might thus be likely targets for Katanin severing. The long and densely packed MT bundle observed in TRNs seem inconsistent with them being subject to heavy severing however. It may be the case that TRNs express high levels of pti-1/TAU (this possibility has been recently confirmed by microarray analysis (Topalidou, personal communication) providing them protection from Katanin or it may be that the absence of Katanin activity would result in even more TRN MTs which might be detrimental to TRN function in some way. Alternatively, the absence of MEI-1 activity at the plus end of MTs in developing TRNs might lead to abnormal process growth or synapse formation though no evidence for such an effect was observed by MEC-2 antibody staining. I did not notice any gross disruption of acetylated α-tubulin antibody staining in mei-1 mutants or RNAi conditions though published images of severed MTs reveal only small breaks which I might not have noticed since small discontinuities in 6-11b-1 staining are not uncommon in WT TRNs. Electron microscopy of mei-1 animals could illustrate whether or not TRN MT number is increased by a lack of MEI-1 activity. Though MEC-2 antibody staining of mei-1 mutants yielded no gross morphological defects, mei-1 RNAi in strains carrying TRN synaptic
GFP markers might yield subtle defects attributable to a lack of MEI-1 activity at sites of TRN synapse formation.

*C. elegans Phocein homologs*

The phocein family of proteins regulates cell polarity, cytoskeletal integrity, and dendritic vesicular trafficking (Bailly et al., 2007; Liu et al., 2009). Mutations in two of the three *C. elegans* phocein homologs result in mechanosensory defects, disrupted acetylated α-tubulin, and MEC-2 antibody staining (though C30A3.3 was not stained for MEC-2). My results are consistent with the MT disruption observed in other cell types (Schulte et al., 2010). It is unclear if the mechanosensory defect observed in Phocein mutants is attributable to a general cellular pathology or effects more specific to mechanosensory components. Phocein proteins are limited to soma and dendrites in mammalian neurons (Blondeau et al., 2003; Haeberlé et al., 2006) though it is unknown where they localize in *C. elegans* neurons. Phocein mutations in *D. melanogaster* result in over-proliferation of neuro-muscular junction synapses. Phoceins may act to couple MTs with vesicular transport in the TRNs with their loss resulting in mechanosensory defects. *C. elegans* homologs of the phocein interacting proteins Striatin (CASH-1) and Dynamin (DYN-1) are expressed in the ALM neurons along with the phocien homolog C30A3.3. Study of *dyn-1* and *cash-1* mutants might help clarify whether phocein mutant phenotypes are due to gross cellular pathology or some effect such as channel complex localization that could be tied to the Phoceins. Overexpression of *dyn-1* and *cash-1* in F09A5.4 or C30A3.3 animals might serve to determine which aspect of phocein function; MT integrity or vesicle cycling, is important for TRN function.
**Tubulin Deglutamylases**

I tested two putative tubulin deglutamylases *ccpp-1* and *ccpp-6* for mechanosensory defects. TRN-specific *ccpp-1* RNAi and *ccpp-1* mutants display mechanosensory defects whereas *ccpp-6* mutants do not. Tubulin glutamylation is necessary for aspects of cilia function (Gaertig et al., 2008) and affects MAP binding (Bonnet et al., 2001). Tubulin glutamylation can both stabilize and destabilize MTs depending on other modifications present (Wloga et al., 2010). Tubulin glutamylation also moderates the affinity of the MT severing enzyme Spastin for MTs (Lacroix et al., 2010)

How could the absence of CCPP-1 mediated deglutamylation in TRNs result in a mechanosensory defect? *ccpp-1* mutants show disrupted 6-11b-1 and intact MEC-2 antibody staining suggesting that axonal transport is intact in these animals despite apparent MT disruption. Overexpression of TAU or creation of a *ptl-1; ccpp-1* double mutant might address the possibility of a MT stability defect being the basis of *ccpp-1* mechanosensory phenotypes. Overexpression of TAU would be expected to suppress *ccpp-1* phenotypes whereas the double mutant would be expected to have an accentuated mechanosensory defect. Excessive tubulin glutamylation caused by the absence of CCPP-1 function might result in excessive Spastin activity and a reduction in the number of TRN MTs leading to mechanosensory defects. MEC-7 antibody staining might be used to examine this possibility though previous results indicate that MEC-7 and 6-11b-1 antibody staining is inadequate to identify gross TRN MT loss. Electron microscopy of *ccpp-1* mutants might be necessary to address the possibility of MT reduction in *ccpp-1* mutants.

**Summary**

My TRN-specific RNAi results illustrate previously unrecognized mechanosensory defects for several genes encoding proteins relevant to MTs. One shortcoming of my screen was a failure to examine the F1 generation. Several genes, which exhibited no RNAi
phenotypes in the P0 animals, might be expected to show phenotypes in the F1 generation such as *pfd-1*. Such expanded analysis might reveal mechanosensory functions for genes that showed none in my experiments. Examining F1 animals would help overcome the problems of protein perdurance and possible early developmental significance that clouded my RNAi results.

**MEC-17 and other regulators of α-tubulin acetylation**

I have illustrated that animals lacking MEC-17 activity show a dramatic reduction in overall TRN MT numbers. It is unknown if *mec-17(u265)* animals display the same degree of TRN MT loss. TRN MT loss is most likely the basis of *mec-17(ok2109)*-induced mechanosensory defects though the reasons for this are not completely clear. MEC-2 antibody staining appears similar to WT in *mec-17(ok2109)* animals suggesting that the few remaining TRN MTs are adequate for mechanosensory channel complex localization or that MT loss occurs after mechanosensory channel complex localization. TRN MT reduction may interfere with mechanosensory channel gating. A TRN process lacking the dense bundle of cross-linked MTs will most likely have physical properties differing from wild-type TRNs and may be less able to open mechanically gated channels in response to membrane perturbation though both the tethering and membrane tension models could be invoked to explain this as there would be fewer points for channel tethering and presumably less MT-exerted membrane “tautness”. TRN MT reduction might also result in transcriptional perturbations similar to those seen in *mec-7* and *mec-12* mutants. No apparent reduction in MEC-2, MEC-7, or MEC-18 protein levels have been observed in *mec-17(ok2109)* animals though protein level assessment may not be sensitive enough to detect transcriptional changes especially considering the 6-11b-1 results which show normal levels of acetylated α-tubulin staining in *mec-17(ok2109)* animals despite a nearly five-fold reduction in TRN MT number observed by electron microscopy. Unstable *gfp*
driven by promoters expressed in the TRNs or microarray analysis could be used to address the possibility of transcriptional repression in mec-17(ok2109) TRNs.

The significance of MEC-17 mediated α-tubulin acetylation remains unclear as does the basis of the dramatic MT loss seen in mec-17(ok2109) mutants. A lack of TRN α-tubulin acetylation might result in decreased MT stability and thus fewer TRN MTs leading to TRN dysfunction though the idea that acetylation stabilizes MTs rather than follows stabilization is still a matter of debate. This hypothesis could be addressed by overexpression of the C. elegans TAU homolog ptl-1 that serves to stabilize MTs and might suppress a MT-instability induced mechanosensory defect in mec-17(ok2109) animals. If it is the case that a lack of α-tubulin acetylation results in fewer TRN MTs, then ATAT-2 is unable to compensate for the loss of MEC-17 activity despite its ability to acetylate the same tubulin residues as MEC-17. atat-2 may be expressed at levels inadequate to compensate for MEC-17, which is highly expressed, or atat-2 may be expressed at different developmental stages. Expression of atat-2 driven by the mec-17 promoter in mec-17(ok2109) animals could address the possibility of atat-2 temporal or expression level inadequacy. If p_mec-17 ATAT-2 were unable to rescue the mec-17(ok2109) phenotype then it would be possible to infer that a lack of α-tubulin acetylation may not be the basis of the TRN MT loss observed in mec-17(ok2109) mutants. A lack of mec-17(ok2109) rescue by p_mec-17 ATAT-2 would lend support to the hypothesis that MEC-17 has other substrates or that it may play a structural role in MT assembly or maintenance. Precedent has been established for the notion that regulators of α-tubulin acetylation may also serve structural roles with catalytically inactive versions of HDAC-6 still capable of regulation MT dynamics (Zilberman et al., 2009)

TRN overexpression of the deacetylase encoding genes hdac-6 or sir-2 driven by the mec-17 promoter in wild type animals could be employed to address the significance of α-
tubulin acetylation. If α-tubulin acetylation is essential for the establishment or maintenance of appropriate TRN MT numbers then overexpression of known α-tubulin deacetylases should result in a mechanosensory defect and fewer TRN MTs. Both HDAC-6 and SIR-2 deacetylate other substrates including histones and, in the case of SIR-2, transcription factors (Daitoku et al., 2004). Deacetylase overexpression experiments might therefore require the use of constructs lacking nuclear localization signals. *hdac-6* overexpression in D-type motor neurons successfully rescues the unc phenotype caused by *mig-2*(gm38)-induced hyperacetylation of MEC-12 in these cells (Solinger et al., 2010) though a similar rescue of mechanosensory defects was not observed in *mec-17*(ok2109); *mig-2*(gm38) animals. *mig-2*(gm38)-induced hyperacetylation is presumably indirect and may act through MEC-17 in TRNs and ATAT-2 in D-type motor neurons or it may simply be inadequate to rescue the *mec-17* defect. Motor neurons have many fewer MTs than the TRNs and *mig-2*(gm38)-induced hyperacetylation may be adequate to produce a phenotype where there is a low level of MEC-12 substrate but not in the TRNs where there is much more MEC-12 present. This view would be consistent with *mig-2*(gm38) inducing α-tubulin hyperacetylation through ATAT-2. HDAC-6 and SIR-2 overexpression experiments might be confounded by the observation that enzymatically inactive HDAC-6 can alter MT dynamics (Zilberman et al., 2009).

What might be the significance of TRN MT luminal density and its disappearance in *mec-17*(ok2109) mutants? MT luminal density has been observed in a variety of neurons including frog olfactory neurons (Burton 1984) and rat sensory neurons. In *C. elegans*, MT luminal density appears to be unique to, or at least much more prevalent in, TRN MTs. MT luminal density is of unknown composition and significance. The observation that TRN MT luminal density disappears in *mec-17*(ok2109) mutants and that it is not observed in any other neurons (which all lack *mec-17* expression) suggests that TRN MT luminal density may be composed of MEC-17 protein. This is not unreasonable considering that MEC-17 appears to
favor polymerized α-tubulin as a substrate and α-tubulin acetylation faces the MT lumen. Though MEC-17 is only 262 amino acids long, up to 15 MEC-17 proteins could in theory be in close proximity in the MT lumen at maximum acetylation levels perhaps resulting in the electron density observed in EM sections. MEC-17 may also be in a complex with other proteins such as ATAT-2 perhaps resulting in enough protein to account for the observed electron density. Overexpression of mec-17 in C. elegans motor neurons which express atat-2 and mec-12 but not mec-17 could be performed to address this hypothesis. Electron microscopy could be performed on such animals to determine whether or not ectopic expression of mec-17 results in motor neuron MT luminal density.

Could the simple lack of MEC-17 in MT lumen lead to disruption of TRN MTs and the overall reduction in MT number observed in mec-17(ok2109) mutants? It is conceivable that the presence of multiple MEC-17 proteins in close proximity could produce steric interference in the MT lumen causing the normal 11-protofilament MT structure to be energetically unfavorable leading to the large diameter 15-protofilament MTs seen in WT TRNs. MEC-17’s poor processivity on free tubulin (Shida et al., 2010) may suggest that MEC-17 stays adherent to free tubulin during the assembly process resulting in large diameter MTs via a steric interference mechanism. If TRN MT luminal density is composed of MEC-17 and ATAT-2 acetylates the same α-tubulin residues as MEC-17 then why is no ATAT-2 MT luminal density observed in mec-17(ok2109) mutants? If atat-2 is expressed at lower levels than mec-17 or at different developmental stages, it may not be present in adequate amounts to result in MT luminal electron density. The MEC-17 structural hypothesis would be further bolstered by ectopic mec-17 expression yielding large diameter MTs in other neuronal cell types. The answers to these questions will have to await ectopic mec-17 expression experiments and careful measurement of mec-17(ok2109) TRN MT diameter and probably tannic acid staining of thin sections, which allows protofilament number to be counted by EM. These experiments are ongoing.
How might a lack of MEC-17-mediated α-tubulin acetylation or a loss of a putative structural role for MEC-17 result in TRN morphology defects? The evidence related to α-tubulin acetylation and neuronal morphology is mixed. Ciliagenesis is impaired in the absence of MEC-17 activity suggesting that MT acetylation is necessary for process outgrowth (Shida et al., 2010). Inhibition of HDAC-6 activity impairs axonal outgrowth in cultured hippocampal neurons implying that de-acetylated MTs are necessary for this process (Tapia et al., 2010). Lack of Elongator complex-mediated α-tubulin acetylation results in increased synaptic bouton expansion and ectopic axonal growth at D. melanogaster neuromuscular junctions (Singh et al., 2010). Interference with Elongator complex tubulin acetylation impairs sprouting and migration of cortical neurons (Creppe et al., 2009). MT acetylation deficiencies correlate with neuronal sprouting and growth deficiencies in studies of neuronal micro RNAs (Yu et al., 2008). In some cases, decreased MT acetylation results in expansion of neuronal sprouting or axon growth whereas in other cases the opposite is true. The sprouting observed in mec-17(ok2109) animals may be due to a decrease in TRN MT acetylation though this has not been conclusively established and other causes could include decreased MT number, transcriptional perturbances based on MT loss, or deregulation of as yet unknown MEC-17 substrates.

Examination of MEC-17 truncations might help clarify MEC-17’s role in TRNs. mec-17 encodes a 262 amino acid protein with illustrated acetyltransferase activity that is required for mechanosensation. The less severe u265 allele carries two missense mutations at positions 46 and 72 whereas the deletion eliminates all but the first exon sparing only 28 amino acids. It is unknown if the small truncation fragment is translated in mec-17(ok2109) animals. Both mec-17 mutant strains exhibit mechanosensory and TRN morphology defects though the null mechanosensory phenotype is more severe. uls31 rescues the morphology defect of mec-17(u265) animals but not that of the null. The uls31 GFP reporter construct contains the first 220 amino acids of MEC-17 fused to GFP and is only lacking the last 42 amino acids of MEC-
17. *ul531* does not rescue the mechanosensory defect of either *mec-17* strain. The conserved gcn5 acetyltransferase domain of MEC-17 spans amino acids 11-176. It is unlikely that the last 42 amino acids of MEC-17 are involved in acetyltransferase activity since only nematode homologs of MEC-17 possess this small domain.

*ul531* includes the entire MEC-17 gcn5 acetyltransferase domain yet it does not rescue the Mec phenotype of either *mec-17* mutant suggesting that: 1) the last 42 amino acids of MEC-17 which are outside of the gcn5 motif are necessary for rescue of the mechanosensory defect or 2) the presence of the GFP fused to truncated MEC-17 inhibits its acetyltransferase function. Possibility two seems unlikely since *ul531* rescues the morphology defect of *mec-17(u265)* animals suggesting that the truncated version of MEC-17 present in *ul531* is active at least in regards to morphology. The rescue of the (u265) morphology defect but not the touch defect by *ul531* suggests that the two phenotypes may be separable and attributable to different parts of the MEC-17 protein. It may be the case that the last 42 amino acids of MEC-17 are needed for proper sub-cellular localization, MEC-17 interaction with other proteins, MEC-17 stability, or some other function independent of its acetyltransferase activity (though the last 42 amino acids not similar to any known protein domains of this type). The apparent significance of the MEC-17 C-terminus suggests that MEC-17’s α-tubulin acetyltransferase activity alone is not adequate for TRN function supporting the idea that MEC-17 may have other substrates or a structural role in MT assembly or maintenance.

Several experiments can be performed to address the significance of the MEC-17 C-terminus. MEC-2 antibody staining should be performed on *mec-17(u265); ul531* animals to determine whether or not the truncated MEC-17 present in *ul531* can rescue the apparent channel localization defect of *mec-17(u265)*. MEC-17 constructs lacking the last 42 amino acids can be used to rescue *mec-17(u265)* and *mec-17(ok2109)* mutants to eliminate the
possibility that the GFP part of *uls31* interferes with MEC-17’s acetyltransferase activity in some way. If such rescue experiments yielded results similar to the *uls31* rescue of *mec-17(u265)* morphology defects, it would be possible to draw the conclusion that MEC-17’s C-terminus is required for proper MEC-17 function. *In vitro* α-tubulin acetylation experiments analogous to those performed by (Shida et al., 2010) could be performed with WT and truncated MEC-17 to determine if the lack of the C-terminus effects MEC-17’s enzymatic activity in some way. Pull down experiments using WT vs. truncated MEC-17 or the MEC-17 C-terminus alone could be performed to determine whether or not the C-terminus is important for MEC-17’s interaction with other proteins. Such interactions might be important for proper sub-cellular localization or some other process essential to proper MEC-17 function.

The *mec-17(u265)* phenotype has not been characterized to the same extent as that of *mec-17(ok2109)*. It would therefore be useful to make the *mec-17(u265); atat-2* strain and determine if the mechanosensory defect becomes as severe as that seen in the *mec-17(ok2109); atat-2* strain. If an equally severe mechanosensory defect were observed in *mec-17(u265); atat-2* animals, this might suggest that it is indeed a lack of α-tubulin (or other substrate) acetylation that forms the basis of *mec-17* mechanosensory defects. *mec-17(ok2109)* animals display a marked reduction in the overall number of TRN MTs though those present are still acetylated presumably by ATAT-2. The less severe mechanosensory defect observed in *mec-17(u265)* animals as compared to *mec-17(ok2109)* might be attributable to a less dramatic loss of TRN MTs. Such a determination can only be made by electron microscopy since antibody staining was inadequate to illustrate MT loss in the *mec-17(ok2109)* strain. These experiments are ongoing.

It is unclear if MEC-17 or ATAT-2 target proteins other than α-tubulin for internal lysine acetylation. Shida et al. (2010) showed that MEC-17 fails to acetylate histones in vitro though
this does not rule out other substrates. The full complement of *C. elegans* proteins regulated by internal lysine acetylation is unknown though it probably numbers in the hundreds based on estimates of internally acetylated proteins in other systems. Results in other systems indicate that likely candidates might include enzymes involved in metabolism (Wang et al., 2010; Zhao et al., 2010) or transcription factors (Daitoku et al., 2004; Yang et al., 2005). Methods similar to those employed in other systems to identify internally acetylated proteins could be applied to *C. elegans* though there might be complications based on the fact that only a small minority of *C. elegans* cells express *mec-17* or *atat-2*. Sorted TRNs could be used to obtain a more homogenous tissue sample though it might be difficult to obtain enough sorted cells for biochemical procedures. The *C. elegans* FOXO transcription factor DAF-16 is regulated by acetylation with its deacetylation depending upon SIR-2.1 activity (Giannakou et al., 2004) and initial results seemed to indicate that *daf-16* deletion mutants may suppress the *mec-17(ok2109)* morphology but not mechanosensory defects (this result in currently being replicated). Should the *daf-16* induced suppression of *mec-17(ok2109)* morphology defects prove to be true, it would lend strong support to the hypothesis that MEC-17 has other acetylation substrates and that the morphology and mechanosensory defects are independent phenomena. The *daf-16* transcriptome (Ron Tepper thesis, 2010) is known and likely morphology altering candidates could be targeted for further study.
MEC-2 Is Recruited to the Putative Mechanosensory Complex in C. elegans Touch Receptor Neurons through Its Stomatkin-like Domain

Shifang Zhang, Johanna Arnadottir, Charles Keller, Guy A. Caldwell,1 C. Andrea Yao,* and Martin Chalfie*
Department of Biological Sciences
Columbia University
New York, New York 10027

Summary
Background: The response to gentle body touch in C. elegans requires a degenerin channel complex containing four proteins (MEC-2, MEC-4, MEC-6, and MEC-10). The central portion of the integral membrane protein MEC-2 contains a stomatin-like region that is highly conserved from bacteria to mammals. The molecular function of this domain in MEC-2, however, is unknown.
Results: Here, we show that MEC-2 colocalizes with the degenerin MEC-4 in regular puncta along touch receptor neuron processes. This punctate localization requires the other channel complex proteins. The stomatin-like region of MEC-2 interacts with the intracellular cytoplasmic portion of MEC-4. Missense mutations in this region that destroy the interaction also disrupt the punctate localization and degenerin-regulating function of MEC-2. Missense mutations outside this region apparently have no effect on the punctate localization but significantly reduce the regulatory effect of MEC-2 on the MEC-4 degenerin channel. A second stomatin-like protein, UNC-24, colocalizes with MEC-2 in vivo and coimmunoprecipitates with MEC-2 and MEC-4 in Xenopus oocytes; unc-24 enhances the touch insensitivity of temperature-sensitive alleles of mec-4 and mec-6.
Conclusion: Two stomatin homologs, MEC-2 and UNC-24, interact with the MEC-4 degenerin through their stomatin-like regions, which act as protein binding domains. At least in the case of MEC-2, this binding allows its nonstomatin domains to regulate channel activity. Stomatkin-like regions in other proteins may serve a similar protein binding function.

Introduction
Mechanosensation underlies such diverse senses as touch, hearing, balance, and proprioception [1]. Compared with what is known for other senses, we know relatively little about the molecular machinery that transduces mechanical stimuli: what molecules constitute the mechanosensory machinery, how they are organized, and how they function. Genetic studies of mechanosensory transduction in C. elegans, Drosophila, zebrafish, and mouse, however, have begun to identify molecules that are either the components of such mechanosensory machinery or necessary for developing specialized mechanosensory cells and circuits. These studies suggest that ion channels formed from either degenerin/epithelial sodium channel (DEG/ENaC) or transient receptor potential (TRP) proteins are essential for mechanosensation [2–8]. Most models of mechanosensation suggest that mechanosensitive channels are activated by mechanical tension either through alteration of membrane properties or through coupling with cellular and/or extracellular components [1, 9].

In C. elegans, six touch receptor neurons sense gentle body touch [10]. These cells, whose sensory processes are closely attached to the body wall, are distinguished from other C. elegans neurons because they are packed with 15–20 filament microtubules and have prominent extracellular matrix. Saturation genetic screens for touch-insensitive mutants have identified several genes needed for the function of these cells. Four genes encode membrane-associated proteins that interact with each other and form an amiloride-sensitive sodium channel complex in heterologous cells [2, 3, 11, 12]. These proteins are the two degenerin channel subunits MEC-4 and MEC-10, the stomatin-like protein MEC-2, and the paraxonase-like protein MEC-6. Two other proteins needed for touch neuron function, the α-tubulin MEC-12 and β-tubulin MEC-7, are needed to form touch neuron-specific 15-filament microtubules [13, 14]. These other proteins, a collagen (MEC-5) and two Kunitz and EGF domain-containing proteins (MEC-1 and MEC-9), are components of the extracellular matrix [L. Emtage et al., in press] [15]. Here, we report on the roles that different domains of MEC-2 have in its localization and function. The mec-2 gene encodes a 481 amino acid polypeptide needed for touch sensitivity [16]. The sequence from amino acid 114 to 363 is 64% identical to human stomatin, a protein that has been implicated in regulating red blood cell conductance [17]; the sequences that lie N- and C-terminal to the stomatin-like region are unique to MEC-2. MEC-2 activates the MEC-4 degenerin channel in Xenopus oocytes and coimmunoprecipitates with the other members of the touch receptor degenerin complex [11]. Previous research has suggested that the MEC-2-specific N terminus is needed for the distribution of MEC-2 along the touch receptor processes [16]. Here we show that MEC-2 interacts in vitro and colocalizes in vivo with MEC-4 through its stomatin-like domain. The MEC-2-specific C-terminal domain is required for self association. A second protein, UNC-24, which has an N-terminal stomatin-like region and C-terminal domain similar to nonspecific lipid transfer proteins [18], also colocalizes and coprecipitates with components of the complex. Functionally, mutation of unc-24 enhances the touch insensitive phenotype produced by temperature-sensitive mec-4 and mec-6 alleles.

Results
We generated separate rabbit polyclonal antibodies against the MEC-2-specific N terminus and the remain-
Figure 1. MEC-2 Localization
The right panel shows the enlarged portion corresponding to each boxed area on the left.
(A) MEC-2 puncta in PLM (lateral), I and PVM (ventral) processes. The average distances
between puncta in different touch receptor neuron processes were ALM: 3.4 ± 0.2 µm
(mean ± SEM, n = 12), PLM: 4.3 ± 0.2 µm (n = 16), AVM: 3 ± 0.2 µm (n = 16), and PVM:
2.3 ± 0.1 µm (n = 10).
(B) MEC-2 (red) and MEC-4::YFP (green) puncta colocalize in an ALM touch receptor
neuron in a young adult.
(C) The regular punctate pattern of MEC-2 in ALM processes is disrupted in mec-4(e253),
mec-6(e950), and mec-10(e20) young adults.
(D) MEC-4::YFP puncta in an ALM process are not affected in a young mec-2(b37) adult.

The recruitment of MEC-2 to the degenerin complex

der of the protein and purified them by preabsorption
to extracts from animals with the mec-2 null mutation
u37. These antibodies bound exclusively to the six touch
receptor neurons in a punctate pattern that depended
on the presence of MEC-2 (Figure 1). Previously, mec-2
β-galactosidase and GFP reporters driven by 3.5 kb of
upstream promoter sequence were expressed in these
cells plus additional neurons in the head and tail [16].
We confirmed this additional expression with a full-
length GFP protein fusion by using 16.5 kb of mec-2
genomic DNA (including the same 2.5 kb of 5' upstream
sequence). In contrast a GFP promoter fusion with just
the 5' upstream sequence was only expressed in the
touch receptor neurons (data not shown). Together,
these data suggest that the additional gene expression
is controlled by sequences outside this 5' upstream
region (perhaps in one or more of the large mec-2 in-
trons), but this expression does not lead to detectable
amounts of protein. The exclusive production of MEC-2
in six touch cells is consistent with the touch insensitive
phenotype, the only known defect, of mec-2 mutants.

The MEC-2 puncta are regularly spaced along the
touch receptor processes with the average spacing
between puncta being larger in the posterior lateral pro-
cesses than in the ventral processes (Figure 1A). The
MEC-2 puncta colocalized with those from a full-length
MEC-4::YFP fusion (Figure 1B), supporting the hypothe-
sis that MEC-2 and MEC-4, which functionally interact
and coimmunoprecipitate in Xenopus oocytes [11], form
a complex in vivo along the touch processes.

All three other components of the putative mechanos-
sensory channel complex (MEC-4, MEC-6, MEC-10) are
needed for the localization of MEC-2 in puncta; dis-
persed rather than regular punctate staining was seen
in mec-4(u253), mec-6(u450), and mec-10(u20) animals
(Figure 1C). In contrast, the null mec-2(u37) mutation
did not disrupt the punctate localization of a full-length
MEC-4::YFP fusion protein (Figure 1D). Thus, MEC-2
appears to be recruited to the degenerin channel com-
plex by the other components. Although MEC-2 binding
to MEC-6 may be important in this recruitment, the effect
of the mec-6 mutation is also likely to be indirect be-
cause mec-6 is needed for MEC-4 localization [12].

We have identified mutations in at least two mec-2
alleles (two had been identified previously) [16, 18],
Figure 2A and Table 1). Twenty-three of the 47 sequenced
alleles are missense mutations; 16 are nonsense muta-
tions; six are splice junction mutations; and two are
transposon insertions. Twenty-one of the missense mu-
tations are located within the stomatin-like domain (Fig-
ure 2A). In contrast to the wild-type protein or the prod-
uct from the remaining two missense mutations, the
MEC-2 protein from missense alleles gave a more-dis-
persed immunostaining with the MEC-2 puncta being
reduced or eliminated (Figure 2B). The touch insensitive
phenotype of mec-2 mutants ranges from weak to com-
plete touch insensitivity [10, 20]. For the missense muta-
tions within the stomatin-like region, the severity of the
touch insensitive phenotype correlated with the disrup-
tion of the punctate pattern (Figure 2C) but not with the
position of the mutation within the domain (data not
shown). Taken together this correlation, the presence
of MEC-2 puncta in animals with mec-2 mutations out-
side the stomatin-like region, and the presence of
MEC-4 puncta in the absence of MEC-2 suggest that
association of MEC-2 with the degenerin channel com-
plex occurs through the stomatin-like region.

MEC-2 and MEC-4 produced in Xenopus oocytes or
CHO cells commounprecipitate [11, 12]. We found that myc-tagged MEC-4 immunoprecipitated the stomatin-like region of MEC-2 (amino acids 114–363, tagged with HA) in Xenopus oocytes (Figure 3A). Because MEC-2 and stomatin are thought to be anchored in but not cross the plasma membrane [16, 21], MEC-2 is likely to interact with the cytoplasmic or membrane spanning regions of MEC-4. Both structural modeling and in vivo functional analysis of the N-terminal portion of MEC-4 (amino acids 1–108) suggest that this region is the possible site for cytoplasmic interaction with other proteins [16, 23–24], GST-MEC-4(1–108), but not GST alone, pulled down full-length MEC-2 and the MEC-2(88–379) stomatin-like region when these proteins were expressed in bacteria (Figures 3B and 3C). This region no longer bound GST-MEC-4(1–108) if the MEC-2 sequence contained any of the changes (R184C, A207T, and P357L) found in the three missense mutations that most severely disrupted the MEC-2 punctate pattern in vivo (Figure 3D). This result suggests that the loss of interaction with the degenerin channel causes the mutant MEC-2 proteins to become distributed in a more diffuse pattern in vivo. These same mutations abolish at least 95% of MEC-2–increased MEC-4d current in Xenopus oocytes (Figure 2D), indicating that the interaction mediated by stomatin-like region is also essential for the regulatory function of MEC-2.

The interaction of the stomatin-like region of MEC-2 with MEC-4, however, is necessary but not sufficient for MEC-3 function because previous experiments showed that the stomatin-like region of MEC-2 or human stomatin had little effect on the MEC-4d current in Xenopus oocytes [11]. In addition, we found that two missense mutations outside the stomatin-like region, R385H (u26) and D387Y (u177), which virtually abolish touch sensitivity in vivo, reduced but did not abolish the activation of MEC-4d by MEC-2 in oocytes (Figure 2D).

Human stomatin has been reported to oligomerize in red blood cells [25], and interallelic complementation within mec-2 suggested that MEC-2 also forms oligomers in vivo [10, 20]. We expected, therefore, that the stomatin-like region of MEC-2 would be sufficient to associate with MEC-2 when both proteins were expressed (without MEC-4) in Xenopus oocytes. We found, however, that the stomatin-like region was insufficient; the MEC-2–specific C terminus was also needed to immunoprecipitate full-length MEC-2 (Figure 3E). (We could not test whether the C terminus was sufficient for oligomerization because we could not express it in oocytes.)

MEC-2 is not the only stomatin-like protein that is expressed in the touch receptor neurons. UNC-24 is also expressed in these and other cells (T. Barnes and S. Hekimi, personal communication) [26]. If the stomatin-like region of UNC-24 also localizes the protein to the
### Table 1. Point Mutations in mec-2

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Mutation positions are numbered according to the mec-2 genomic sequence (GenBank Accession U26736) and the full-length MEC-2 amino acid sequence (GenBank Accession Q27435, Huang et al., 1995). The asterisk indicates a stop codon.

degenerin complex, we would expect a punctate pattern for an UNC-24::GFP fusion in the touch receptor neurons. The fusion is indeed distributed in puncta that colocalize with those of MEC-2 (Figure 4A). unc-24 mutants are touch sensitive, but we could uncover a role for unc-24 in touch sensitivity using sensitized backgrounds [19]. Specifically, unc-24(e138) enhanced the touch insensitive phenotype of mec-6(u247) animals at 15°C and mec-4(u465) at 21°C (Figure 4B). unc-24 mutants also displayed a slight harsh touch phenotype in the head (Figure 4C), but this was not enhanced in the mec double mutants.

Expression of UNC-24 in Xenopus oocytes did not replace MEC-2 with regard to the increase of the MEC-4d current (Figure 5A). Moreover, addition of UNC-24 to MEC-4d and MEC-2 caused a 50.6% reduction in the current at ~85 mV. The significance of this partial reduction is unclear. Nonetheless, UNC-24 interacts with both of these proteins in Xenopus oocytes. HA-tagged UNC-24, the stamin-like region of UNC-24, but not the C-terminal lipid transfer domain immunoprecipitated MEC-2 (Figure 5B) and Myc-tagged MEC-4d (Figure 5C). Neither MEC-2 nor MEC-4 was needed for or interfered with the binding of the other protein to UNC-24 (data not shown). These results suggest that all three proteins can form a complex and that the reduction in the MEC-4/ MEC-2 current does not result from competition for binding of MEC-2 and UNC-24.

**Discussion:**

Electrophysiological studies and genetic interactions [19] have suggested that a degenerin channel complex formed with MEC-2, MEC-4, MEC-6, and MEC-10 underlies mechanosensation in the touch receptor neurons. Our results further support this model by showing that MEC-2 colocalizes with MEC-4 in vivo. Because MEC-4 puncta form in the absence of MEC-2, MEC-2 does not
initiate complex formation. The production of the MEC-2 punctate pattern, however, does require the other proteins of the degenerin complex, suggesting that it may be recruited late in the formation of the complex. In contrast, the distribution or maintenance of MEC-4 along the process requires MEC-8 [12], a difference that may explain why touch cell degeneration caused by dominant mutations in MEC-4 requires MEC-6 but not MEC-2 [27].

The association of MEC-2 with the complex (and of MEC-2 with the N terminus of MEC-4 in vitro) depends on the stomatin-like region of MEC-2. The distribution of MEC-2 along neuronal processes does not require this region [16] or the other members of the degenerin complex; instead the MEC-2-specific N-terminal region is sufficient for the dispersal of MEC-2 [16]. MEC-2-specific termini appear to be important for the activation of the channel complex [11] and for the oligomerization of MEC-2 (this paper). We propose that the stomatin-like domain of MEC-2, by interacting with the degenerin channel, positions the N and C termini of MEC-2 in the complex so they can activate channel activity. Several stomatin-like proteins in C. elegans (STL-1 and STO-5; S.Z. and M.C., unpublished observations), bacteria (HfIK and HfIC), yeast (Pho2p), and mammals (podocin) have additional domains that could be similarly localized through the binding of their stomatin-like regions. Roselli et al. [28] recently reported that many mutations in the stomatin-like region of podocin prevented its localization to the plasma membrane. The colocalization of UNC-24 with MEC-2 in the touch receptor neurons, the enhancement of the mechanosensitive defect in mec-4ts and mec-4ts animals, and the immunoprecipitation of MEC-2 and MEC-4 by UNC-24 suggest that UNC-24 is also a member of the degenerin complex. Our data suggest that the stomatin-like domain of this protein brings it to the complex. Other eukaryotic stomatin proteins may also interact with DEG/ENaC proteins.

Both prokaryotes and eukaryotes have stomatin-like genes [29]. The C. elegans genome has ten stomatin-
like genes [30] of which only mec-2, unc-24, and unc-1 have been characterized. The unc-1 gene is widely expressed in the nervous system, and it has been implicated in anesthetic sensitivity [31, 32]. Promoter-containing transcriptional GFP reporters for the six of the other stomatin-like genes express predominantly in neurons (S.Z. and M.C., unpublished data). One possibility is that these proteins interact with many other degenerin proteins in C. elegans.

The ability to bind and regulate other proteins appears to be a conserved feature of stomatin-like proteins. As with the degenerin channel complex in the C. elegans touch receptor neurons, some complexes contain more that one stomatin-like protein. The bacterial stomatin-like proteins HIKK and HIKC complex with a metalloprotease, FtsH, and regulate its activities [33]. The yeast stomatin-like proteins Pho1p and Phb2p complex with the m-AAP protease and negatively regulate its activity [34]. In C. elegans the wild-type unc-24 gene is needed for the proper localization of UNC-1 [35]. In rat, stomatin associates with the GLUT1 glucose transporter and appears to regulate glucose transport in red blood cells [36, 37].

Mammalian stomatin proteins are associated with a cholesterol-rich, Triton X-100-insoluble fraction from the plasma membrane lipid rafts. Human stomatin is abundant in erythrocyte lipid rafts [38]; it is thought to act as a protein scaffold at the cytoplasmic surface [39]. In mouse, the Stomatin Related Offactory protein (SRO) of offactory sensory neurons is associated with caveolin-1, a protein associated with lipid rafts [40]. Finally, human podocin, a stomatin-like protein expressed in kidney podocytes, is also associated with lipid rafts [41]. Building on these observations, we suggest that MEC-2, UNC-24, and by extension the mechanosensory channel complex, may be associated with a specialized lipid environment that may influence channel activity.

Experimental Procedures

Strain Growth and Characterization
C. elegans strains were cultured at 20°C unless otherwise indicated [42]. Wild-type (N2) [43], mec mutants [10, 43], and unc-24(n138)
References:


