THE MECHANISM AND SIGNIFICANCE OF MICROTUBULE ACETYLATION

by

JYOTHI SHILPA AKELLA

(Under the Direction of JACEK GAERTIG)

ABSTRACT

Lysine acetylation of α-tubulin is a conserved post-translational modification that is present on microtubules in many structures including cilia, neuronal projections and the spindle apparatus. The enzyme that acetylates microtubules and the significance of microtubule acetylation has been unknown. *mec-17* is a gene highly expressed in the touch receptor neurons of *C. elegans* and was identified in a screen for genes required for touch sensation. *mec-17* encodes a highly conserved protein with distant homology to the GCN5 family of histone acetyltransferases. MEC-17 is expressed in all organisms with acetylated microtubules. Using complementary genetic approaches in *Tetrahymena thermophila, Danio rerio* and *C. elegans,* we showed that MEC-17 is an α-tubulin acetyltransferase whose function is conserved among diverse species. Surprisingly, the catalytic activity of MEC-17 as an acetyltransferase for the K40 residue of the a-tubulin expressed in the touch receptor neurons, MEC-12, is not required for touch sensation. *C.elegans* touch receptor neurons are filled with wide diameter microtubules with 15 protofilaments that were suggested to be specifically adapted for touch sensation. Other

groups showed that the enzymatic function of MEC-17 is not required for touch sensation. We used a suppressor strategy to explore the role of MEC-17 in touch sensation. We identified *sma-2* as a suppressor of *mec-17* mediated touch insensitivity. *sma-2* encodes a receptor activated SMAD in the TGF β pathway in *C.elegans* that plays a critical role in the regulation of body size. Using a mutant in another pathway that contributes to size regulation in *C.elegans*, we show that touch insensitivity in mutants with severe microtubule defects can be rescued by making the animal smaller, presumably because the axon of the touch receptor neuron is shorter. We also show that the suppression of microtubule defects by animal size reduction requires a functional mechanosensitive channel containing MEC-4. We propose that microtubules are needed for touch sensation when the axon reaches a certain length. Microtubules could play a role in connecting the individual ion channels, perhaps by making the membrane more rigid. Alternatively, microtubules could be required for propagation of the touch stimulus along the length of the axon to the synapse.

INDEX WORDS: Microtubules, Tubulin Post-translational modifications, Acetylation, Touch Receptor Neurons, 15 protofilament microtubules, Touch sensitivity.

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DEDICATION

I dedicate this dissertation to my beloved uncle and grandmother.

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ABBREVIATIONS

TRNs- Touch Receptor Neurons

PTMs- Post Translational Modifications

Pf- Protofilament

TTL- Tubulin Tyrosine Ligase

TTLL- Tubulin Tyrosine Ligase Like

SSM- Selective Stabilization Model

CLDM- Cell Length Dependent Model

EB-End Binding

AIS- Axon Initial Segment

CCP- Cytosolic Carboxypeptidase

CCPP- Cytosolic Carboxypeptidase

MRC- Mechanoreceptor Current

K- Lysine

Y-Tyrosine

R-Arginine

Q-Glutamic acid

D-Aspartic acid

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction to microtubules

Microtubules are long and flexible cytoskeletal hollow tubes that are present in all eukaryotic cells. Microtubules are polymers of α-and β-tubulin heterodimers. Tubulin was originally identified as a colchicine-binding protein that was highly abundant in cilia, mitotic apparatus, brain tissue and spermBorisy and Taylor [1,2,3]. α- and β-tubulin are both GTP-binding proteins but only β-tubulin has a GTPase activity. The α/β tubulin dimers associate in a head to tail fashion to form a longitudinal protofilament. Protofilaments interact laterally to form a microtubule. The number of protofilaments in a microtubule varies depending on the microtubule type and cell type[4-6]. The structural basis of the protofilament diversity will be discussed in the last part of this thesis. While microtubules are a key element of the cytoskeleton of eukaryotic cells, bacteria have a homolog of tubulin called FtsZ, a GTPase that forms the contractile ring and is required for cell division[7]. Thus, tubulins are among the most ancient proteins. Microtubules are required for a variety of functions including cell division, cell motility, intracellular transport, and cell differentiation including the polarization of neurons.

Microtubule structure, assembly and dynamic instability:

Microtubule structure:

The 3D structure of the α/β tubulin heterodimer was obtained using electron tomography of zinc-induced tubulin sheets[8,9]. The 3D structure of a microtubule was later reconstructed at a 8A resolution [10]. Each tubulin monomer has three main domains: 1) an N-terminal nucleotide-binding domain (residues 1-206), 2) an intermediate region (residues 206-381) and 3) a helical C-terminal region. The nucleotide-binding pocket is formed by a number of loops (T1-T6) at the N-terminal end of the core helix H7. The core helix connects the nucleotide-binding domain with a second domain that comprises three helices (H8-H10) and a mixed β sheet (S7-S10). The intermediate domain contains loops that are present on the inside surface of microtubules and contribute to lateral protofilament interactions. This region also contains the M-loop that makes up the taxol-binding pocket of β tubulin. Finally, the C-terminal region is formed by two antiparallel helices H11 and H12 that are located on top of the N terminal and intermediate domains. The C-terminal outside surface is the primary interface for binding of microtubule-associated proteins (MAPs) including motor proteins: dyneins and kinesins.

My dissertation is focused primarily on the mechanism and function of a biochemical process, lysine acetylation, which occurs on the luminal surface of the microtubule. There are important differences in the polypeptide structure between the luminal and outside surface of the microtubule. The external surface of microtubules is highly ordered due to the presence of a number of helices except for the most C-terminal region (known as the tubulin tail) that is highly acidic (for this reason sometimes referred

to as the E-hook). The external surface of microtubules forms the binding surface for motor proteins and so called structural MAPs (e.g. tau and MAP2 abundant in neurons), proteases and antibodies[11]. On the other hand, the internal or luminal surface of the microtubule comprises mostly of loops rather than helices[11]. These loops include the H1-S2 loop and the H7-S9 loop also known as the microtubule or the M loop (the taxol binding site)[11]. The M loop interacts with the H1-S2 and H2-S3 loops of the adjacent protofilament acting like glue that holds the protofilaments together. Importantly, the H1-S2 loop is where the K40 residue of α-tubulin is present[11]. This is the residue that undergoes reversible acetylation, the topic of this dissertation. *In vivo*, microtubules can exist in a number of conformations with different numbers of protofilaments. Most commonly microtubules have 13 protofilaments when assembled *in vivo*. However, microtubules assembled *in vitro* have a variable number of protofilaments ranging from 8 to 17[12] indicating that the same tubulin can form tubes with a variety of diameters and that *in vivo,* mechanisms must exist that control the fidelity of microtubule protofilament organization. While most microtubules are made of 13 pf in vivo, there are important exceptions. For e.g., doublet microtubules found in cilia have 13 protofilaments in the A tubule and 10 protofilaments in the B tubule that is fused to the side of tubule A[13]. Triplet microtubules in centrioles/basal bodies have one complete tubule A and two incomplete 10 protofilament tubules B and C[14]. Microtubules that have 15 protofilaments are rare but are found in the touch receptor neurons of *C. elegans* (a subject of this thesis)[15] but also in macronuclei of ciliates *Tetrahymena* and *Paramecium* and in the mammalian cochlear epithelial cells[16]. The N-terminal part of the M loop is suggested to act as a hinge that helps accommodate varying protofilament number due to its flexibility[11].

Microtubule polarity

Microtubules have an intrinsic polarity with one end of the microtubule having an exposed α-tubulin (called minus) and the other end having a terminal a β-tubulin (called plus)[17]. *In vivo*, the minus end is often anchored to a "microtubule organizing center" (MTOC) such as the centrosome or basal body[18]. MTOCs are places from which microtubules grow out that also function to anchor the minus ends of microtubules. However, microtubules can also form without visibly associating with an MTOC. For example, plant cells and mammalian oocytes lack obvious MTOCs and assemble microtubules[18]. One example where microtubules form in the cytoplasm is the axon of neurons and this will be discussed later. Regardless of the location, microtubules are nucleated by a complex whose main component is a ring of another tubulin called g-tubulin, that acts as a nucleator and a cap of the minus end (reviewed in[18]). The end of the microtubule with the β-tubulin exposed is called the plus end. For microtubule polymerization to occur, a critical concentration (Cc) of tubulin dimers needs to be reached. *In vitro*, tubulin can be added to both plus and minus ends. However, the Cc for the plus end is much lower as compared to the Cc for the minus end that requires a very high concentration of free tubulin dimers to be extended[17]. Thus practically, microtubules grow primarily at the plus end. *In vivo,* the minus end can be anchored or it can depolymerize. In vertebrates, a protein called Patronin keeps the minus end stable by preventing its depolymerization[19]. Due to its lower Cc, the plus end is the end that is extended when the microtubule polymerizes[17]. Microtubule polarity is not only important during end assembly. Because the surface of the microtubule is polarized due to a uniform arrangement of tubulin dimers, this imparts microtubules with an inherent

polarity that is a key determinant that regulates the activity of motor proteins that move to either plus or minus end.

Dynamic instability

A growing plus end of microtubules has a layer of newly added dimers on which the GTP on β-tubulin has not yet been hydrolyzed (the so called GTP cap). The size of the GTP cap varies from 13-200 subunits in vitro to ~750 subunits in vivo[20] and depends on the rate of addition of new subunits and the rate of hydrolysis of GTP on β-tubulin of dimers that are already in the lattice. The GTP cap is lost when the addition of new dimers to the plus end is too slow and cannot exceed the rate of GTP hydrolysis. When GDP subunits are present at the very end of the microtubule, plus end depolymerization takes place. There is a structural difference between the dimers containing GTP and those containing GDP on β-tubulin. GTP dimers are straight along the longitudinal axis while in the GDP dimers, α - and β-tubulin slightly tilt forming bent dimers[21]. When the GTP cap is gone, the microtubule depolymerizes as a result of weakening of lateral interactions of GDP tubulin protofilaments that curl outward (and eventually get peeled off). GTP tubulin protofilaments are straight and hence have stronger lateral interactions that provide a more stable landing surface for incoming tubulin[22]. Importantly, at the free tubulin concentrations not far above the Cc, the plus end fluctuates between the periods of assembly and disassembly and this phenomenon is called dynamic instability and occurs both *in vivo* and *in vitro*[17]. A "catastrophe" is a transition of a plus end between polymerization and depolymerization and a "rescue" is a transition from depolymerization to polymerization. The pattern of dynamic instability appears

stochastic; it is not possible to predict the future behavior of an individual microtubule. Presumably, dynamic instability reflects fluctuations in the concentration of tubulin dimers around the plus end that would oscillate above or below the Cc[17]. However, *in vivo* there are proteins that promote either the polymerization (such as XMAP215 a plus end polymerase)[23] or depolymerization (such as kinesin-13)[24]. Dynamic instability is believed to play an important role in searching of a space by growing ends of microtubules, that facilitates making contacts with targets of microtubule ends (such as kinetochores of chromosomes during mitosis or adhesion plaques in moving cells) or simply allows for microtubules to expand into an area of the cytoplasm free from obstacles[25]. This simple consequence of dynamic instability plays a role in the forming axon of neuron and will be discussed later.

Regulation of dynamics of microtubules *in vivo*

A large number of microtubule associated proteins (MAPs) modify microtubule dynamics. These include structural MAPs such as MAP1a, MAP2 and Tau and also tip regulators such as XMAP215, kinesin-13 and CLASPs.

One of the critical regulators of microtubule dynamics *in vivo* is XMAP215. XMAP215 was initially identified in *Xenopus* eggs where it was observed that incubating centrosomes with XMAP215 greatly increased the rate of microtubule polymerization to around 10 fold [23]. More recently, it was identified that XMAP215 is a processive microtubule polymerase. Through *in vitro* assays, it was observed that XMAP215 binds to the ends of microtubules and speeds up the addition of multiple tubulin subunits without falling off[26].

Another key regulator of microtubule dynamics is kinesin-13. Kinesin-13 is an unusual kinesin that uses the energy from ATP hydrolysis to destabilize the protofilaments and cause end depolymerization[24]. *In vitro* kinesin-13 reaches microtubule ends by diffusing through the microtubule lattice[24]. As explained earlier, curved protofilaments depolymerize due to a weakening of lateral interactions between neighboring subunits. Interestingly, the microtubule depolymerizing activity of kinesin-13 is affected by a post-translational modification of tubulin called detyrosination that is based on removal of the C-terminal Tyrosine (Y) residue[27,28]. *In vitro,* tyrosinated microtubules (the native unmodified state) were shown to be more susceptible to microtubule depolymerization by kinesin-13[28]. More recently, it was found that the suppression of the microtubule depolymerization by kinesin-13 is one of the first steps in microtubule remodeling during axon regeneration[29]

Several classes of proteins that bind to the plus end of microtubules exist. These proteins called +TIPs include the end binding (EB) proteins and CLASPs or CLIP-170 Associated Proteins. +TIP proteins influence dynamic instability[30]. Several +TIP binding proteins have been implicated in selective microtubule stabilization including CLASPs and CLIP-170[31]. +TIPs mediate linking microtubules to intracellular structures including the actin cytoskeleton[32]. It is interesting to note that a +TIP CLIP-170 preferably binds to subunits that contain tyrosinated tubulin that also tends to be enriched near the microtubule ends[27]. These interactions play an important role during axonal growth and pathfinding[33].

Another important class of MAPs that regulate microtubule dynamics *in vivo* are the structural MAPs such as Tau and MAP2. Structural MAPs promote microtubule assembly and also regulate bundling of microtubules. *In vitro*, incubating microtubules with Tau promoted microtubule assembly and stability[34]. Structural MAPs have a conserved C-terminal microtubule binding domain and a variable N-terminal projection domain. The size of the bundle generated by specific structural MAPs is dependent on the length of the N terminal projection domain[35]. *In vivo*, phosphorylation of these MAPs reduces their affinity to microtubules. Phosphorylation of structural MAPs is one of the most common converging points for many proteins involved in specification of neuronal polarity; for example, a major kinase that regulates neuronal polarity[36], GSK3β, phosphorylates Tau[37]. Structural MAPs also contribute to polarity by being enriched in one compartment in the cell and not in the other for e.g., Tau is enriched in axons whereas MAP2 is enriched in dendrites[38]. Mice with a knockout of Tau are nearly normal, but Tau is partially functionally redundant with MAP1B and both contribute to neuron elongation and migration[39]. Tau undergoes hyperphosphorylation in the brains of Alzheimer's patients and contributes to the disease phenotype[40]. Mutations in Tau lead to tauopathies, neurodegenerate disorders that are caused by accumulation of abnormal Tau protein aggregates.

Tubulin isotypes

Microtubules differ greatly in their tubule structure (singlet, doublet or triplet), protofilament number, length, orientation, extent of bundling, flexibility and the level of dynamics. From the point of interest of this thesis, it is important to consider that

microtubules in each neuronal compartment (cell body, dendrite and axon) have vastly different properties. In fact, how microtubules can be differentially controlled in different neuronal compartments is largely an unanswered question. Thus, I will discuss briefly the mechanisms that contribute to the diversity of microtubules.

One of the ways to make different populations of microtubules is by using multiple isotypes (primary amino acid sequence variants) of tubulin. Many organisms including mammals express multiple isotypes of α - and β-tubulin, often in the same cell type (reviewed in[41]). The tubulin isotypes differ mostly in the sequence of their C-terminal tails[42]. The tubulin tail domains play a critical role in interactions with MAPs and are essential for survival based on a Tetrahymena study[43] [44]. There are a growing number of diseases that are caused by mutations in a single tubulin isotype. Mutations in TUBA1A α -tubulin cause lissencephaly, a "smooth brain" disease associated with abnormal neuron migration[45]. Mutations in TUBA1A, TUBB2B and TUBB3 cause polymicrogyria (a brain with an excessive number of folds) [45-47]. A mutation in TUBB5 causes microcephaly (small brain) [48]. At the cell level, a mutation in TUBB3 leads to disturbances in microtubule dynamics, interactions with kinesin motors and strongly affects the axon guidance[47]. A mutation in the other β-tubulin isotype that is highly expressed in the nervous system; TUBB2B causes defects in cell migration that are not observed in TUBB3 mutants[46]. TUBB2B and TUBB3 are 90% homologous except for major differences in their C terminal tails. These many observations showing that mutations in specific tubulin isotypes lead to unique phenotypes open up a possibility that isotypes have evolved to perform non-identical functions. In PC-12 neuronal-type cells grown *in vitro,* out of the five isotypes of b-tubulins available, isotypes I and II were highly

expressed in differentiating neurites[49]. However, the isotype effects can also be explained by their unique patterns of expression and different quantities of each isotype in the common tubulin pool. A functional proof that a neural isotype has a specialized function has not been performed yet for a mammalian model. An ideal experiment would involve swapping the coding region of an important isotype with the sequence of another isotype. Coding sequence-swapping experiments in fungi showed that their tubulin isotypes are functionally equivalent. In mice 3T3 cells, a chimeric tubulin with the amino terminal portion of chicken b- tubulin and the C terminal tail of *S.cerevisiae* co-assembled into microtubules with the endogenous tubulin showing that major sequence variations in the C-terminal tails of tubulin do not disrupt microtubule assembly[50]. However, there is one well-documented case of isotype non-equivalence. In *Drosophila* a testis-specific β-tubulin has evolved to make the axoneme in the sperm and its function cannot be substituted by another β-tubulin expressed in the same species[51]. One can therefore speculate that microtubules having different ratios of different tubulin isotypes differ in the dynamics or ability to interact with motors or other MAPs. While the role of tubulin isotype diversity in the mammalian nervous system remains unclear, *C. elegans* offers a potential case of tubulin isotype specialization.

mec-7 and *mec-12* encode b- and α -tubulins that are expressed mostly in the touch receptor neurons, the only cells that assemble unique 15 protofilament microtubules[4,52,53]. Mutations in either *mec-7* or *mec-12* make animals touch insensitive and many strong alleles also prevent the formation of 15 pf microtubules (instead the touch neurons assemble 11 pf microtubules that are typical in other cell types in *C. elegans*) [4]. However, as is the case of mammalian models, a formal proof of unique functionality of *mec-7* and

mec-12 based on a coding sequence swaps has not been performed. We will discuss the role of tubulin isoforms in the touch neurons of *C. elegans* further (see below).

Microtubule Post-translational modifications

Another way to generate functionally diverse microtubule populations is through selective post-translational modifications (PTMs) of tubulin subunits. The most common post-translational modifications of tubulin include glycylation , glutamylation , detyrosination , D2 (removal of EY from the C-terminus of a-tubulin and lysine acetylation (as reviewed in[54]). These PTMs are reversible. One of the ways PTMs contribute to microtubule diversity is by spatial differences in their levels of accumulation. These differences could be established between cell types, between different compartments of the same cell type or even on the same microtubule (as reviewed in[54]). This way, microtubules can be fine-tuned to perform different functions. An example of an accumulation of a modification only in a certain cell type is tubulin glycylation that is abundant in cells with cilia and mostly within the axoneme where it plays an important role in the assembly and maintenance of cilia [55,56]. An example of functional specialization between microtubules in different compartments of the same cell type is the accumulation of different post-translational modifications between axons and dendrites in neurons where dendrites are enriched in native unmodified tyrosinated tubulin whereas axons are enriched in acetylated, glutamylated and detyrosinated tubulin[42] . These differences impart different properties to microtubules in axons and dendrites. The differences between microtubules in axons and dendrites shall be discussed in great detail in the next section. Microtubule PTMs could be used to impart a

specific function to only one part of a microtubule for example, in the doublet microtubules of axonemes, glutamylation of tubulin is observed only on the B tubule where it regulates the activity of inner arm dynein[57].

One of the ways post-translational modifications of tubulin help achieve functional specialization is by regulation of the proteins that interact with microtubules such as motors and structural MAPs[54]. Most post-translational modifications of tubulin occur on the C terminal tails of a and b tubulin, which are present on the external surface of microtubules[11,54]. The external surface of microtubules forms the binding surface for many microtubule interacting proteins including motor proteins, severing and depolymerizing factors, structural MAPs and +TIP proteins[11 54]. However, α-tubulin K40 acetylation is unique in that it occurs inside the lumen of the microtubule[58]. This unique location of microtubule acetylation makes it puzzling to understand how a modification in the lumen is translated to an event outside the microtubule. Possibly, tubulin acetylation regulates an unknown luminal activity or affects the external surface indirectly. The factors that recognize the structural change imparted by microtubule acetylation (if there's any change to the external surface of microtubule) have not been identified yet. Below, I will discuss some of the most prevalent PTMs on microtubules and the specialized functions that they tune microtubules to perform. Later, I will focus on microtubule lysine (K40) acetylation, a unique biochemical modification of microtubules that is a major topic of this dissertation.

Post translational modifications on the C-terminal tails of tubulin

As noted earlier, the most prevalent PTMs that are observed on the C-terminal tails of α and β tubulin include detyrosination, glutamylation and glycylation. Tubulin is also subjected to other modifications including phosphorylation, methylation and sumoylation (as reviewed in [54]). Below, I will discuss some general aspects of tubulin PTMs. The roles of PTMs in neurons shall be discussed in detail in later sections of this chapter.

Some tubulin PTMs play important roles in the maintenance of microtubule stability and dynamics. In most eukaryotes, the C-terminal Yon a-tubulin is removed by an unknown enzyme in the process known as detyrosination that occurs on the microtubule[59]. The enzyme tubulin tyrosine ligase (TTL) puts back a Y on the C terminus tail of a-tubulin on an unassembled tubulin dimer[60]. Detyrosination of tubulin inhibits the tubulin depolymerizing enzyme MCAK kinesin-13. *In vitro,* MCAK kinesin-13 depolymerized tyrosinated microtubules more efficiently compared to detyrosinated microtubules[28]. Also, detyrosination inhibits a +TIP CLIP-170[27]. A knockout of TTL is embryonically lethal and disturbs neuronal development in a way similar to a knockout of MCAK kinesin-13[61].

Another important tubulin is polyglutamylation, a PTM based on generation of a branched peptide made of multiple Es anchored to a primary polypeptide E via its g-carboxyl group based isopeptide bond[62]. Polyglutamylation is mediated by enzymes related to TTL called TTLLs including TTLL1, TTLL4, and TTLL6[54]. *In vitro* and *in vivo*, polyglutamylated microtubules are a preferred substrate targeted by microtubule severing complexes spastin and katanin[63]. Overexpression of the tubulin

polyglutamylase enzyme TTLL-6 in HeLa cells led to a complete loss of the microtubule pool due to severing by spastin[63]. In axons, Tau protects the highly glutamylated microtubules from severing by spastin[42]. This once again points to the sophisticated machinery that cells have to impart select properties only to certain microtubules.

Another conserved PTM that plays a major role in the maintenance of cilia is tubulin glycylation, a PTM structurally similar to glutamylation except that the modification branched peptides are made of glycine. Tubulin glycylation is mediated by the enzymes TTLL3 (chain initiation/elongation) and TTLL10 (chain elongation)[54]. In *Tetrahymena thermophila,* removing TTLL3 leads to a mild shortening of cilia and slow growth[55]. shRNA against the tubulin glycylases TTLL3 and a closely related TTLL8 in mice destabilized ependymal cilia[56].

Tubulin PTMs also play a role in the regulation of motor activity. The beating cycle of cilia involves bending of axonemal microtubules generated by dynein arm motors. *Tetrahymena* and *Chlamydomonas* mutants lacking either TTLL6 or TTLL9 tubulin polyglutamylase show abnormal ciliary beating and are affected specifically in the activity of inner dynein arms that control the cilia waveform [57,64]. Interestingly, in both models, when the mutant axonemes were isolated and reactivated with ATP, the microtubules underwent sliding with increased velocities, suggesting that tubulin glutamylation acts as a "brake" in the cilium that limits sliding of microtubules[57]. Respiratory cilia in knockout mouse lacking TTLL1 tubulin glutamylase fail to generate a proper power stroke [65]. Male mice mutant in TTLL1 are also sterile and have disrupted sperm axonemes suggesting that tubulin glutamylation is also needed for proper assembly

of axonemal microtubules[66]. Moreover, in neurons, tubulins PTMs play critical roles in navigating motor proteins [67,68]. This helps in the maintenance of polarity.

General mechanism of lysine acetylation

Although lysine acetylation on histones and tubulin is very well known, this PTM is emerging as one of the most prevalent modifications *in vivo* with many substrates including metabolic enzymes, components of the cytoskeleton etc[69]. In fact, the number of proteins with lysine acetylation *in vivo* is now rivaling those that are phosphorylated and ubiquitinated[69]. Lysine acetylation is mediated by a K-acetyltransferase and the modification is removed by a deacetylase enzyme.

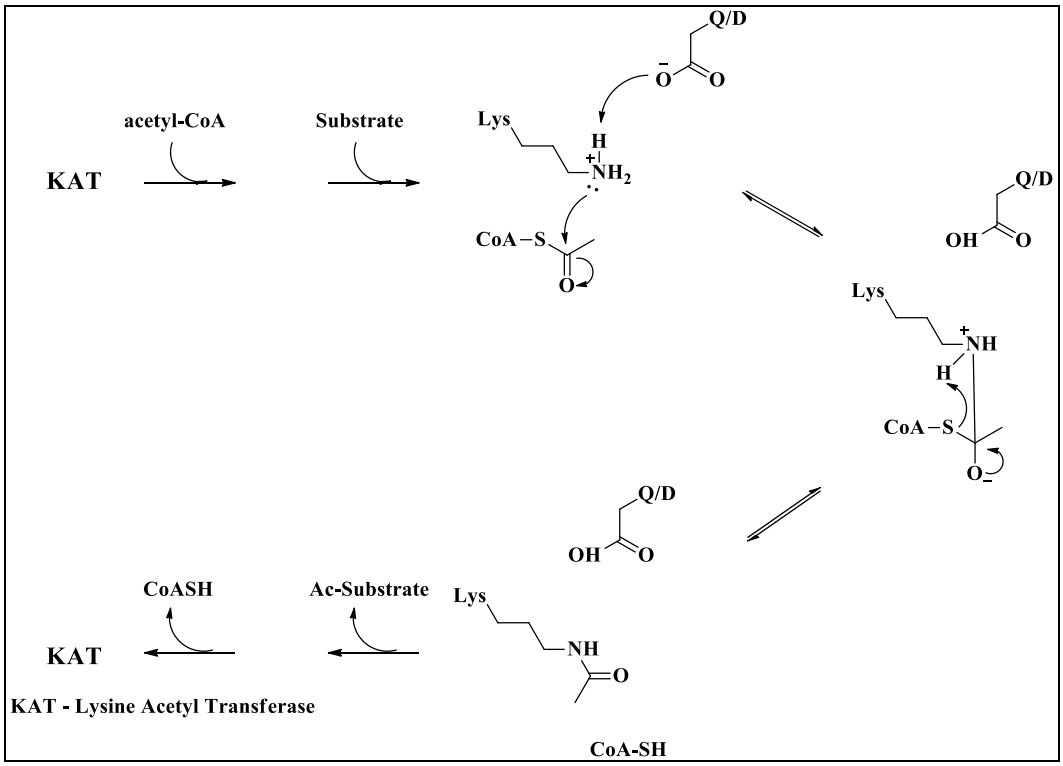

Figure 1: Mechanism of Lysine acetylation. Modified from [70] .

Q/D- Glutamic acid/Aspartic acid.

Typically, for a lysine (K) acetylation reaction to occur, the following are the steps based on our knowledge of histone acetylation[70]. First, in the K-acetyltransferase there is a residue that acts as the initiating base for catalysis. Typically, this is an amino acid that is negatively charged. The lysine acetyltransferase can bind to both the protein substrate with an accessible lysine and acetyl-coA as an acetate group donor. The catalytic base attacks the lysine of the substrate and deprotonates its epsilon amino group. The deprotonated amino group then attacks the acetyl-coA resulting in the form of an unstable intermediate product. Then the acetate is transferred onto the amino group of the lysine and the CoA is released as a product.

Microtubule acetylation

Microtubule acetylation was one of the first discovered tubulin PTMs. First, altered gel mobility showed that α-tubulin in *Chlamydomonas reinhardtii* was modified during flagellar assembly and that the modified α-tubulin was the predominant form observed in the flagella [71]. Radioactive labeling using tritiated acetate indicated that α-tubulin was acetylated and subsequent chemical approaches localized acetylation to the epsilon amino group of a lysine [72]. Thin layer chromatography on smaller fragments of α-tubulin then showed that α-tubulin acetylation occurs on lysine 40[73]. Antibodies against acetyl-K40 α-tubulin epitope (6-11B-1), revealed that acetylated microtubules are present in cilia, basal bodies, neurites, a subset of cytoplasmic microtubules and the mitotic spindles[74]. Acetylated microtubules were observed to differ from non-acetylated microtubules in their decreased turnover rate and resistance to microtubule destabilizing agents such as nocodazole and colchicine[75,76]. This does not mean though, that acetylation of K40 stabilizes microtubules. Instead, the evidence so far, points at K40 acetylation accumulating on long-lived microtubules that are stabilized by other factors[77,78].

Although K40 acetylation of α-tubulin is conserved from *Chlamydomonas* to mammals, it was surprising that a K40R α-tubulin strain of *Tetrahymena thermophila* was indistinguishable from *wild-type* cells[79]. Some eukaryotic species do not have K40 or do not seem to acetylate K40 on a-tubulin including yeast, and *Dictyostelium*. *Plasmodium falciparum* has an α-tubulin with K40 but lacks detectable acetylated tubulin (as reviewed in[79]). There are other K residues that can be acetylated beside K40 on a-tubulin at least in mammalian cells [69] and so the results of mutations of K40 or

detection by the antibodies specific to acetyl-K40 could be telling only a part of the functional story.

Although the microtubule acetyltransferase enzyme had not been identified until this study (see next chapter), the enzymes that deacetylate microtubules have been known for a while. Histone Deacetylase 6 (HDAC6) and Sirtuin-2 (SirT2), both members of the histone deacetylase family were identified as tubulin deacetylases[80,81]. Interestingly, HDAC6, although part of the histone deacetylase family, shows no deacetylating activity towards histones[42]. However, HDAC6 has other substrates besides a-tubulin namely, cortactin and HSP90. Several studies have addressed the significance of tubulin deacetylation by perturbing the levels of the deacetylating enzymes. This was done either by lowering or elimination of the expression levels of deacetylases through gene knockouts and knockdowns or by inhibiting the enzymatic activity of HDAC6 with drugs such as Tubacin and Trichostatin A (TSA). These studies led to several indirect evidences supporting a role for microtubule acetylation in cell motility and microtubule stability[77,82,83]. *In vitro* binding assays suggested that motors preferentially bind to acetylated microtubule[84,85]. Increasing tubulin acetylation levels by inhibiting HDAC6 activity led to a rescue in the transport deficits observed in the cortical neurons of a Huntington disease model[84]. This led to a suggestion that microtubule acetylation plays a role in regulating intracellular transport. On the basis of the accumulation of microtubule acetylation in axonal microtubules, this modification was suggested to play a role in neuron polarization[86]. However, this interpretation came into question recently when it was found that longer lived microtubules, such as those in axons, accumulate modifications such as acetylation and that acetylation *per se* does not contribute to

neuronal polarization[78]. This suggestion was further supported by observations from an experiment where treatment of non-polarized hippocampal neurons with Tubacin, a drug that selectively inhibits the tubulin acetylating activity of HDAC6 and thus leads to an increase in microtubule acetylation did not perturb neuronal polarization[86,87].

A role for microtubule acetylation in neuronal migration was also suggested when an overexpression of a K40A α-tubulin in cortical projection neurons of mice embryos led to neuron migration defects[88]. The functions attributed to microtubule acetylation by the indirect methods mentioned above should be treated with caution since the phenotypes observed could be due to an importance of the K40 residue that is being changed to Alanine or Arginine or due to the effect of a loss of deacetylation of other substrates of HDAC6 such as Hsp90 and Cortactin. Thus, to identify the *in vivo* functions of microtubule acetylation, the identification of the enzyme responsible was very important. Moreover, the candidates put forth as the tubulin acetyltransferases including ELP-3[88](a component of the elongator complex) and ARD-1/NAT-1 N-acetyltransferases[89] were not unequivocally shown to be the tubulin acetyltransferase enzymes. Thus, there is a need for the identification of the tubulin acetyltransferase enzyme.

Role of microtubules in the development and maintenance of axons

Neurons are filled with a dense network of microtubules. In fact, brain tissue is one of the best sources for purification of tubulin by cycles of polymerization and depolymerization induced by cold. In the neuron, microtubules play a role in both cell differentiation (when the neurites are formed) as well as maintenance by providing tracks for the long-range

transport. In the sensory touch neurons of *C. elegans* microtubules may also have an additional role in sensing, signal transduction or propagation (see below).

Importance of microtubules for axon growth

Microtubules are necessary for growth and maintenance of an axon. Treatment of the dorsal root ganglion cells with colchicine (a drug that depolymerizes microtubules) led to the retraction of the axon in an hour[90]. Depletion of all the microtubules in a touch receptor neuron (TRN) in *C. elegans* either by colchicine treatment or through mutations in the TRN-specific *mec-12* α-tubulin and *mec-7* β-tubulin, prevented microtubule assembly and inhibited the TRN process formation[4].

Where are microtubules nucleated in axons?

Typically, in animal cells, microtubules are nucleated at an MTOC, commonly the centrosome located well inside the cell, often near the nucleus. However, neurons represent an exception. In the neuron cell body there is a centrosome that nucleates most if not all cell body microtubules. Microtubules that are found in the axon and dendrites are not connected to the centrosome and lack a MTOC at their end (as reviewed in[91]). In neurons, microtubules are nucleated from the centrosomes only in the very early stages of neuron development (up to 2 days *in vitro)*[91]. Until recently, a widely- accepted model was that the axonal and dendritic microtubules are nucleated in the centrosome, undergo severing and are transported into the axon and dendrites by motor proteins. In support of this model, injection of an antibody specific to a microtubule severing protein, -p60 subunit of katanin, caused accumulation of centrosome-attached microtubules in the

neuron's cell body [92]. However, this model is most likely incorrect because, a complete laser ablation of the centrosome in cultured hippocampal neurons did not prevent neuronal polarization and microtubule accumulation in the neurites[93]. Furthermore, in addition to γ-tubulin that is associated with the centrosome there is also a pool of peripheral g-tubulin inside neurites[93]. Thus, the axonal and dendritic microtubules are most likely nucleated at multiple points along the axon and dendrite on soluble g-tubulin nucleated complexes[93]. Thus, neurons do what plant cells have been known to do for a long time, nucleated microtubules without an MTOC (as reviewed in[18]).

Whether tubulin is transported as heterodimers, oligomers or polymers has been a matter of debate. Observations on transport of fluorescently labeled tubulin in giant squid axons suggested that tubulin is transported by kinesin-1 in the form of oligomers[94]. However, it was later observed that tubulin heterodimers alone could also be carried by kinesin-1 via their association with CRMP-2[95]. CRMP-2 is a protein that promotes microtubule assembly and then gets incorporated into the microtubules it has helped assemble[96]. CRMP-2 is a promoter of axon specification in mammalian neurons and is required for axon guidance and elongation in *C. elegans*. Mutations in *unc-33*, the *C. elegans* CRMP-2 lead to altered neuronal polarity and misdirection of axonal motors into dendrites[97].

How is the axon specified?

A neuron is comprised of a cell body, a usually single axon projection that transmits signals (to other cells usually through a synapse located at the axon end) and multiple dendrites that receive signals from other cells. Axons and dendrites differ in many

aspects including their microtubule organization and protein composition[91]. Importantly, there is a difference in the orientation of microtubules in both these compartments. In the axon all microtubules are plus end out whereas in dendrites, the microtubule polarity depends on the species and the proximity of the microtubule to the cell body. In mammalian neurons, the microtubules in the part of the dendrite closer to the cell body have a mixed polarity whereas the microtubules in the part of the dendrite farther to the cell body are generally plus end out. In *C. elegans*, all microtubules in the dendrites have a uniform minus end out polarity[98,99].

Initial studies on neuron polarization observed that a neuron is polarized in 5 stages[100]. During stage 1 and 2, all neurites grow at the same rate. At stage 3, one of the neurites starts growing longer and eventually becomes an axon[36,100]. In stages 4 and 5, the axons and dendrites acquire all their specialized structural features such as pre and post-synaptic structures. Generally, a neurite is considered to have turned into the axon when it is 15 μm longer than the rest of the neurites[101]. There have been many studies aimed at understanding how one neurite gets chosen to be the axon. One of the approaches taken to do this was by knocking out or knocking down a gene and looking for defects in axon formation[36]. Another way researchers tried to identify critical regulators of neuronal polarity was by overexpressing a certain gene and looking for the development of multiple neurites displaying axon like qualities[36]. The main problem with both these approaches is a lack of distinction between whether a protein is needed to specify and axon or whether it is needed to initiate growth. However, many factors contributing to axon specification have been identified and the connections among them are gradually emerging.

Extracellular cues for axon specification

During neuron polarization, extracellular cues (which are mostly secreted proteins) play a major role in determining which neurite turns into the future axon. In *C. elegans*, UNC-6 (Netrin), a secreted protein and UNC-40 (the receptor for UNC-6) were shown to play an instructive role in the specification and guidance of an immature Hermaphrodite Specific Motor Neuron (HSN neuron). *unc*-6 and *unc*-40 mutants fail to break symmetry to put out a ventrally projecting axon. Not only did they have defects in neuronal polarity but also, in the axon guidance[102]. Also, mutations in *lin-44* and *lin-17*, the *C. elegans* homologs of the secreted proteins Wnt and Frizzled lead to a complete reversal of polarity in TRNs[103]. Four TRNs (e.g. PLM, ALM) make a long anteriorly directed axon process emerging from the cell body. In the *lin-44* and *lin-17* mutants the axon process grows in the reverse direction. Thus, Wnt signaling plays a role in axon specification and guidance[103].

Intracellular cues for axon specification

There are several intracellular cues that direct axon specification. Interestingly, many of these intracellular cues converge on the cytoskeleton, whose remodeling is key to axon specification[36]. Although initial studies had identified a critical role for the regulation of actin cytoskeleton in the specification and growth of an axon[104], more recently, a role for microtubules in the establishment of polarity has emerged. It was found that many polarity regulators contribute to establishment of polarity by regulating microtubule dynamics[36].

It needs to be mentioned that the actin cytoskeleton plays a major role in the establishment of polarity. Localized actin depolymerization using cytochalasin D in stage 2 unpolarized hippocampal neurons can help turn the neurite in which actin was depolymerized into the axon. Possibly a localized depolymerization of the actin network is needed to create more room for microtubules to protrude into this area[104,105].

One of events that occur during neuron polarization is the selective enrichment of certain proteins in one of the neurites that will become the axon[36]. Some of these selectively enriched proteins seem to promote the formation of an axon via regulation of microtubule dynamics. One such protein that is selectively enriched in the future axon and promotes axon formation via regulation of microtubule dynamics is AKT[36]. AKT is a kinase that phosphorylates and inactivates GSK3β [106]. GSK3β plays a role in establishment of polarity by regulating the microtubule affinities of proteins such as Tau and CRMP2 [107] [37]. Tau and CRMP-2 may promote axon formation by their role in promoting microtubule assembly and stability. Selective enrichment of AKT in the future axon could promote microtubule assembly and stability in one neurite and thus help establish polarity. What biased this selection to this one neurite is not known yet. This selective enrichment could possibly be achieved either by degradation of the protein in all neurites except the future axon. Another way the cell could achieve a protein enrichment is through localized translation of mRNA in the future axon[36].

Another polarity regulator that works by regulating the microtubule affinity of a cytoskeleton-associated protein is Brain Derived Neurotrophic factor (BDNF). BDNF seems to promote microtubule assembly by preventing the phosphorylation and

subsequent reduction in the microtubule affinity of CRMP-2, a factor that binds to tubulin heterodimers and promotes microtubule assembly[37]. Interestingly, in immature hippocampal neurons growing on a patterned substrate, the neurite that first came into contact with the BDNF stripe turned into the axon[36]. BDNF prevents phosphorylation of CRMP-2. Thus, it seems that the levels of BDNF and GSK3b need to be tightly regulated to mark only one neurite as the axon.

Role of microtubules in axon specification

One of the earliest events that occur prior to neuron polarization is a selective accumulation of a microtubule motor, kinesin-1, in the neurite that eventually turns into the future axon[101]. This is surprising considering that the differential orientation of microtubules is not present at this early stage[101]. Jacobson *et al* found that in unpolarized stage 2 neurons kinesin-1 shuttles between different neurites. At some point, one neurite starts to accumulate kinesin-1 and turns into the axon. What are the cues that promote the retention of kinesin-1 in one of the neurites is a key question. In the future axon, kinesin-1 may interact with microtubules that form the proximal region called the initial segment. A recent study showed that the microtubules that form the initial segment are enriched in GTP-tubulin[108], and thus their plus end caps could be much longer or these microtubules could have internal segments of GTP-subunits. In vitro studies showed that kinesin-1 binds more strongly to GTP-tubulin enriched microtubules[109]. How this GTP tubulin accumulation occurs in the future axon is not yet known. Are the microtubules in the future axon altered by some unknown factors that prevent GTP hydrolysis? Is there more free tubulin in the future axon that prevents the loss of GTP caps? Are the

microtubules in the future axon being rescued more? These are some of the important questions to follow. It has been suggested that the increased amount of GTP-tubulin could reflect an increase in the frequency of rescue events thus resulting in the more robust growth of microtubules inside the future axon. Once the microtubules show increased growth, and longer GTP-tubulin regions, this could stimulate binding and motility of kinesin-1, which could bring in factors that mediate microtubule assembly such as tubulin heterodimers in a complex with CRMP-2. Thus a positive feedback loop could operate between microtubule growth and kinesin-1 that is mediated by accumulation of GTP-tubulin.

The observation that there is more GTP-tubulin in the future axon indicates that microtubules there are more stable or more likely to grow. Strikingly, making microtubules more stable by an artificial means is sufficient to turn a neurite into an axon. In one important study, application of caged taxol followed by local uncaging in one of the neurites of nonpolarized hippocampal neurons conferred an axonal identity to that particular neurite as suggested by accumulation of axonal markers such as Tau[86]. This observation suggested that local microtubule stabilization is necessary and sufficient for neuron polarization. These observations led to the "Selective Stabilization Model". This model suggests that neuronal polarization occurs by selective stabilization of the microtubules in one of the neurites and this neurite eventually becomes the axon. Further support for this model comes from the observation that many mutants of polarity regulators such as SAD and GSK3 show altered levels of microtubule stability as suggested by their resistance to microtubule depolymerizing drugs such as nocodazole[86]. An alternative hypothesis is known as the Cell Length Dependent Model[78]. According to

this model, microtubule assembly parameters between the future axon and the rest of the neurites remain the same in time and space and microtubules have a constant arrival and departure rate that is dependent on the length of the neuron[78]. Therefore, the longer the axon, the more microtubules it will have. This high number of microtubules can bring in more factors required for elongation and differentiation[110]. If there are no differences between microtubules in the future axon and the rest of the neurites, then how is one neurite being chosen for a selective accumulation of GTP tubulin? What are the consequences of this selective accumulation that lead to the neurite being conferred an axonal identity? What else are the events that GTP tubulin accumulation leads to apart from the recruitment of KIF-5? The CDLM is in agreement with Verhey *et al*'s observations that there is no difference between microtubule stability between axons and dendrites at stage 3 of neuronal polarization[87]. But, the CDLM does not explain the selective accumulation of GTP tubulin in the future axon. Possibly both models are applicable and two mechanisms work in parallel. Alternatively, the CDLM model could be responsible for the very early stage of polarization. Once the future axon is specified, the SSM mechanism could promote the axon elongation and maintain its differentiated state. At this moment, it does seem that to a certain extent, neurite elongation could contribute to axon specification. It is known that the future axon is 15mm longer than the rest of the neurites[101]. But how neurite elongation is achieved only in one neurite remains unknown.

Microtubule polarity

Axons and dendrites also differ in the orientation of microtubules. In most organisms, axons have microtubules with their plus ends out (i.e. microtubules growing away from the cell body of the neuron), whereas the dendritic microtubule orientation differs between organisms and between microtubules in different cell types. In *C. elegans,* axonal microtubules have their plus ends out whereas dendrites have their minus ends out (i.e. microtubules growing towards the cell body of the neuron)[98]. Microtubules in distal dendrites (region of the dendrite farther away from the cell body of the neuron) are plus ends out in mammalian neurons. During neuron polarization in hippocampal neurons, all neurites have a plus end out organization until stage 4[111]. Only by stage 5, after forming presynaptic and post-synaptic structures do they get their final polarity with axons and distal dendrites (region of the dendrite farther to the cell body of the neuron) having a plus end out organization and proximal dendrites having mixed polarity.

Recent studies in *C. elegans* have highlighted the importance of microtubule polarity in the establishment of neuronal polarity. A *C. elegans* mutant of *unc-116*, which encodes a kinesin-1 heavy chain, showed improper localization of motor proteins where UNC-104, another neuronal kinesin heavy chain was not localized to the axon in *unc-116* mutants and seemed to be improperly accumulated in the distal dendrites. Moreover, in *unc-116* mutants dynein motors, which are normally enriched in the dendrites were mislocalized[99]. End binding proteins (EB) proteins are +TIPs that can be used as a marker to visualize growing microtubules and based on the directionality of the EB comets (which bind to the growing plus ends), one can determine the direction the microtubule is

growing. Imaging using EB-GFP on these animals led to the observation that microtubules in the dendrites of the *unc-116* kinesin-1 mutants there were more plus end out than minus end out microtubules compared to wild-type[99]. *In vitro* studies showed that UNC-116 kinesin-1 can slide microtubules against each other[99]. UNC-116 may contribute to the orientation of neuronal microtubules by sliding microtubules that have plus ends out away from the dendrite into the cell body, resulting in a uniform minus end out polarity in the dendrite (using minus end out microtubules as a track for the UNC-116 plus end directed motility)[99]. This uniform organization in turn, probably helps in promoting the polarized transport of neuronal cargoes. This relatively novel role of kinesin in microtubule sliding, different from its role in transport, was also recently implicated to play a role in initial neuron growth[112].

Thus, the UNC-116 kinesin-1 dependent sliding model explains how the dendrite becomes enriched in the minus end out microtubules but it does not explain how the axon can be composed exclusively of plus end out microtubules. One possibility is that microtubules with a minus end out are prevented from entering the axon by the filter that is located in the initial segment (see below). This filter could prevent UNC-116 from entering the axon while carrying a minus end out microtubule, which in principle would be the same as the one that the same motor moves out of the dendrite. It is agreeable that all the studies so far point to kinesin-1 playing an important role in the specification and maintenance of polarity. The events upstream of selective GTP tubulin accumulation in the future axon and immediately downstream of kinesin-1 recognizing the future axon remain unknown. Moreover, it is not clear yet whether kinesin-1 contributes to axon specification and maintenance by sliding plus ended microtubules in the axon (thus

bringing in axonal cargo) or whether by promoting assembly or both. In dendrites, kinesin-1 clearly seems to play a role in the maintenance of polarity via its sliding role.

Axon Initial Segment

The axon initial segment (AIS) is a physical barrier that arises after polarization that acts as a filter to prevent mixing of components between the axonal and dendritic compartments. The AIS is an F-actin and Ankyrin based structure that not only prevents random diffusion of molecules (the AIS was shown to aid the diffusion of smaller 10 kD labeled dextran but, not a 70 kD labeled dextran) but also has selectivity towards certain motors wherein it allows for transport of cargo carried by certain motors. Indeed, it was observed using swapping experiments that the axon initial segment in a mammalian neuron allowed for transport by KIF5 but not KIF17 kinesin[113]. Thus, the axon initial segment helps in the maintenance of distinct protein composition between different compartments.

Role of post-translational modifications of tubulin in the neuron

Axonal microtubules are not only more enriched in GTP-tubulin as discussed earlier but also have higher levels of PTMs on tubulin. The PTMs that accumulate on axonal microtubules are greatly different from the PTMs accumulating on dendritic microtubules[42] thus, microtubules in axons have different properties compared to dendritic microtubules. Axonal microtubules show an enrichment of PTMs that are known to accumulate on older stable microtubules such as acetylation, detyrosination and glutamylation. Dendritic microtubules, on the other hand, are still in their native

unmodified state i.e. enriched in tyrosinated microtubules. There are differences in microtubule composition not only between different compartments of the neuron, but within the same compartment as well. It was observed that the microtubule shaft is enriched in the acetylated, detyrosinated and glutamylated microtubules whereas; the growth cone is enriched in tyrosinated microtubules[42,86]. Interestingly, although FRAP studies haven't established differences between the microtubules in axons and dendrites, there have been differences observed between microtubule dynamics in the axon shaft and the growth cone and this has been extrapolated to differences between axons and dendrites due to the similar microtubule composition between growth cone and dendrites[86].

For example, the axon is highly enriched in acetylated tubulin[86]. An early study showed that kinesin-1 binding to acetylated microtubules is stronger as compared to microtubules lacking acetylation at K40[85]. Overexpression of a K40A alpha tubulin in E17.5 cortexes of mice led to impaired migration of cortical projection neurons[88]. However, treating hippocampal neurons with tubacin, a drug that selectively inhibits the tubulin deacetylating activity of HDAC6 led to an increase in the tubulin acetylation levels in the dendrites, but this alone did not affect the selective targeting of KIF-5 to the axon[86,87]. The discovery of the enzyme that acetylates tubulin (see next chapter) helped to clarify the role of tubulin acetylation in the neuron and it is a relatively unimportant modification in mammals (see Discussion). However, tubulin acetylation could be required for normal assembly of axonal microtubules (see Chapter 4 and Discussion). Moreover, newer in vitro studies failed to detect an effect of tubulin acetylation on the in vitro motility by kinesin-1[87].

The axon is enriched in microtubules with detyrosinated tubulin while tyrosinated tubulin is enriched on microtubules in the dendrites[86,87]. Microtubules in the dendrites and in the growth cone are also more dynamic as compared to the dendrites [86,87]. This dynamic nature of microtubules is extremely important especially in the case of the growth cone, which is required for axons to probe an area for growth. KIF5 binds preferentially to detyrosinated microtubules in vitro[67]. In the hippocampal neuron, mutations in the region of KIF-5 that gives it decreased specificity for binding to detyrosinated microtubules turn KIF5 into a bidestination motor where it was observed to localize to dendrites as well as axons [67]. RNAi knockdown of TTL, the enzyme that tyrosinates tubulin in hippocampal neurons lead to increased transport of KIF-5 into dendrites[67]. Furthermore, mice lacking TTL die with neural development defects[61], but this could also be an effect of excessive detyrosination on other motors and kinesin-13 end depolymerizer.(see below).

The dendritic and growth cone microtubules are more dynamic, enriched in tyrosinated tubulin that is selectively depolymerized by kinesin-13[28]. Tubulin Tyrosine Ligase or TTL is the enzyme that puts a tyrosine back on to the C-terminus of detyrosinated tubulin[60]. TTL knockout mice displayed altered neuronal organization and cultured TTL null neurons displayed premature axonal differentiation and growth[61]. Tyrosinated tubulin is crucial for interactions between microtubules and +TIP binding proteins such as CLIP-170[27]. Alterations in these interactions result in the accelerated growth observed in the cultured TTL null neurons[27]. Moreover, it was recently found that tyrosinated tubulin plays an important role in the formation, growth and remodeling of the growth cone, a structure rich in actin and microtubules that an axon extends during

growth and path finding[33]. It was suggested that defects in the remodeling of the actin and microtubule cytoskeleton of the growth cone led to the axonal pathfinding defects observed in the TTL knockout mice.[33]. Moreover, the accumulation of extremely stable tubulin in the absence of TTL could have also led to premature axonal differentiation in the TTL null cultured neurons.

Polyglutamylation of tubulin has also been identified to play important roles in navigating motor proteins to maintain distinct protein composition between axons and dendrites. Low levels of polyglutamylated tubulin in a ROS22 mice model has been shown to selectively reduce the amount of KIF1a kinesin-3 motor in neurites[68]. However, the abundance of two other motors KIF-5- a kinesin-1 and KIF2a-a kinesin-2 are unaffected[68]. This further supports the idea that tubulin PTMs can act selectively on specific motors.

CCP-1 is the enzyme that shortens the polyglutamylation side chains[114]. Mice with a mutation in CCP-1 (pcd mutant) have excessive levels of polyglutamylated tubulin on microtubules in the neurons and these mutants and display neurodegeneration in of the cerebellum[115]. The neurodegeneration defects in the CCP-1 mutant mice can be rescued by RNAi of the tubulin glutamylating enzyme TTLL-4 that restores the normal levels of tubulin glutamylation[114]. One possible reason for progressive structural defects in mutants of CCP-1 could be that the hyperglutamylated microtubules in these mutants are destabilized by severing enzymes such as katanin[63]. In C. elegans, the *ccpp*-6 mutants (CCPP-6 is the homolog of CCP1 in *C. elegans)* show age related degeneration of

chemosensory neurons[116] and this phenotype could be rescued by reducing polyglutamylation levels in these animals.[117]

Model organisms chosen for my thesis

For my project, I have used three different model organisms namely the ciliate *Tetrahymena thermophila,* zebrafish *Danio rerio* and nematode *C. elegans.* These three organisms have complementary experimental advantages which we have tapped to identify the tubulin acetyltransferase and to further probe the role of the microtubule acetyltransferase and microtubules in touch sensation. Below are a few reasons why these organisms are great for studies on gene function and for studying microtubules.

Advantages of *Tetrahymena thermophila*

Tetrahymena thermophila, a unicellular ciliated protist is a freshwater organism. *Tetrahymena* cells are very easy to grow and maintain in a laboratory setting. The prevalence of homologous recombination makes *Tetrahymena* particularly great for reverse genetics. One can make germline or somatic knockouts because of the availability of two nuclei. More recently, a lot of work is being done to establish forward genetics strategies in *Tetrahymena*. Gene expression in *Tetrahymena* can also be conditionally induced or repressed with the help of certain promoters. The presence of a number of cilia also makes it great for biochemical studies on microtubule-rich organelles. *Tetrahymena* is excellent for studying microtubules and their modifications. Although *Tetrahymena lacks* tubulin heterogeneity, it assembles diverse populations of microtubules (close to 18 types). These diverse microtubules are generated by post-

translationally modifying the one α-tubulin and two β-tubulins available in this one celled organism.

Danio rerio as a model organism

Danio rerio, commonly known as zebrafish is a fresh water fish that makes a very good model system to study development. We chose zebrafish to study microtubule modifications for a couple of reasons.

It is a vertebrate organism with many genes and pathways having homologs in humans. Fertilization is ectopic and the embryo is transparent thus enabling easy visualization of changes occurring during development. Zebrafish are a great model for genetic studies with both forward and reverse genetics strategies worked out. Gene expression can be easily knocked down using morpholinos that are modified oligonucleotides. Morpholinos lower gene expression levels by interfering with translation initiation or by blocking splicing[118,119]. Although morpholinos have many side effects, they are a quick and easy first step in studies aimed to identify gene function. More recently, a TALEN system has been popular for genetic manipulations in zebrafish[120]. TALENs have a DNA binding domain fused to a FokI endonuclease. By designing TALENs that bind to either side of the target sequence, one can specifically excise the target sequence by FokI heterodimers. This results in insertions or deletion mutations being induced due to the non-homologous end joining repair.

C. elegans as a model organism

C. elegans, a free living nematode makes a great model system for doing genetic studies. They are cheap to maintain, require no special care and most strains can be grown and maintained easily at room temperature. *C. elegans* is particularly favorable to study the nervous system. The connectivity of all the 302 neurons in *C. elegans* is already mapped and most of the genes have homologs in vertebrates[121]. We chose *C. elegans* TRNs as a model to study microtubules and the function of tubulin acetylation for a couple of reasons. Most importantly, TRNs are highly enriched in acetylated tubulin and loss of *mec-12*, the only a-tubulin containing acetylable K40, leads to loss of touch sensation. Thus, the specific tubulin that is acetylated is also important and its activity can be easily measured simply by touching the mutant animal. The loss of microtubules in the TRNs leads to a loss of touch sensitivity, an easily scorable phenotype which could be used for genetic studies[122]. Second, the microtubules of the TRNs are not essential for the animal's growth. Third, one can selectively affect touch sensitivity without affecting any other general functions of microtubules in neuron growth and in transport[123]. Thus, we could pursue a novel role of microtubules in mechanosensory transduction that is different from its traditional role in growth and transport.

Mechanosensation in *C. elegans*

Mechanosensation is an essential sensory function that is critical for survival. Mechanosensation involves sensing a change in pressure imposed by an external object. In mammals, mechanosensation is important in many locations including the skin (sensation of touch), ear (detection of sound) and kidney (detection of fluid flow). In the

invertebrate *C. elegans*, gentle touch sensation is important to navigate through the environment, to sense food and to sense a mate[124]. *C. elegans* uses two mostly distinct molecular pathways and groups of neurons to sense either harsh or gentle touch. This thesis is involved with the gentle touch sensation pathway that is mediated by six touch receptor neurons (TRNs). The TRNs sense gentle touch along the entire animal body (reviewed in[125]). There is also a mostly separate mechanism that senses gentle touch at the front of the head[125], which will not be discussed here.

A response to gentle touch of the body results in an avoidance response. Thus a forward-moving animal touched below the head rapidly retracts and the reverse behavior is observed when the animal is touched near the tail. This response to touch is carried out through the six TRNs, ALMR, ALML, PLMR, PLML, AVM, and PVM neurons. These TRNs were identified as neurons involved with gentle touch sensation through laser ablation studies[122]. The synaptic circuitry of TRN s includes the six TRNs mentioned above, five pairs of interneurons and 69 motor neurons. The TRNs signal to the interneurons using both chemical synapses and gap junctions. The interneurons signal on the motor neurons mostly through gap junctions[126]. Interestingly, the TRNs do not receive synaptic input from other neurons [126]. The TRNs are strategically positioned close to the cuticle of the animal's body with only a thin layer of hypodermis separating the neuron from the cuticle. Perhaps this arrangement enables TRNs to generate a quick response to touch. The TRNs have special features including a layer of extracellular matrix (mantle) which envelopes the neuron plasma membrane and is in contact with the mechanosensitive ion channels[122]. Remarkably, the TRN processes have specialized unique wide diameter microtubules made of 15 protofilaments. All other neurons have 11

protofilament microtubules while cilia have doublets that are made of a 13 pf tubule A and 10 pf tubule B, a typical organization found in cilia of other organisms[4]. The microtubule ultrastructure of the TRN makes them easily identifiable in TEM cross sections. It is noteworthy that the TRNs were originally called "microtubule cells" due to their prominent microtubules[127]. The lateral TRNs have one long continuous process emerging from the cell body that runs anteriorly covering half the length of the worm's body. The TRN microtubules will be discussed in greater detail in later sections.

Genes involved in touch sensation were found through genetic screens for mutations that cause touch insensitivity. The genes identified were called mechanosensory abnormal (mec). There are 18 mec genes that were identified so far, and the molecular identity of most of them is known. Further, there are those genes which when mutated cause touch insensitivity indirectly for e.g., mutations in *unc-54*, a myosin heavy chain in the body wall muscle lead to touch insensitivity because they twitch excessively and this causes constant stimulation of the TRNs and the TRNs eventually stop to respond[122,128].

The specification of TRN s requires *unc-86* a homeobox containing transcription factor. Mutations in *unc-86* lead to alterations in the pattern of divisions of the Q cell lineages that normally give rise to TRN progenitors. Mutations in *unc-86* can result in a complete failure to specify TRNs. Most genes expressed in TRN have an *unc-86* binding site in their promoters[129]. This binding site may facilitate transcription by *mec-3*, the master regulator of all the genes expressed in TRNs. MEC-3 forms a heterodimer with UNC-86 to facilitate the transcription of many genes expressed in the touch receptor neurons including its own (in a feedback loop)[129]. MEC-3 is required for process

development as suggested by the observation that *mec-3* mutants fail to extend a TRN process[122]. However, these animals do have the cell body of TRNs suggesting MEC-3 is required for the development of the process rather than specification of the touch cell fate. Moreover, unlike other mec mutations which lead to progressive degeneration of the TRN process, *mec-3* mutants never form a process[122].

The most distinguishing features of a TRN are a very darkly staining extracellular mantle and wide diameter microtubules that are arranged in bundles[122]. MEC-1, MEC-5 and MEC-9 form the darkly staining mantle[122]. MEC-1 and MEC-9 have an EGF/Kunitz domain (EGF is a domain present in secreted proteins and Kunitz is a domain present in protease inhibitors) and MEC-5 encodes a collagen[122]. Together, these three genes encode proteins required for the secretion of the mantle that is characteristic of the TRNs. The TRN axon processes, when formed, are between the hypodermis and the body wall muscle. Later in development, these processes get engulfed by the hypodermis[122]. Improper positioning of the TRNs due to mutations in genes encoding the mantle results in a loss of touch sensitivity. MEC-1 is not only important for the secretion of the mantle but also for the localization of the mechanosensitive channel. Mutations in *mec-1* also cause a displacement of the TRNs from their attachment site on the body wall. This leads to a loss of sensory activity and eventually causes premature aging in the TRNs[130]. Aging TRNs display morphological defects such as a long ectopic process being extended from the cell body and also branches growing off the main TRN process[131]. The most intriguing finding on the *mec-1* mutants was that unlike many mutations that affect organism aging in general, *mec-1* mutations specifically affect TRN aging[131].

The genes encoding the components of the mechanosensitive channel include *mec-4* (Degenerin), *mec-10* and *mec-6*. *mec-4* and its paralog *mec-10* encode a mechanosensitive channel[132-135]. A gain of function mutation in *mec-4* causes constant activation of the channel thus leading to the death of the TRN[136]. *mec-6*, encodes a component required for the proper localization of the channel. Electrophysiological recordings on Xenopus occytes expressing various components of the channel suggested a role for MEC-6 in the amplification of the current passing through MEC-4 and MEC-10 channel complex[132]. Furthermore, this current was even more amplified upon expression of MEC-2[133]. MEC-2 contains a stomatin like domain is also part of the channel complex and genetically interacts with MEC-4 [137]. The 15 pf microtubule assembly in TRNs requires MEC-12 α-tubulin and MEC-7 β-tubulin[52,53]. The exact role of these microtubules in the TRN will be discussed further. However, it should be noted that microtubule components, *mec-12* and *mec-7*, genetically interact with the channel components although there is no evidence of a physical association between the mechanosensitive channel and 15 protofilament microtubules[137,138]. Since mutations in *mec-2* can be suppressed by mutations in *mec-7* or *mec-12* and certain mutations in *mec-4* are enhanced by mutations in *mec-7* β-tubulin and *mec-12* α-tubulin, this has led to the suggestion that MEC-2 acts as a linker between the mechanosensitive channel and the inside of the cell perhaps through interactions between MEC-2 and the 15 protofilament microtubules[137].

A model for mechanotransduction in *C. elegans.*

The current model for mechanotransduction, the conversion of a mechanical force into a biochemical signal is as follows (reviewed in[124]). When a touch stimulus is applied to the cuticle, the MEC-4 channel is forced inside the cell. This displacement of the channel causes a change inside the phospholipid bilayer holding the channel. This triggers a conformational change in the channel forcing it to open and allowing for a flux of sodium ions, thus generating a mechanoreceptor current (MRC)[123]. The MRC leads to activation of the TRNs and the stimulus then propagates in both directions from the site of touch along the axon. It is suspected that there is a mechanism that sustains the stimulus so that its magnitude does not diminish as it propagates. For example, the mobility response is roughly equal regardless of the position of touch or how far it is from the synapse[125].

The 15 protofilament microtubules

Microtubule protofilament number is variable and ranges between 9 and 16 *in vivo*. *In vitro*, microtubules can form with protofilament size ranging from 8 to 17. *In vivo*, 13 is the most common number of protofilaments. However, variations in the protofilament number exist with significant differences in number observed in the 15 protofilament microtubules in *C. elegans* TRNs, to 12 protofilaments in cockroach epidermis to 15 in crayfish sperm[4]. Because of the variation in protofilament size, the diameter of the lumen of the microtubules also varies considerably. 13 protofilament microtubules have an outer diameter of 25nm and inner diameter of 15nm. The mechanism of regulation of the protofilament number *in vivo* is not clear at all. One would think that something regulates the number of the g-tubulin units in the nucleating ring but so far there is no direct

evidence in support of such a "nucleation-based protofilament determination model". Alternatively, the protofilament number could be regulated post-nucleation by binding of certain MAPs to reorganize the microtubule wall. Interestingly, *in vitro* when microtubules form without a nucleating template, a protein called doublecortin, a stabilizing MAP, promotes selective formation of 13 protofilament microtubules[139].

In *C. elegans,* in cell types other than TRNs, microtubules have11 protofilaments (11p). Interestingly, purified tubulin and MAPs from *C. elegans* selectively formed microtubules with 9-11 protofilaments *in vitro* in contrast to bovine tubulin, which formed 13 protofilament microtubules[140] Sensory cilia though, have doublet microtubules made of 13 protofilaments in the A tubule and 10 protofilaments in the B tubule similar to other systems suggesting axonemal structure has a strict protofilament number requirement perhaps by offering the cilium stability. TRNs however, have unusually large 15p microtubules that are assembled from MEC-12 α-tubulin and MEC-7 β-tubulin[52,53]. The TRNs in *C. elegans* have around 450 15 protofilament microtubules which form bundles. These microtubules form arrays that are discontinuous. The plus ends of these microtubules are located on the outside of the bundles and at least some appear attached to the plasma membrane[127]. Interestingly, the number of 15 pf microtubules steadily increases during development as the animal body increases in size and the axons of TRNs lengthen. L1 larvae have very few 15 pf microtubules but are fully touch sensitive (the TRN axons in L1 have fully formed)[127]. It has been suggested that the discontinuous array could offer flexibility to the bundle so that the microtubules can just slide past each other[127]. The numbers and the length of the microtubules in TRNs increase until 12 hours after hatching. From 12 to 36 hours of development that

corresponds to L1 to young adult stages, there is a great increase in the number of microtubules. However, the average length of microtubules stays the same[4]. Also, the number of microtubules in the bundle drops in the segment of the axon close to the cell body[127].

The 15 protofilament microtubules in TRNs differ in many of their properties from the 11p microtubules expressed in other cell types. 15 protofilament microtubules are selectively destabilized by colchicine whereas the same drug does not affect the 11 protofilament microtubules of other cell types[4]. On the other hand, benomyl treatment (another microtubule depolymerizing drug that binds to tubulin at a site distinct from colchicine) leads to a loss of the 11 protofilament microtubules[15]. Mutations in *mec-12* α-tubulin and *mec-7* β-tubulin lead to a loss of the 15 pf microtubules in TRNs. In severe loss of function mutants of either *mec-12* or *mec-7*, ~100 11 pf microtubules are present in place of the normal 15 pf microtubules and likely thanks to their activity, the touch neurons have a proper morphology but, these animals can't sense touch [4,141]. This piece of data alone would indicate that the determination of the 15 pf diameter occurs post-nucleation and requires that *mec-12* and *mec-7* tubulins are present in the microtubule lattice along with other tubulins that have not been characterized yet. The presence of 15 pf microtubules in only one cell type suggests a specialized role in mechanosensation rather than axon development. The low number of microtubules formed in the absence of MEC-7 and MEC-12 could be due to the low amount of the other tubulin isotypes in TRNs that assemble into these 11 protofilament microtubules. It is currently unknown whether heterodimers made of *mec-7* and *mec-12* can be assembled into 11 protofilament microtubules or have an intrinsic ability to assemble into 15 pf microtubules. It is very

likely that at least in some of the severe *mec-12* and *mec-7* mutants, the 11 pf microtubules form from tubulins other than *mec-12* or *mec-7* (*C. elegans* expresses 9 isotypes of α and 6 isotypes of β-tubulin). Thus, it is possible that in TRNs, MEC-7 and MEC-12 normally copolymerize with other tubulin isotypes and their presence in the copolymer somehow specifies the 15 pf organization.

It was originally thought that bundled 15 protofilament microtubules maybe required for increased rigidity of the axon so that the plasma membrane becomes more sensitive to deformation[123]. Thus, microtubules would play a role in transmitting the deformation of the plasma membrane onto the mechanosensitive channel. It is worth mentioning that the 15 pf microtubules are predicted to be stiffer as compared to the 11 pf microtubules[123]. Also, it is intriguing that the 15 pf microtubules are also found in the pillar cells of cochlea that are also mechanoreceptors[16]. Similar to TRN microtubules, microtubules in these pillar cells are bundled. Perhaps this stiff nature of their microtubules makes them more sensitive to sensory stimuli An alternative possibility is that 15 pf microtubules play a generic role in motor-based transport to supply the axon with all of the required components (most notably microtubules and ion channel components). However, this hypothesis does not explain why the unique 15 pf organization of microtubules would be present as 11 pf microtubules fulfill the transport function just fine in other neurons. Moreover, mammalian neurons with often much longer and thicker axons rely on 13 pf microtubules for transport[142]. Further support for a non-transport or sensory role for 15 protofilament microtubules came from an observation that colchine treatment of adults (at the concentration known to selectively depolymerize only15 pf microtubules in TRNs) caused touch insensitivity without affecting the distribution of the

mechanosensitive channel component MEC-4, suggesting that the transport along microtubules is not critical in mature TRNs[123]. The problem with this experiment is that it was not possible to determine whether the channel subunit composition or structure remains normal without microtubules. Microtubules are not required for channel gating as there is a base mechanoreceptor current that passes through the channel even in *mec-7* and *mec-12* mutants that lack the 15 protofilament microtubules. However, the 15 protofilament microtubules seem to be required for the passage of an optimal amount or amplitude of a MRC as seen in the *mec-12(e1605)* strain that has the 15 protofilament microtubules but, is touch insensitive[123].

A more direct role for the 15 protofilament microtubules in mechanotransduction emerged through studies on a unique mutant, *mec-12(e1605)* that has a seemingly normal density of 15 protofilament microtubules but can't sense touch[123]. However, this experiment also has a weakness because it is possible that a general transport function is compromised despite the presence of microtubules in the *mec-12(e1605)* mutant. Importantly, electrophysiological data suggested that the 15 protofilament microtubules are involved in generating optimal amplitude of MRC[123]. *mec-7* and *mec-12* mutants have reduced MRC amplitudes and reduced adaptation time of the channel suggesting 15 protofilament microtubules could be required to keep the channel open longer and delay adaptation (adaptation is when the animal stops responding after repeated touches and occurs through redistribution of forces on the membrane)[123]. Alternatively, the MRC amplitude could depend on the number of channels open simultaneously. It is calculated that 14-25 channels open up for a MRC of optimal amplitude to pass so as to elicit a touch response[134]. Although microtubules don't make any direct contact with the channel

component MEC-4[138], the great number of attachments they make with the plasma membrane coupled with the bundled nature of these 15 protofilament microtubules might make them well suited for the propagation of the signal[137]. It is interesting to note that touch insensitive mutants of *mec-12* and *mec-7* can still respond when touched near the synapses suggesting that the closer the site of stimulus to the synapse; the easier it is for the signal to be propagated along the axon without the 15 protofilament microtubules[52]. Moreover, the 15 protofilament microtubules might act in touch sensation through MAPs present only in TRNs such as PTL-1 (Protein Tau Like-1) and ELP-1(Elongator Like Protein-1)[143-145].

Our understanding of the *in vivo* functions of a conserved modification of microtubules- acetylation is very poor due to a lack of good tools. In the next section, I will describe how we identified MEC-17 as an alpha tubulin acetyltransferase enzyme to enable studies aimed at identifying the functions of microtubule acetylation. We chose MEC-17 as a candidate for the alpha tubulin acetyltransferase due to a presence of an acetyltransferase domain[146] and conservation across all organisms with microtubule acetylation and cilia. Most importantly, MEC-17 is expressed in the TRNs of *C. elegans* and the gene was first identified in a screen for genes required for touch sensation[147]. Remarkably, the TRNs are one of the very few cell types in *C. elegans* that express MEC-12, the only alpha tubulin in *C. elegans* with a K40 residue[53]. These characteristics provided us with enough justification to suspect that MEC-17 could be the a-tubulin acetyltransferase. We utilized the exclusive homologous recombination in *Tetrahymena* to generate knockouts of *mec-17*. Furthermore, we exploited the biochemical utility of *Tetrahymena* to purify tubulin and perform *in vitro* acetylation assays. We also tested

how conserved the function of this enzyme was by knocking down the expression of *mec-17* in a vertebrate, zebrafish. We tapped some of the best features of zebrafish: clear embryos that are easy to visualize and the ease of knocking down genes using morpholinos to test whether the enzymatic activity of MEC-17 is important for zebrafish development. Finally, we exploited the touch insensitivity phenotype of *mec-17* mutants in *C. elegans* to test whether the loss of touch sensation in these animals is due to a lack of microtubule acetylation. If this was the case, we planned on using this easily observable and scorable phenotype of touch insensitivity to probe the function of microtubule acetylation *in vivo.* Additionally, we have exploited the rich genetic resources of *C. elegans* to identify a role of the 15 protofilament microtubules in touch sensation. Finally, we have successfully made a *mec-17* knockout zebrafish that could be used for future studies on axon regeneration.

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CHAPTER 2

IDENTIFICATION OF MEC-17 AS AN a-TUBULIN ACETYLTRANSFERASE*

*** MEC-17 is an α-tubulin acetyltransferase**

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SUMMARY

In most eukaryotic cells, subsets of microtubules are adapted for specific functions by post-translational modifications (PTMs) of tubulin subunits. Acetylation of the e-amino group of K40 on a-tubulin is a conserved PTM on the luminal side of microtubules[1] that was discovered in the flagella of *Chlamydomonas reinhardtii*[2,3]. Studies on the significance of microtubule acetylation have been limited by the undefined status of the a-tubulin acetyltransferase. Here, we show that MEC-17, a protein related to the Gcn5 histone acetyltransferases[4] and required for the function of touch receptor neurons in *C. elegans*[5,6], acts as a K40-specific acetyltransferase for a-tubulin. *In vitro*, MEC-17 exclusively acetylates K40 of a-tubulin. Disruption of the *Tetrahymena* MEC-17 gene phenocopies the K40R a-tubulin mutation and makes microtubules more labile. Depletion of MEC-17 in zebrafish produces phenotypes consistent with neuromuscular defects. In *C. elegans*, MEC-17 and its paralog W06B11.1 are redundantly required for acetylation of MEC-12 a-tubulin, and contribute to the function of touch receptor neurons partly via MEC-12 acetylation and partly via another function, possibly by acetylating another protein. In summary, we identify MEC-17 as an enzyme that acetylates the K40 residue of a-tubulin, the only PTM known to occur on the luminal surface of microtubules.

RESULTS AND DISCUSSION

Acetyl-K40 marks are enriched on a subset of microtubules that turnover slowly (reviewed in[7]). The K40 residue of a-tubulin is not required for survival in protists, such as *Tetrahymena*[8], or *Chlamydomonas*[9] but appears to be important in vertebrates. In neurons, axonal microtubules have higher levels of K40 acetylation than dendritic

microtubules[10]. Neurons that overexpress a K40A mutant a-tubulin show altered motor-based trafficking and cell differentiation[11,12]. Kinesin-1, a motor that is preferentially targeted to the axon[13], has higher affinity for acetylated as compared to non-acetylated microtubules[11,14,15]. Two histone deacetylase-related enzymes, HDAC6 and SIRT2, deacetylate a-tubulin[16,17]. The a-tubulin acetyltransferase (aTAT) has been partially purified[18] but the identity of the catalytic subunit remains unknown. Recently Steczkiewicz and colleagues reported that the conserved protein domain DUF738 has weak amino acid sequence homology to the catalytic domain of the Gcn5 histone acetyltransferases[4]. Among the DUF738 proteins is MEC-17, whose activity is required for the maintenance of touch receptor neurons (TRNs) in *C. elegans*[5,6]. Intriguingly, in *C. elegans,* acetylated a-tubulin (MEC-12) is enriched in the TRNs[19]. These observations opened the possibility that MEC-17 is involved in K40 acetylation on a-tubulin.

MEC-17 homologs are present in most eukaryotes with exception of fungi and plants (Supplementary Fig. 1). We used DNA homologous recombination to disrupt the gene encoding MEC-17, MEC17*,* in the ciliate *Tetrahymena thermophila* (Supplementary Fig. 2). Immunofluorescence with 6-11 B-1, a monoclonal antibody (mAb) that is specific for acetyl-K40 on a-tubulin[20] showed a marked loss of acetyl-K40 in *Tetrahymena* cells lacking MEC17 (MEC17-KO) (Fig. 1a-c). Western blots with 6-11 B-1 mAb showed a nearly complete loss of acetyl-K40 a-tubulin in MEC17-KO cells, comparable to cells carrying a K40R substitution in a-tubulin (Fig. 1g,h). Consistently, 2D SDS-PAGE showed that MEC-17-KO a-tubulin isoforms are more basic than wild-type isoforms (Supplementary Fig. 3). On a western blot with pan-acetyl-K antibodies bands corresponding to a-tubulin and its proteolytic fragments were missing in the MEC-

17-KO and K40R cell extracts, while a few non-tubulin bands (including histones) were present (Fig. 1g,h). In wild-type cells analyzed by immunofluorescence, the pan acetyl-K antibodies strongly labeled microtubules and nuclei (Fig. 1d). In the MEC-17-KO and K40R cells, acetyl-K was not detected on microtubules, but nuclei remained labeled (Fig. 1e,f). We conclude that in *Tetrahymena*, a-tubulin is the major if not the only substrate of MEC-17-dependent K acetylation.

The MEC-17-KO *Tetrahymena* cells had a normal growth rate (results not shown). However, the MEC-17-KO cells grew more slowly than *wild-type* on medium with the microtubule depolymerizing compound oryzalin. In MEC-17-KO cells treated with oryzalin, most axonemes depolymerized or were shorter than similarly-treated wild-type cells (Supplementary Fig. 4). Conversely, the MEC-17 KO cells grew faster than wild-type cells in medium with paclitaxel, a microtubule-stabilizing drug (Fig. 1i). This drug phenotype is consistent with an increase in dynamics of microtubules in MEC17-KO cells[21]. *Tetrahymena* cells with K40R a-tubulin had a similar drug phenotype (Fig. 1i, Supplementary Fig. 4). These observations indicate that in *Tetrahymena*, MEC-17 regulates the dynamics of microtubules by acetylation of K40 on a-tubulin.

MEC-17 is required for the maintenance of TRNs in *C. elegans*[5,6]. The W06B11.1 gene encodes a protein closely related to MEC-17[5,6]. Using 6-11 B-1 mAb, we confirmed that *C. elegans* wild-type adults have a strong signal for acetylated a-tubulin in the six TRNs[19] (Fig. 2a). Single *mec-17* or W06B11.1 mutants retained normal levels of acetylated microtubules in the TRNs (Fig. 2c,d). However, double *mec-17* and W06B11.1 mutants lacked an acetyl-K40 signal in the TRNs similar to *mec-12* a-tubulin mutants (Fig. 2e,f). Thus, MEC-17 and W06B11.1 are redundantly required for acetylation of

K40 on MEC-12 a-tubulin. W06B11.1 or *mec-17* single deletion mutants had reduced touch responsiveness, and a loss of both genes reduced the touch responsiveness further (Fig. 2g). Next, we investigated the role of MEC-17-dependent acetylatable K40 of a-tubulin. MEC-12 is the only a-tubulin with K40, and *mec-12(e1607)* (probable null allele[22]) worms have greatly reduced touch responses. Using Mos1 transposon excision repair[23], we integrated single transgenes encoding MEC-12 with either wild-type K40 or K40R or K40Q substitutions into the *mec-12(e1607)* mutant. The MEC-12-K40 transgene restored the levels of touch response to ~ 80% that of *wild-type* (Fig. 2g), while animals with either MEC-12-K40R or MEC-12-K40Q showed reduced touch response. With the limitation that the wildtype MEC-12 transgene does not fully restore touch sensation, and taking into account that *mec12(e1607)* mutants have a basal level of touch response, we calculate that a non-acetylatable MEC-12 is 30-33% less efficient than wild-type MEC-12. Nevertheless, animals with K40 substitutions on MEC-12, do respond to touch more frequently than animals lacking MEC-17 and W06B11.1. Thus we surmise that MEC-17 and W06B11.1 contribute to touch sensation partly by acetylating a-tubulin on K40, and through a second mechanism, likely by acetylation of a non-tubulin substrate(s).

We used zebrafish to test whether MEC-17 is required for a-tubulin acetylation in vertebrates. Acetyl-K40 a-tubulin is enriched in cilia[24] and axons of neurons in zebrafish[25]. Zebrafish has a single MEC-17 ortholog, zgc:65893 (*mec17*). We injected wild-type zebrafish embryos with morpholinos (MOs) that target either the translation initiation region or a predicted splice junction of *mec17*. The splice junction MO caused a severe reduction in the levels of *mec17* mRNA, possibly by nonsense-mediated mRNA

decay (Supplementary Fig. 5). Both MOs produced similar developmental defects, including curved body shape, short body axis, hydrocephalus, small head and small eyes (Fig. 3a,b and results not shown). The vast majority of control embryos injected with random sequence MOs or 5 bp mismatched MOs appeared normal (Supplementary Table 1,2). The *mec17* morphants often did not respond or had slow startle response when probed with a needle, consistent with neuromuscular defects (Table 1,2, Supplementary videos S1-3). Immunofluorescence of wild-type embryos with 6-11 B-1 showed that acetyl-K40 carrying microtubules are abundant in the nervous system, including the brain, optical nerves, spinal cord, and axons of peripheral nerves (Fig. 3c-c'') and in cilia (Fig. 3c"). Strikingly, *mec17* morphants showed a nearly complete loss of 6-11 B-1 signal in neurons (Fig. 3d-d'), but not in cilia (Fig. 3d"). The axons of primary motor neurons in the trunk were strongly labeled by the 6-11 B-1 mAb in controls but not in morphants (Fig. 3c', d'); synaptotagmin 1 localization at synaptic termini[26] indicates that the morphants do contain axons (Fig. 3e,f). Depletion of human MEC-17 (C6orf134) in HeLa cells using siRNAs reduced the levels of acetyl-K40 a-tubulin (Fig. 4i), indicating that MEC-17 is also required for a-tubulin acetylation in mammals.

Overexpression of GFP-Mec17p in *Tetrahymena* greatly increased acetylation of microtubules (Fig. 1j-l). Expression of a murine homolog, MmMEC-17 (Q8K341), in PtK2 cells (which have naturally low acetyl-K40 a-tubulin), induced massive acetylation of cytoplasmic microtubules (Fig. 4a-h). The above observations indicate that either MEC-17 has intrinsic aTAT activity or is an activator of aTAT. To test whether MEC-17 alone can mediate K40 acetyltransferase activity, we established a tubulin acetylation assay using axonemes purified from *Tetrahymena* MEC17-KO, with acetyl-CoA. A crude

GFP-Mec17p-enriched fraction (obtained from transgenic *Tetrahymena*) had K40 aTAT activity *in vitro* that was dependent on the presence of acetyl-CoA (results not shown and Fig. 5a, lanes 1-3). Next, we assayed a recombinant MmMEC-17 (expressed in *E. coli* as a GST fusion, supplementary Fig. 6) on MEC17-KO axonemes. GST-MmMEC-17, but not GST, mediated a robust aTAT activity *in vitro* (Fig. 5a, lanes 4,5). To test whether the MEC-17 activity is specific to the K40 residue, we assayed GST-MmMEC-17 with axonemes from either a MEC17-KO *Tetrahymena* (K40 a-tubulin) or from a K40R *Tetrahymena* mutant (R40 a-tubulin) and used pan acetyl-K antibodies to detect acetyl modification of any K residue. GST-MmMEC-17 modified K40 axonemes (Fig. 5b, lanes 3,4) but failed to acetylate R40 axonemes (Fig. 5b, lane 5). Thus, the activity is specific to K40. Since axonemes are composed of tubulin and MAPs, there is a possibility that *MEC-17* activates another protein that is an axoneme-bound aTAT. When MEC-17-KO axonemes were pretreated with 1M salt to remove MAPs, no loss of activity was detected, suggesting that MEC-17 does not require an axoneme-associated cofactor (Supplementary Fig. 7a). To test whether MEC-17 has intrinsic activity, we performed an *in vitro* acetylation assay with highly purified tubulin obtained from *Tetrahymena* MEC17-KO cells (Fig. 5d). GST-MmMEC-17 mediated a robust K40 acetylation activity on purified tubulin that was comparable to the level of activity seen with axonemes (Fig. 5c, lanes 4,8). The activity of GST-MmMEC-17 was stimulated when purified tubulin was exposed to GTP to promote tubulin polymerization (Fig. 5c, lanes 3,4). Paclitaxel also stimulated MEC-17 activity, likely by promoting microtubule polymerization (Fig. 5c, lanes 8,9 and Supplementary Fig. 7b). These data indicate that MEC-17 has an intrinsic a-tubulin acetyltransferase activity. The K40 residue of a-tubulin is located on

the luminal surface of the microtubule[1]. When the MEC-17-KO *Tetrahymena* axonemes were subjected to *in vitro* acetylation by GST-MmMEC-17, the acetyl-K40 signal was observed near one or both axoneme ends, often as a decreasing gradient from the microtubule end (Supplementary Fig. 8). This supports the model that MEC-17 enters the microtubule lumen from the microtubule end.

To conclude, we identified MEC-17 as an a-tubulin K40 acetyltransferase. We show that MEC-17 is important in the nervous system in both vertebrates and invertebrates. Importantly, another aTAT enzyme likely exists. MEC-17 sequences, are absent from *Chlamydomonas reinhardtii*, an organism that has aTAT activity[3,18] and zebrafish embryos depleted in MEC-17 showed a dramatic loss of acetyl-K40 in neurons but not in cilia. A recent study revealed that ELP3, a conserved histone acetyltransferase, is required for normal levels of K40 acetylation and the differentiation of cortical neurons in the mouse[12]. However, an ELP3 expressed in insect cells and partially purified was associated with only weak aTAT activity *in vitro*[12]. Moreover, TRN microtubules remain highly acetylated in *C. elegans elpc-3* mutants, which lack the sole ELP3 homolog (Fig. 2b and[27,28]). NAT1-ARD[29] and NAT10[30] are also associated with acetylated microtubules, but it is not known whether these proteins have intrinsic aTAT activity. Thus, the identity of the second aTAT remains uncertain.

Tetrahymena cells lacking a-tubulin acetylation are resistant to paclitaxel and sensitive to oryzalin, consistent with an increase in microtubule dynamics[21]. Based on these studies, MEC-17-mediated K40 acetylation could mildly stabilize microtubules. It remains to be determined whether changes in microtubule dynamics are a direct effect of acetyl-K40 or are mediated by microtubule effector proteins. We show that in *C. elegans*,

MEC-17 contributes to TRN function partly by acetylating K40 on MEC-12 a-tubulin and partly by other means. For example, MEC-17 could acetylate another protein, or act as a MAP, possibly inside the microtubule lumen.

METHODS

To disrupt the MEC-17 gene in *Tetrahymena*, we used homologous DNA recombination with a fragment carrying the *neo4* marker that replaced the coding region. MEC-17 was overexpressed in *Tetrahymena* using the *MTT1* cadmium-dependent promoter. In *C. elegans*, MEC-12-K40, MEC-12-Q40, and MEC-12-R40 transgenes were introduced into a single site on chromosome II in the EG4322 strain. Animals homozygous for a MEC-12 transgene and homozygous for the *mec-12(e1607)* allele were obtained by standard crosses. All touch sensation assays in *C. elegans* were done using blind scoring. To deplete human MEC-17 (C6Orf134) mRNA in Hela cells, we introduced MEC-17-specific siRNAs (ON-TARGETplus pool, Dharmacon) using Oligofectamine (Invitrogen). To knockdown *mec17* expression in zebrafish, MOs designed to target the MEC-17 mRNA (Open Biosystems) were injected into early embryos. ATG-MEC17 MO targets the translation initiation site of *mec17* mRNA. SP-MEC17 MO targets the exon3/intron3-4 splice junction, and is expected to result in an aberrant splicing isoform of exon2 to exon 4, producing a frameshift mutation and associated protein truncation. As a negative control, we injected MO with a random sequence (oligo-25N, Gene Tools) or a 5bp mismatch to the ATG-MEC17 MO.Live embryos were scored for phenotypes at 48 hpf. To produce a recombinant MEC-17 protein, the cDNA sequence of the murine MEC-17 (BF135007, Open Biosystems) was subcloned into pGEX-3X plasmid (GE

Healthcare), expressed in BL21 *E. coli* cells as a GST fusion and purified using GST-Bind kit (Novagen). The *in vitro* acetylation assays were performed in 50 mM Tris-HCl pH 8.0, 10 mM glycerol, 0.1 mM EDTA, with purified *Tetrahymena* MEC-17-KO axonemes or tubulin (purified using DEAE chromatography), recombinant GST-MmMEC-17 enzyme and 10 mM acetyl-CoA. The reaction was detected by western blotting using anti-acetyl-K antibodies.

Tetrahymena.

For disruption of the Tetrahymena MEC17 gene the two targeting fragments (1.4 kb of 5' UTR and 2.0 kb of the coding region with 3' UTR) of the MEC17 locus were designed and subcloned on the sides of the *neo4* selectable cassette[31]. The fragments were amplified with the addition of restriction sites with the following pairs of primers: 5'-ATTGT**GGGCCC**TAGCATTTCTGGAAGATTCATTC-3' (ApaI), 5'-AATA**CCCGGG**CAATTGAATGTATGTGCTGAT-3' (SmaI) and 5'-AAATT**CTGCAG**TTAGTACTTTAGAAGTGATGCT-3' (PstI), 5'-AAATT**GAGCTC**TCTAGTTGACTATATTATGCATTC-3' (SacI). The fragments were designed to remove a small part of the 5'UTR and most of the coding region and insert the *neo4* resistance cassette in reverse orientation. CU428 and B2086 mating cells were biolistically transformed as described[32]. Heterokaryons with a germline disruption of *MEC17* and progeny cells homozygous for the disruption in the micronucleus and macronucleus were obtain by a heterokaryon x heterokaryon mating[33].

For overexpression of GFP-Mec17p, the coding region of MEC17 was amplified with primers 5'-ATATT**ACGCGT**CATGGAGTTTAACTTCATCATTAATAG-3' and 5'-ATATT**GGATCC**TCATTTTTTGTAGTATGTGTAGTGAT-3' and subcloned

between the MluI and BamHI sites of pMTT1-GFP plasmid[34] and the MTT1-GFP-MEC17-BTU1 fragment was integrated into the *BTU1* locus by biolistic bombardment and paclitaxel selection[35]. The expression of GFP-Mec17p under the MTT1 promoter was induced with 2 mg/ml $CdCl_2$ for 2 hr.

For immunofluorescence, cells were prepared as described (Wloga et al., 2006) and stained overnight with the following antibodies: anti-acetylated K40 a-tubulin 6-11 B-1 mAb 1:200 dilution[36]; pan anti-acetyl-K antibodies (ImmuneChem, ICP0380) at 1:150 dilution; anti-a-tubulin 12G10 mAb ([37], Developmental Studies Hybridoma Bank at 1:25; and polyclonal anti-tubulin antibodies (SG, 1:600). To compare the levels of tubulin acetylation side-by side, *wild-type* cells were marked by feeding for 10 min with India Ink and mixed with MEC17-KO cells.

For western blotting studies, *wild-type* (CU428), MEC17-KO and K40R mutant cells[8] were grown to the mid-log phase. The cytoskeletal fractions were prepared as described[38] except that trichostatin A at 1 mg/ml was added to concentrated cells prior lysis. Total extracts of 2 x 10^4 cells, or 5 mg of cytoskeletons per lane were used for western blotting with the following antibodies: 12G10 mAb (1:5000); 6-11 B-1 mAb (1:5000); pan anti-acetyl-K antibodies (1:300); hv1 anti-histone (1:2000).

Zebrafish.

To knockdown MEC-17 expression in zebrafish, two morpholinos designed to target the MEC-17 mRNA (Open Biosystems) were injected into embryos (at 3 ng/embryo): ATG-MEC17 (5'CATTCAGGTCGTAAGGGAAATCCAT-3') and SP-MEC17 (5'-AGAGAAAGCTATTTTACCCGTTCTG-3'). ATG-MEC17 targets the translation initiation site of MEC-17 mRNA. SP-MEC17 MO targets the exon3/intron3-4 splice

junction, and is expected to result in an aberrantly spliced isoform in which exon2 is joined to exon 4. The predicted transcript contains a frameshift mutation and encodes a nonsense protein. As a negative control we injected MO with a random sequence (oligo-25N, Gene Tools) or a 5bp mismatch to the ATG-MEC17 MO (5'-CATTgAcGTCcTAAGGcAAATgCAT-3'). Live embryos were scored for phenotypes at 48 hpf, or fixed processed for immunofluorescence as described[39]. The antibodies were used in the following concentrations: 6-11 B-1 mAb (1: 1000), Znp-1 anti-synaptotagmin 1 mAb (1: 100). After incubation with secondary antibodies (Zymed) overnight at 4^{o}C (1: 500) embryos were mounted in 100 mg/ml of DABCO (Sigma-Aldrich) in PBS and viewed in a Leica TCS SP confocal microscope. Live zebrafish morphants shown in videos S1-S3 were recorded using on the Zeiss Stemi SV11 Apo microscope and a SPOT FLEX camera (Diagnostic Instruments Inc.) at 12 frames per second.

For RT-PCR, mRNA was isolated from 70 embryos 24 hpf using TRIzol reagent (Invitrogen, Carslbad, CA), and total cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules,CA). The sequences of primers used to amplify the *mec17* cDNA were: MEC17EX1F 5'-GGTCGGAAAGCGCATGGGAG-3' and MEC17EX5R2 5'-GAAGTCGAAGAGCTCTGAGCC-3'. The forward primer binds to exon 1 and the reverse primer binds to exon 5 (the splice blocking morpholino binds to the junction between exon 3 and the intron 3-4). For the control amplification of b-actin cDNA, we used the following primers: 5'-GATTCGCTGGAGATGATG-3' and 5'-GTCTTTCTGTCCCATACCAA-3'.

C. elegans.

C. elegans strains were cultured as described[40]. The following strains were used: N2, *wild-type*; CB1607, *mec-12(e1607)* III; RB1696, *mec-17(ok2109)* IV; RB1869, *W06B11.1(ok2415)* X; ET389, *mec-17(ok2109)* IV,*W06B11.1(ok2415)* X; VC1937, *elpc-3(gk2452)* X; ET431, *mec-12(e1607)* III, *ekSi1[Pmec-12::MEC-12::3'UTR mec-12]* II; ET432, *mec-12(e1607)* III, *ekSi2[Pmec-12::MEC-12(K40Q)::3'UTR mec-12]* II; ET433, *mec-12(e1607)* III, *ekSi3[Pmec-12::MEC-12(K40R)::3'UTR mec-12]* II; EG4322, ttTi5605; *unc*-119(ed3).

A MEC-12 plasmid, p*MEC-12*, was constructed using a MEC-12 cDNA and genomic sequence as described[19]. Using overlapping PCR, we mutated the K40 codon to either a Q or R codon on pMEC-12 along with silent substitutions creating restriction sites (PvuI site for R40 plasmid and HindIII site for Q40 plasmid) and confirmed the mutagenesis by sequencing the entire plasmids. The K40, R40 or Q40 derivatives of pMEC-12 were used to prepare targeting plasmids for Mos-SCI[23] as follows. pCFJ151 is a plasmid vector designed to target fragments into the ttTi5605 locus on chromosome II[23]. A 1.7Kb fragment pMEC-12 comprising a part of the MEC-12 cDNA sequence and 3'UTR was amplified using the primers 5'-ATTATGTTTAAACCAAG**CTCGAG**TTCTCCATC-3' (PmeI site is underlined and XhoI site shown in bold) and 5' AATTATGATCACAGCA AAG GAT TCA AGG CTC3' (BclI site is underlined), digested with BclI and BglII and inserted into a modified pCFJ151 lacking its original XhoI site (as a result of earlier XhoI digestion, blunting and religation). The resulting plasmid was digested with PmeI and XhoI and used for insertion of a 5.7 Kb of 5'UTR and a part of cDNA amplified from either pMEC-12-K40,

or p-MEC-12Q40 or pMEC12-R40 using primers 5'-ATAATGTTTAAACCGGCGAGAAGAGCTATCAA-3' (with PmeI site underlined) and 5'-AATTTGGAGAACTCGAGCTTGGCC-3' (with XhoI underlined). The resulting plasmids: pCFJ151-MEC-12-K40, pCFJ151-MEC-12-Q40 and pCFJ151-MEC-12-R40 were used for introduction of single copy MEC-12 transgenes into a site on chromosome II of the EG4322 strain and integrant animals were identified as described[23]. Strains homozygous one of the three *MEC-12* transgene types and homozygous for the *mec-12(e1607)* probable null allele[22] were obtained by standard crosses. All touch assays were done using blind scoring. To determine the touch response level, 30 L4 larvae were isolated on a 5 cm 1x NGM OP50 seeded plate and adult animals were scored for touch responses after 24 hr. Each animal was touched 10 times by moving an eyebrow hair across the body below the anterior and posterior ends. The level of touch response was calculated as an average number of responses per 10 touches.

For immunofluorescence, animals were made permeable by the 'freeze-crack' method, followed by methanol and acetone fixation (10 min at -20ºC for each)[41], and probed with the primary antibody (6-11 B-1 mAb, 1:500 dilution) and the secondary antibody (anti-mouse rhodamine (Cappel, 1:50). Animals were observed with a Zeiss Axioskop microscope equipped for differential interference contrast (DIC) and fluorescence microscopy. Images were captured with a Hamamatsu ORCA-ER digital camera with Openlab 5.0.2 software (Improvision). Images were processed using Adobe Photoshop CS2. Matched images were taken with the same exposure and processed identically. There was no statistical significance between the acetyl-K40 signal intensities over TRNs (after adjacent background subtraction) in *wild-type*, *mec-17*, and W06B11.1

single mutant strains (241±117; 294±111; and 252±162 arbitrary pixel intensity units, respectively) (Fig. 2a,c,d). The signal intensity in *mec-12* mutants or the double mutant *mec-17*; W06B11.1 could not be determined because there was no detectable TRN signal to measure (Fig. 2e,f).

Mammalian Cells

PtK2 rat kangaroo kidney epithelial cells were grown in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine and an antibiotics mix at 37°C with 5% CO_2. For transfection, cells were grown in 24-well plates to 80-90% of confluency, and transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer instructions, using either 20 ng of pEGFP-N1 plasmid (Clontech) plasmid alone or 20 ng of pEGFP-N1 and 800 ng of pCMV-SPORT6-mmMEC17 (Open Biosystems, MMM1013-7510854) for 16-20 h. Following transfection, cells were grown for 48 hr, split onto coverslips and grown for another 24 hrs and subjected to immunofluorescence. Coverslips with cells were rinsed with PBS, fixed in 4% paraformaldehyde in PBS for 12 min and permeabilized in 0.5% Triton-X-100 in PBS for 15 min. After permeabilization, coverslips were incubated in 3% BSA in PBS for 10 min and incubated in primary antibodies diluted in 3% BSA in PBS (6-11 B-1 anti-acetyl-K40 at 1:300 1:10 and polyclonal anti- a-tubulin (Sigma-Aldrich) 1:10 for 2 hr. After 3 x 5 min washes with PBS cells were incubated in secondary antibodies: anti-mouse IgG-Cy3 in 3% BSA in PBS at 1:100 for 1 hr, washed 3 times and mounted with 100 mg/ml DABCO (Sigma) in PBS and viewed in a Leica TCS SP confocal microscope.

To deplete human *MEC-17* (C6Orf134) mRNA in Hela cells, we used ON-TARGETplus siRNAs from Dharmacon as a pool of four siRNAs; the sequences are as follows:

5'-GUAGCUAGGUCCCGAUAUA-3' (#1);

5'-GAGUAUAGCUAGAUCCCUU-3' (#2);

5'-GGGAAACUCACCAGAACGA-3' (#3);

5'-CUUGUGAGAUUGUCGAGAU-3' (#4).

GFP siRNA, 5'-GCUGACCCUGAAGUUCAUCUGdTdT-3' (Invitrogen) was used as a negative control. HeLa cells were grown as above and transfected with 100 nM of siRNAs (in the pool, each siRNA was at 25 nM) using Oligofectamine (Invitrogen) transfection reagent in accordance with the manufacturers' instructions. Transfections were performed three times sequentially, followed by subculturing into the new wells. Fifty hr after the first transfection, 300 nM of Trichostatin A in DMSO or the same volume of DMSO were added to the cell cultures, and cells were grown for another 7 hr. Cells were collected and lysed with boiling Laemmli loading buffer containing 2.5% SDS. Lysates of equal number of cells were analyzed using SDS-PAGE/western blot with mouse antibody against acetylated α-tubulin (6-11B-1, Sigma), 1:1000, and mouse anti-α-tubulin antibody (DM1A, Sigma), 1:10,000.

Substrates for in vitro tubulin acetylation.

To prepare MEC17-KO axonemes, *Tetrahymena* cells were grown to the mid-log phase and deciliated by pH shock[42]. Cilia were suspended in 1 ml of 1% NP-40 in the axoneme buffer (30 mM HEPES, 20 mM potassium acetate, 5 mM $MgSO_4$, 0.5 mM EDTA, pH 7.6) with Complete protease inhibitors (Roche). After 1-2 min on ice, axonemes were collected by centrifugation (20,000 x g, 15 min, 4^{o}C), suspended in the *in vitro*

acetylation reaction buffer (50 mM Tris HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, 1mM DTT) with protease inhibitors and stored at -80^{o}C.

Total tubulin was purified from the MEC17-KO strain of *Tetrahymena* using a protocol modified after Yakovich and colleagues[43]. *Tetrahymena* cells (2 x 10^9) were suspended in 40 ml of PME+P buffer (0.1 M Pipes pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, and 25 μg/ml leupeptin) on ice. Cells were sonicated on ice using a Sonic Dismembrator Model 100 (Fischer Scientific) with ten 30 sec bursts at 25 W with a 2 min cooling interval between each burst. The lysate was incubated on ice for 30 min and centrifuged at 40,000 x g for 30 min at 4°C. The supernatant was filtered through glass wool and loaded into a 10 ml column DEAE-Sepharose Fast Flow Matrix (GE Healthcare, earlier equilibrated with two volumes of PME+P) at a rate of 2.5 ml/min using a peristaltic pump. The column was washed with two column volumes of PME+P and followed by four column volumes PME+P with 0.1 M KCl, 0.25 M glutamate pH 6.9. Tubulin was eluted with two column volumes PME+P and 0.3 M KCl, 0.75 M glutamate pH 6.9. Two and half ml fractions were collected. Fractions 6 through 8 were pooled and supplemented with 10 mM $MgCl_2$, 8% DMSO (v/v), and 2 mM GTP. The tubulin-rich pooled fraction was incubated at 37 °C for 60 min to induce microtubule assembly and centrifuged at 50,000 x *g* at 30 °C for 30 min. The pellet consisting of microtubules was rinsed once with warm PME (~37 °C), and suspended in ~1.5 ml of ice-cold PME. The pellet was solubilized by sonication (thirty ~5 s bursts at 10 W). The tubulin solution was incubated on ice for 30 min, and centrifuged 50,000 x *g* at 4 °C for 30 min. The supernatant containing highly purified dimeric tubulin was stored at −80 °C in 50 μl aliquots.

To polymerize tubulin, 100 ml of purified *Tetrahymena* MEC-17-KO tubulin (5.5 mg/ml) in PME buffer (100 mM Pipes, 1 mM $MgCl_2$, 1 mM EGTA, pH=6.9) was combined with 80 ml of BRB80 buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8), 2 ml of 100 mM GTP and 1 ml of 0.2 M DTT and incubated for 1 hr at 37^{o}C. Microtubules were collected by centrifugation (13,000 rpm, 10 min at room temperature) and the pellet was suspended in the acetylation reaction buffer (see above).

Expression and purification of MEC-17 *enzymes.*

Tetrahymena cells with a GFP-Mec17p encoding transgene under MTT1 promoter were grown without paclitaxel to the density 2 x 10^5 cells/ml (25 ml) and overexpression was induced by incubation with 2.5 mg/ml of $CdCl_2$ for 3 hr. The cells were collected by centrifugation, washed with Tris-HCl buffer, pH7.5, suspended in the cold *in vitro* acetylation buffer with protease inhibitors and gently homogenized on ice using a Dounce tissue grounder. The homogenate was centrifuged for 20 min at 20,000 x g at 4^{o}C and the supernatant stored in aliquots at -80^{o}C.

To express recombinant MEC-17 proteins, the pCMV-SPORT6 plasmid containing a full cDNA sequence of the murine MEC-17 ortholog (BF135007, Open Biosystems) was used as template for amplification of the coding regions with primers: 5'-AAATT GAGCTCTGGAGTTCCCGTTCGATGTGGAT-3' and 5'AATA GAATTCCCGCGGACTAAGCTTTGGCCATGG**TTA**CC-3'. The fragment was subcloned into pGEX-3X expression vector (GE Healthcare). The *E.coli* BL21 cells carrying either pGEX-3X (GST) or pGEX-3X –MmMEC-17 (GST-MmMEC-17) plasmids were grown in 3 ml cultures of LB medium with ampicilin (50 mg/ml concentration) overnight at 37^{o}C with shaking. A 1.5 ml of culture was transferred into

25 ml of LB medium, IPTG was added to 1 mM final concentration and bacteria were grown for 2.5-3 hrs at 37^{o}C with shaking. Bacteria were collected by centrifugation (6000 x g, 10 min), washed with 25 ml of cold washing buffer (20 mM Tris-HCl, pH 8.0, 0.2M NaCl, 10% glycerol, 2 mM EDTA) and centrifuged as above. Bacteria were suspended in 1ml of washing buffer supplied with 50mM b-mercaptoethanol, 0.5mM PMSF, 10 mg/ml leupeptin, 5 mg/ml DNAse I, 10 mg/ml RNAse A, 1 mg/ml lysozyme, subjected to 2-3 rounds of freezing at -80^{o}C (20-30 min each) followed by thawing on ice, followed by 10 passages through a syringe with an 18 gauge needle. The homogenate was centrifuge at 16,000 x g for 20 min at 4^{o}C and GST-tagged recombinant proteins were purified with GST-Bind Kit (Novagen) according to manufacturer instructions. The recombinant proteins were stored aliquoted at -80^{o}C.

In vitro tubulin acetyltransferase assay

The assays were performed in a buffer that was used earlier for histone acetyltransferases containing 50 mM Tris-HCl pH 8.0, 10 mM glycerol, 0.1 mM EDTA, 1 mM DTT [44] in 50 µl volumes that included 5 ml of purified *Tetrahymena* MEC-17-KO axonemes or tubulin, 10 ml of GFP-*Mec-17*p supernatant or purified GST-MmMEC-17 enzyme and 0.5 ml of 1mM acetyl-CoA). Samples were incubated for 60-90 min at 28^{o}C. The reaction was stopped by addition of 5X SDS sample buffer and heating for 5 min at 96^{o}C. Proteins from 10 ml of samples were separated on 10% SDS-PAGE gel and transferred onto nitrocellulose and processed with 6-11 B-1 anti-acetylated K40 a-tubulin mAb (1:15,000) or pan anti-acetyl-K antibodies (1:500) or anti-a-tubulin antibodies (12G10, 1:10)

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FIGURES AND FIGURE LEGENDS

Figure 1. MEC-17 is required for acetylation of K40 on a-tubulin in *Tetrahymena*. a-c, Wild-type (prefed with ink) and MEC17-KO (arrow) cells labeled with anti-acetyl-K40 mAb (6-11 B-1) and anti-tubulin antibodies. **d-f**, Wild-type (d), MEC17-KO (e) and K40R (f) *Tetrahymena* labeled with pan anti-acetyl-K antibodies. **g-h**, Western blots of cells (g) or cytoskeletons (h) probed with 6-11 B-1 mAb, pan anti-acetyl-K, anti-a-tubulin (12G10 mAb) and anti-histone hv1 antibodies. Stars mark non-tubulin proteins. Arrows

mark acetylated histones. **i**, Growth curves of *Tetrahymena*. **j-l**, Wild-type (left) and GFP-Mec17p overproducing (right) *Tetrahymena* cells analyzed for GFP (j) or 6-11 B-1 mAb immunofluorescence (k).

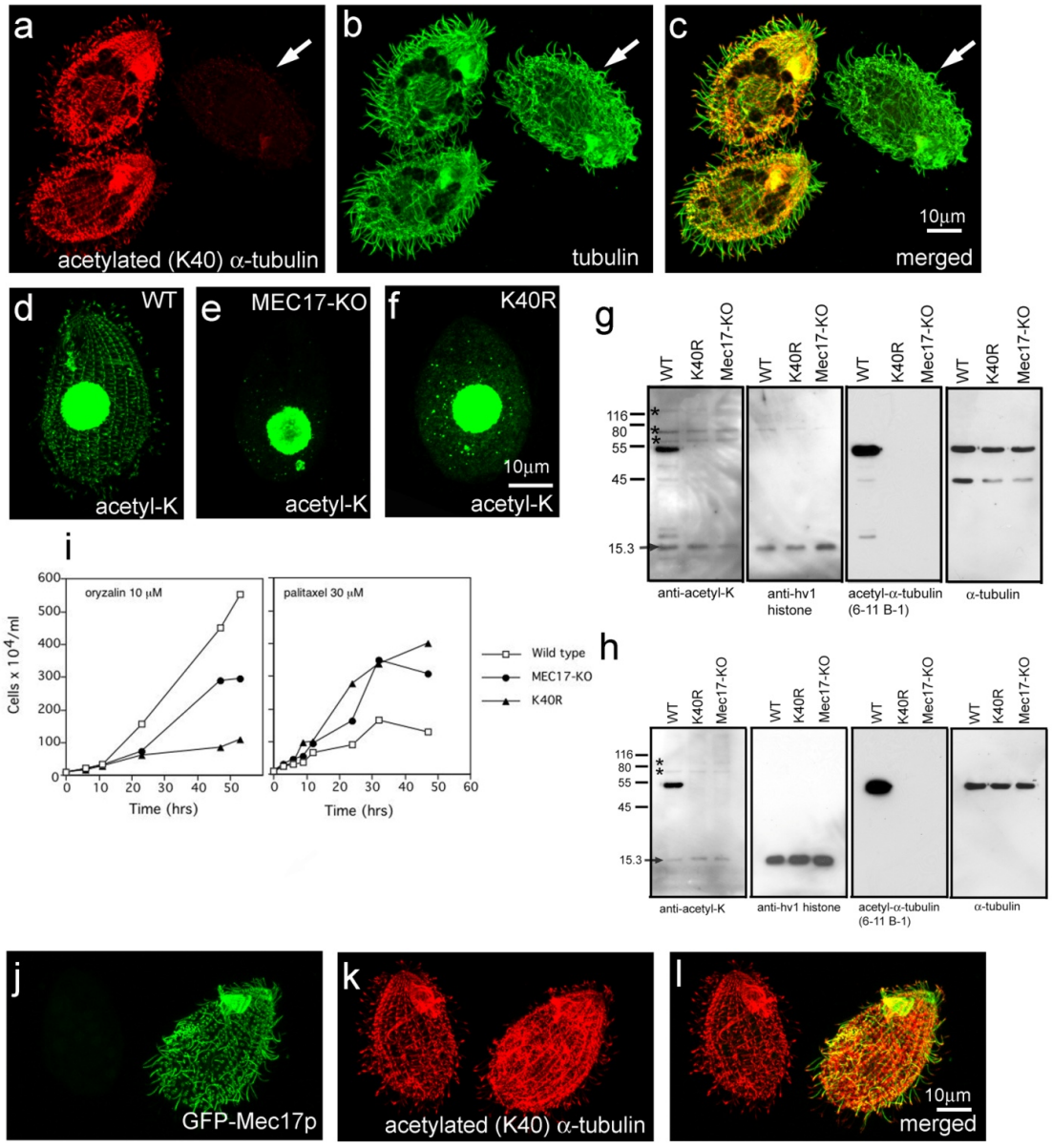
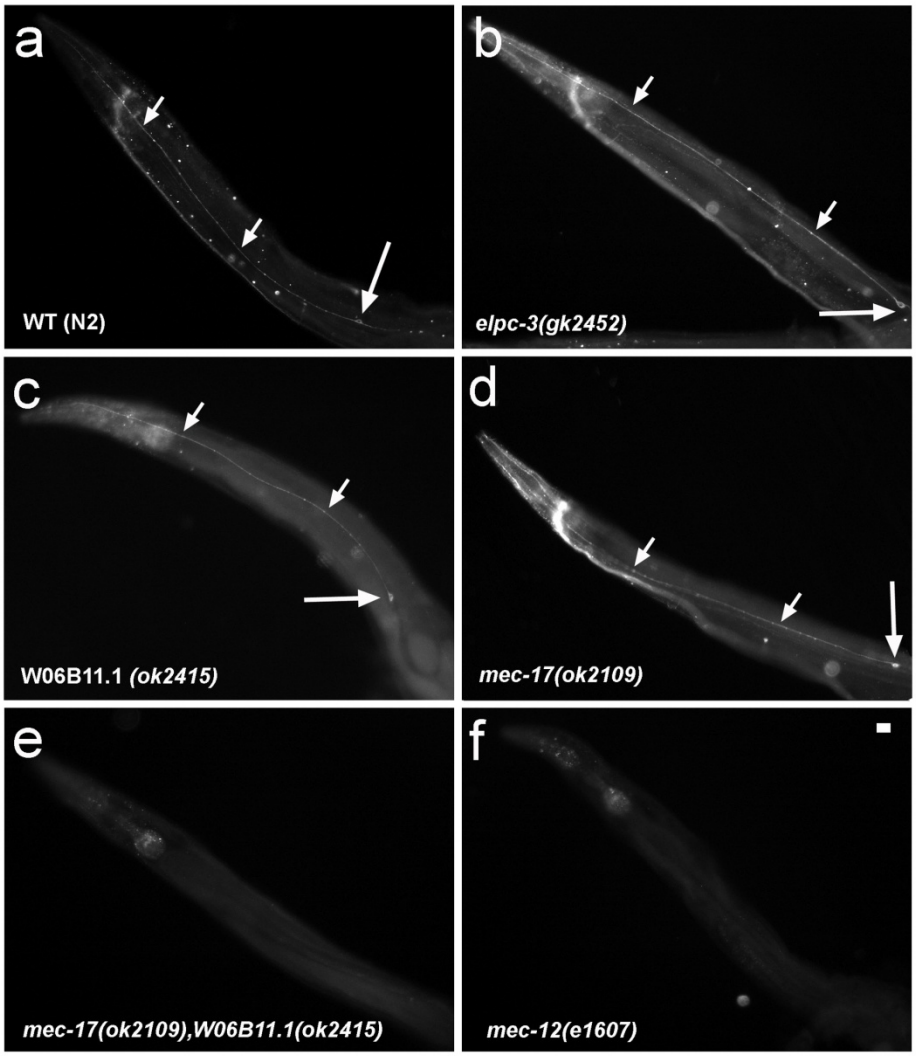


Fig 1

Figure 2. MEC-17 and W06B11.1 are required for acetylation of K40 and contribute to touch sensation in *C. elegans*. a-f, Wild-type and mutant adult hermaphrodites were labeled using 6-11 B-1 mAb. Small and large arrows mark axons and cell bodies of TRNs, respectively. Scale bar 10 mm. **g**, Histogram quantifying touch responses. The error bars represent SEM. Asterisk marks significant difference when compared to K40 transgene *mec-12(e1607)* ($p<0.0001$). The following numbers of animals were tested: *wild-type*, 69; *mec-12(e1607)*, 49; *mec-17(ok2109)*, 44; *W06B11.1(ok2415), 33*; *mec-17(ok2109) W06B11.1(ok2415)*, 140; K40 transgene *mec-12(e1607)*, 84; Q40 transgene *mec-12(e1607)* 78; R40 transgene *mec12(e1607)* 75.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WT (N2)
b
elpc-3(gk2452)
c
W06B11.1 (ok2415)
d
mec-17(ok2109)
e
mec-17(ok2109),W06B11.1(ok2415)
f
mec-12(e1607)
g
average number of positive responses per 10 touches
WT (N2)
mec-17(ok2109)
W06B11.1(ok2415)
mec-17(ok2109); W06B11.1(ok2415)
mec-12 (e1607)
no transgene
K40 transgene
Q40 transgene
R40 transgene
*
*

Fig 2

Figure 3. MEC-17 is required for K40 acetylation in zebrafish and normal embryonic development. Control embryos (a,c,c',c",e) and embryos injected with MEC17-ATG morpholinos, 48 hr post fertilization (hpf). (b,d,d',d",f) were observed live (a,b) or subjected to immunofluorescence 48 hpf using either 6-11 B-1 mAb (c-d") or Znp1 mAb (e,f), which recognizes synaptotagmin 1. c' and d' show higher magnifications of the areas boxed in c and d. c" and d" show higher magnifications of the areas of pronephrons that contain cilia (marked with arrows in c" and d"). In e and f, arrows mark axons of peripheral neurons.

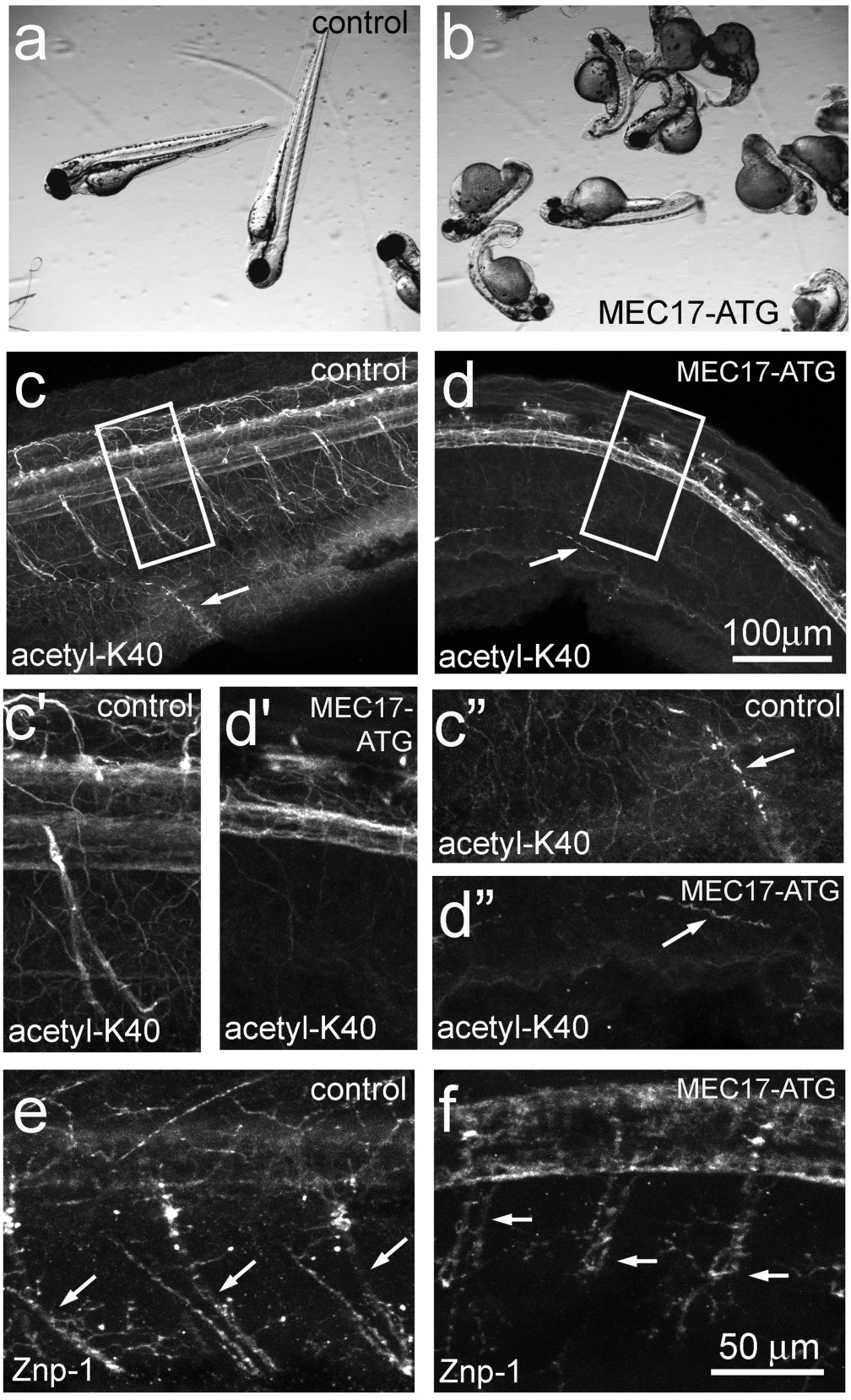

a
control
b
MEC17-ATG
c
control
acetyl-K40
d
MEC17-ATG
100µm
acetyl-K40
c'
control
acetyl-K40
d'
MEC17-
ATG
acetyl-K40
c"
control
acetyl-K40
d"
MEC17-ATG
acetyl-K40
e
control
Znp-1
f
MEC17-ATG
50 µm
Znp-1

Fig3

Figure 4. MEC-17 controls the levels of microtubule acetylation in mammalian cells. **a-h**, Expression of Mm-MEC-17 in Ptk2 cells increases the levels of acetyl-K40 a-tubulin. Cells expressing either EGFP or EGFP and Mm-MEC17 were stained with 6-11 B-1 mAb and anti-a-tubulin antibodies. **i**, Depletion of Hs-MEC-17 in HeLa cells reduces the level of acetyl-K40 a-tubulin. Cells were transfected with either GFP or Hs-MEC17 siRNAs and after 50 hr, treated for 7 hr with either 300 nM trichostatin A (TSA, stock solution in DMSO) or DMSO alone. Cell lysates were analyzed by western blot probed with either 6-11 B-1 mAb (top, middle panels) or anti-a-tubulin mAb (bottom panel).

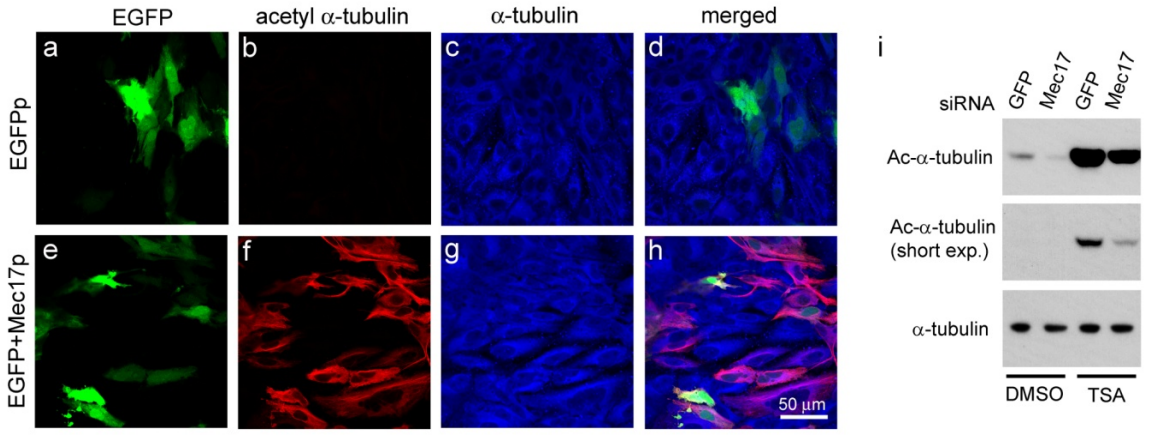


Fig 4

Figure 5. MEC-17 has intrinsic, K40-specific a-TAT activity. a, Crude *Tetrahymena* and recombinant murine MEC-17 were used for *in vitro* acetylation reactions of MEC17-KO axonemes and analyzed by western using 6-11 B-1 and 12G10 mAb. **b**, *In vitro* acetylation assays were performed with GST-MmMEC-17 using axonemes isolated from either the MEC17-KO (K40) strain or a K40R a-tubulin mutant. The marker (M) is acetylated glutamate dehydrogenase (55.6 kD). **c**, Recombinant GST-MmMEC-17 directly acetylates purified tubulin from the MEC17-KO strain *in vitro*. **d**, Coomassie Blue-stained gel with either purified MEC17-KO tubulin (36 ng) or porcine brain tubulin (15 ng, 99% pure, Cytoskeleton Inc).

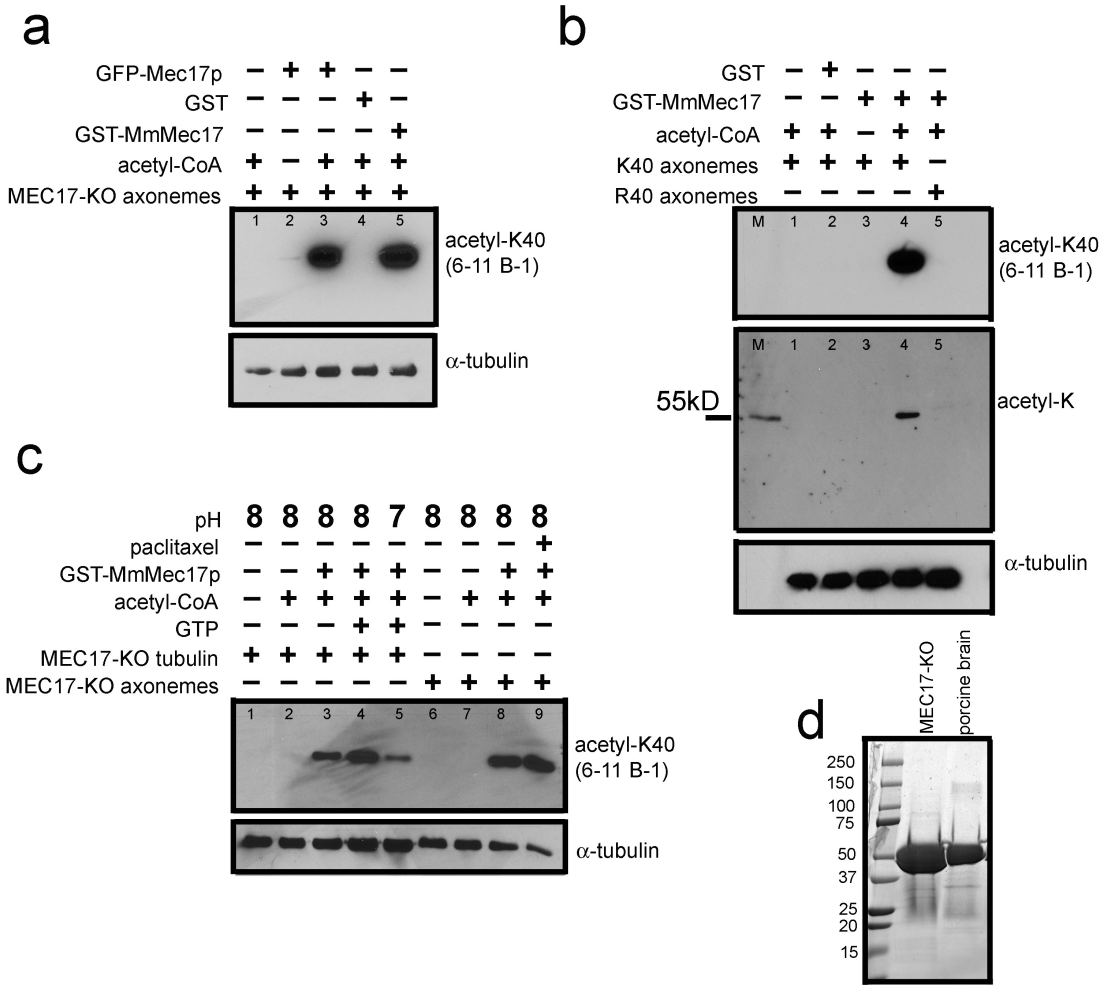

a
GFP-Mec17p
GST
GST-MmMec17
acetyl-CoA
MEC17-KO axonemes
1 2 3 4 5
acetyl-K40
(6-11 B-1)
α-tubulin
b
GST
GST-MmMec17
acetyl-CoA
K40 axonemes
R40 axonemes
M 1 2 3 4 5
acetyl-K40
(6-11 B-1)
55kD
acetyl-K
α-tubulin
c
pH 8 8 8 8 7 8 8 8 8
paclitaxel
GST-MmMec17p
acetyl-CoA
GTP
MEC17-KO tubulin
MEC17-KO axonemes
1 2 3 4 5 6 7 8 9
acetyl-K40
(6-11 B-1)
α-tubulin
d
MEC17-KO
porcine brain
250
150
100
75
50
37
25
20
15

Fig 5

SUPPLEMENTARY INFORMATION

Supplemental Tables.

Table S1. Frequencies of phenotypes observed at 48 hpf in fish injected with either random sequence, or MEC17-ATG or MEC17-SP MOs.

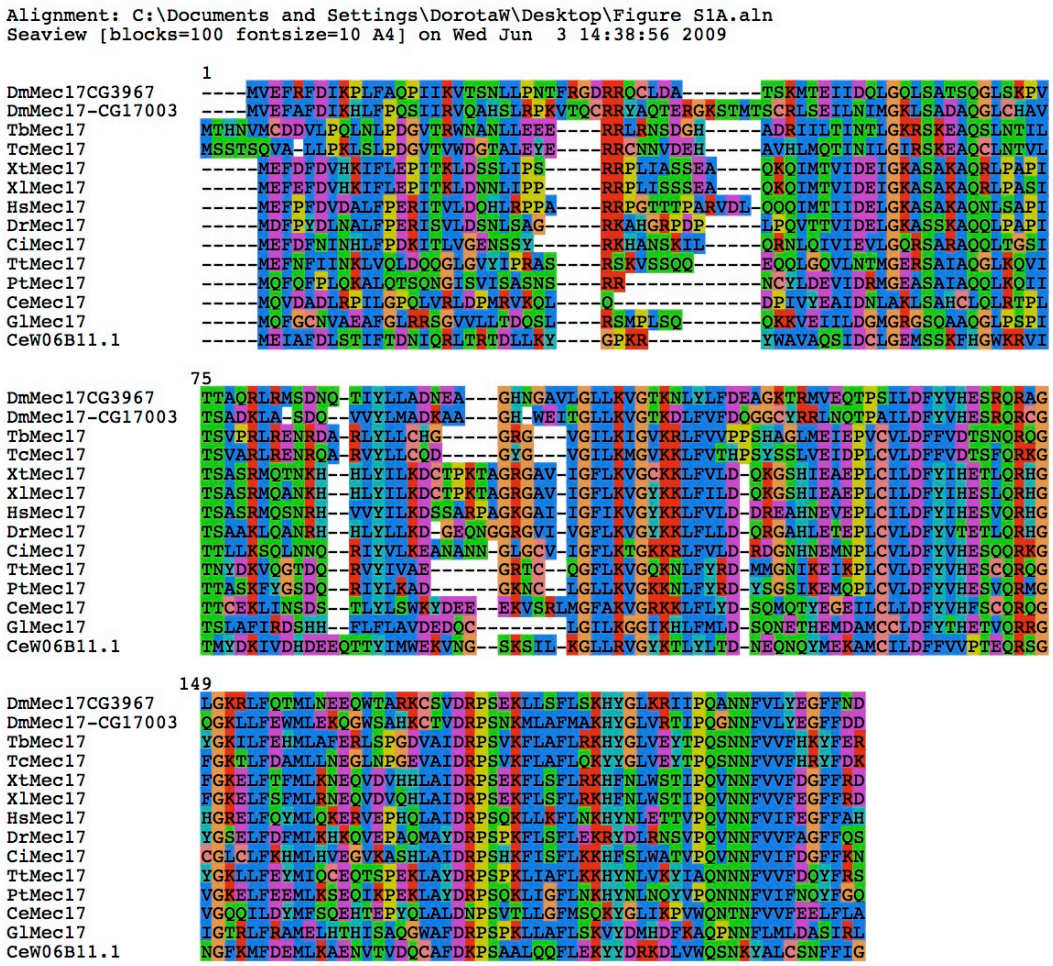
Phenotype	1ng control MO (n=87)	1ng MEC-17 ATG MO (n=84)	1ng MEC-17 SP MO (n=106)
Lethality	11%	7%	28%
Hydrocephaly	0%	15%	33%
Short body axis	4.5%	35.7%	41.5%
Edema of the heart	2%	15.4%	13.2%
Small head/eyes	3.4%	28.5%	47.1%
Reduced mobility	5.7%	41.6%	23.5%
Paralyzed	4.5%	15.4%	15%

Table S2. Frequencies of phenotypes observed at 48 hpf in fish injected with either MEC17-ATG or 5bp mismatch control MOs.

Phenotype	1ng ATG MIS MO (n=77)	1ng Mec-17 MO (n=188)
Lethality	2.5%	3.7%
Hydrocephaly	0%	13.8%
Edema of the heart	3.8%	7.9%
Short body axis	5.1%	27.1%
Small head/eyes	6.4% small heads	79.2% small heads
Mobility	2.5% reduced mobility	17.5% reduced mobility

Supplemental Figures.

Figure S1. Multiple sequence alignment of the catalytic domain of MEC-17 homologs from diverse species. The following species abbreviations were used: Dm, *Drosophila melanogaster*; Tb, *Trypanosome brucei*; Tc, *Trypanosoma cruzi*; Xt, *Xenopus tropicalis*; Xl, *Xenopus laevis*; Hs, *Homo sapiens*; Dr, *Danio rerio*; Ci, *Ciona intestinalis*; Tt, *Tetrahymena thermophila*; Pt, *Paramecium tetraurelia*; Ce, *Caenorhabditis elegans*; Gl, *Giardia lamblia*.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DmMec17-CG17003 ----MVEFAFDIKHLFPQSIIRVQAHSLRPKVTQCRRYAQTERGKSTMTSCRLSEILNIMGKLSADAQGLCHAV
TbMec17 MTHNVMCDDVLPQLNLPDGVTRWNANLLEEE----RRLRNSDGH-----ADRIILTINTLGKRSKEAQSLNTIL
TcMec17 MSSTSQVA-LLPKLSLPDGVTVWDGTALEYE----RRCNNVDEH-----AVHLMQTINILGIRSKEAQCLNTVL
XtMec17 -----MEFDFDVHKIFLEPITKLDSSLIPS-----RRPLIASSEA----QKQIMTVIDEIGKASAKAQRLPAPI
XlMec17 -----MEFEFDVHKIFLEPITKLDNNLIPP-----RRPLISSSEA----QKQIMTVIDEIGKASAKAQRLPASI
HsMec17 -----MEFPFDVDALFPERITVLDQHLRPPA----RRPGTTTPARVDL-QQQIMTIIDELGKASAKAQNLSAPI
DrMec17 -----MDFPYDLNALFPERISVLDSNLSAG-----RKAHGRPDP-----LPQVTTVIDELGKASSKAQQLPAPI
CiMec17 -----MEFDFNINHLFPDKITLVGENSSY------RKHANSKIL-----QRNLQIVIEVLGQRSARAQQLTGSI
TtMec17 -----MEFNFIINKLVQLDQQGLGVYIPRAS----RSKVSSQQ------EQQLGQVLNTMGERSAIAQGLKQVI
PtMec17 -----MQFQFPLQKALQTSQNGISVISASNS----RR-----------NCYLDEVIDRMGEASAIAQQLKQII
CeMec17 -----MQVDADLRPILGPQLVRLDPMRVKQL----Q-----------DPIVYEAIDNLAKLSAHCLQLRTPL
GlMec17 -----MQFGCNVAEAFGLRRSGVVLLTDQSL----RSMPLSQ-------QKKVEIILDGMGRGSQAAQGLPSPI
CeW06B11.1 -----MEIAFDLSTIFTDNIQRLTRTDLLKY----GPKR----------YWAVAQSIDCLGEMSSKFHGWKRVI
75
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DmMec17-CG17003 TSADKLA-SDQ--VVYLMADKAA---GH-WEITGLLKVGTKDLFVFDQGGCYRRLNQTPAILDFYVHESRQRCG
TbMec17 TSVPRLRENRDA-RLYLLCHG-----GRG---VGILKIGVKRLFVVPPSHAGLMEIEPVCVLDFFVDTSNQRQG
TcMec17 TSVARLRENRQA-RVYLLCQD-----GYG---VGILKMGVKKLFVTHPSYSSLVEIDPLCVLDFFVDTSFQRKG
XtMec17 TSASRMQTNKH--HLYILKDCTPKTAGRGAV-IGFLKVGCKKLFVLD-QKGSHIEAEPLCILDFYIHETLQRHG
XlMec17 TSASRMQANKH--HLYILKDCTPKTAGRGAV-IGFLKVGYKKLFILD-QKGSHIEAEPLCILDFYIHESLQRHG
HsMec17 TSASRMQSNRH--VVYILKDSSARPAGKGAI-IGFIKVGYKKLFVLD-DREAHNEVEPLCILDFYIHESVQRHG
DrMec17 TSAAKLQANRH--HLYLLKD-GEQNGGRGVI-VGFLKVGYKKLFLLD-QRGAHLETEPLCVLDFYVTETLQRHG
CiMec17 TTLLKSQLNNQ--RIYVLKEANANN-GLGCV-IGFLKTGKKRLFVLD-RDGNHNEMNPLCVLDFYVHESQQRKG
TtMec17 TNYDKVQGTDQ--RVYIVAE------GRTC--QGFLKVGQKNLFYRD-MMGNIKEIKPLCVLDFYVHESCQRQG
PtMec17 TTASKFYGSDQ--RIYLKAD------GKNC--LGLLKVGKKNLFYRD-YSGSIKEMQPLCVLDFYVHESVQRMG
CeMec17 TTCEKLINSDS--TLYLSWKYDEE--EKVSRLMGFAKVGRKKLFLYD-SQMQTYEGEILCLLDFYVHFSCQRQG
GlMec17 TSLAFIRDSHH--FLFLAVDEDQC---------LGILKGGIKHLFMLD-SQNETHEMDAMCCLDFYTHETVQRRG
CeW06B11.1 TMYDKIVDHDEEQTTYIMWEKVNG--SKSIL-KGLLRVGYKTLYLTD-NEQNQYMEKAMCILDFFVVPTEQRSG
149
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TcMec17 FGKTLFDAMLLNEGLNPGEVAIDRPSVKFLAFLQKYYGLVEYTPQSNNFVVFHRYFDK
XtMec17 FGKELFTFMLKNEQVDVHHLAIDRPSEKFLSFLRKHFNLWSTIPQVNNFVVFDGFFRD
XlMec17 FGKELFSFMLRNEQVDVQHLAIDRPSEKFLSFLRKHFNLWSTIPQVNNFVVFEGFFRD
HsMec17 HGRELFQYMLQKERVEPHQLAIDRPSQKLLKFLNKHYNLETTVPQVNNFVIFEGFFAH
DrMec17 YGSELFDFMLKHKQVEPAQMAYDRPSPKFLSFLEKRYDLRNSVPQVNNFVVFAGFFQS
CiMec17 CGLCLFKHMLHVEGVKASHLAIDRPSHKFISFLKKHFSLWATVPQVNNFVIFDGFFKN
TtMec17 YGKLLFEYMIQCEQTSPEKLAYDRPSPKLIAFLKKHYNLVKYIAQNNNFVVFDQYFRS
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CeMec17 VGQQILDYMFSQEHTEPYQLALDNPSVTLLGFMSQKYGLIKPVWQNTNFVVFEELFLA
GlMec17 IGTRLFRAMELHTHISAQGWAFDRPSPKLLAFLSKVYDMHDFKAQPNNFLMLDASIRL
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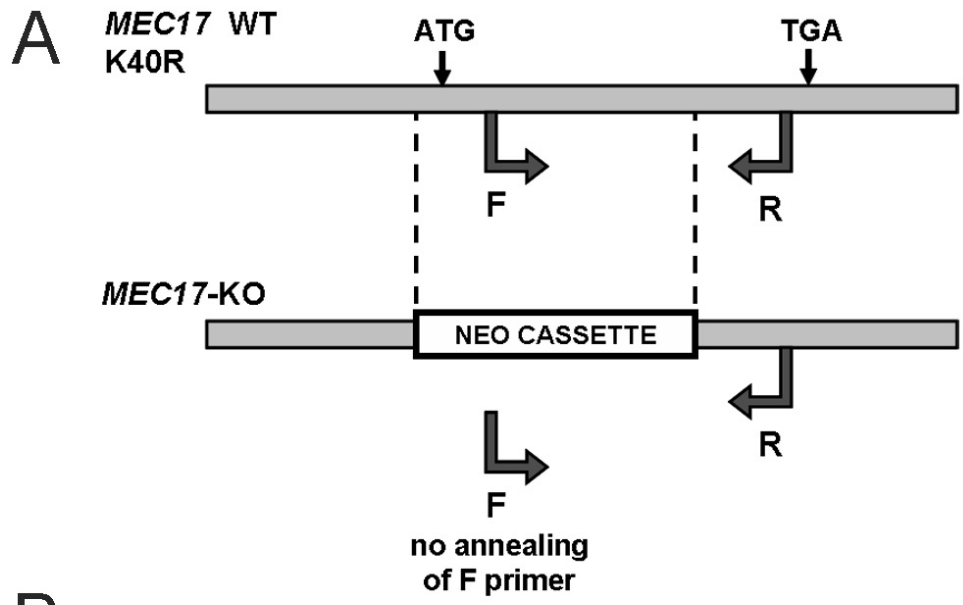
Fig S1

Figure S2. Deletion of the MEC17 gene in *Tetrahymena*. Genomic DNA was purified from a wildtype, MEC17-KO and K40R ATU1 mutant strains and subjected to PCR with a pair of primers that amplify either the *NRK18* sequence (positive control, left panel), or the MEC17 sequence with one primer located in the targeted region intended to be replaced by *neo4* cassette (5'-ACGATAAGGTATAAGGAACAG-3') and another primer located outside of the targeted region of MEC17 (5'-AAGTTATCTATCTTATCCAGG-3') (middle panel), or a mixture of MEC-17 primers and NRK18 control primers that provide an internal control (right panel).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ATG
TGA
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F
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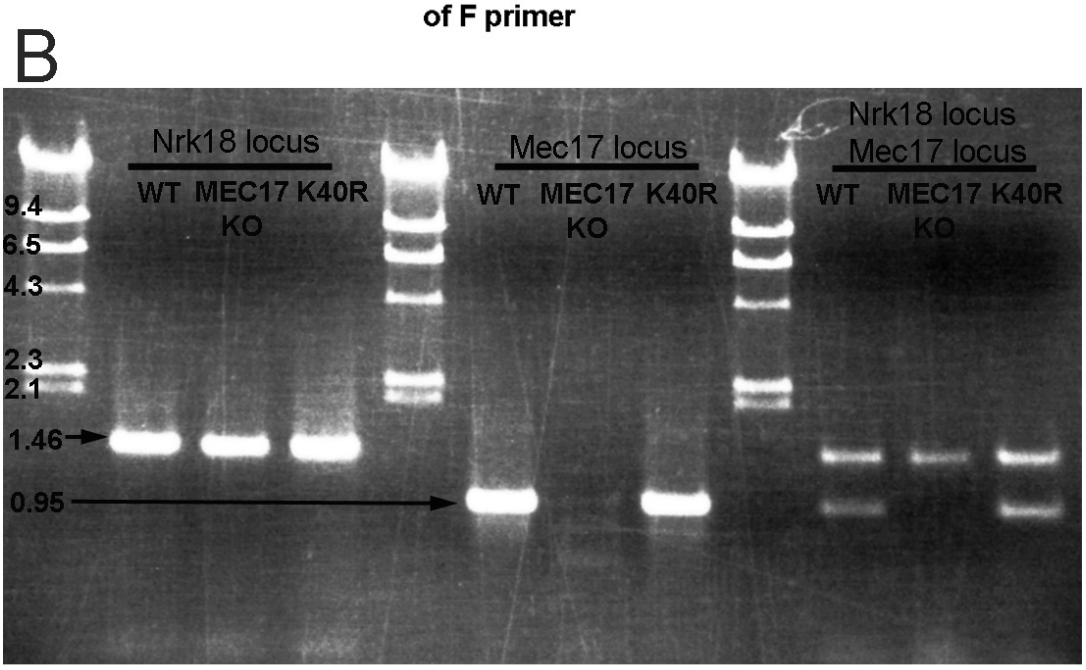
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WT MEC17 K40R
KO
Mec17 locus
WT MEC17 K40R
KO
Nrk18 locus
Mec17 locus
WT MEC17 K40R
KO
9.4
6.5
4.3
2.3
2.1
1.46
0.95

Fig S2

Figure S3. Silver-stained 2D SDS PAGE of ciliary proteins from either wildtype, MEC-17-KO and K40R strains of *Tetrahymena*. Note a shift in the position of a-tubulin isoforms in the MEC-17-KO and K40R sample, consistent with loss of acetylation. The left side represents the more basic part of the gel.

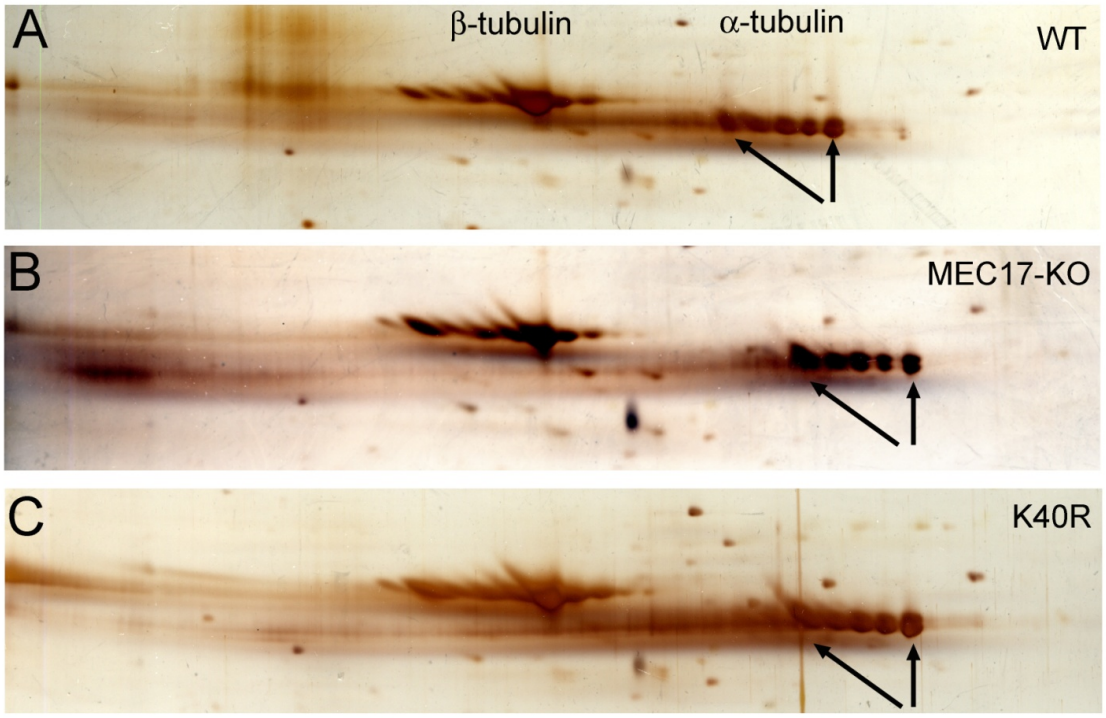


Fig S3

Figure S4. Axonemes are more sensitive to oryzalin treatment in MEC-17-KO and K40R a-tubulin mutants of *Tetrahymena*. The graph on the left side illustrates the responses of wildtype, K40R and MEC-17-KO strains to oryzalin of varying concentration. Cells were suspended at 10^5 cell/ml in SPP medium and incubated with the drug for 22 hr at 30^{o}C and the cell density was determined. On the right side there are images of cells that were either untreated (top row) or treated with 25 mM oryzalin for 12 hr, and subjected to immunofluorescence with an anti-a-tubulin (12G10) mAb.

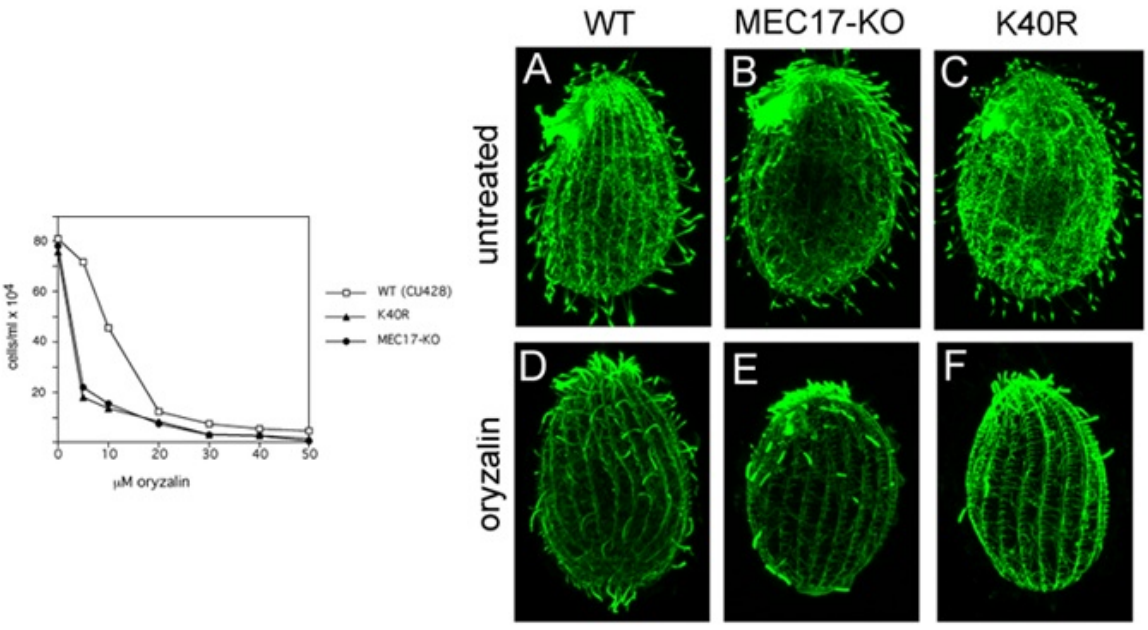
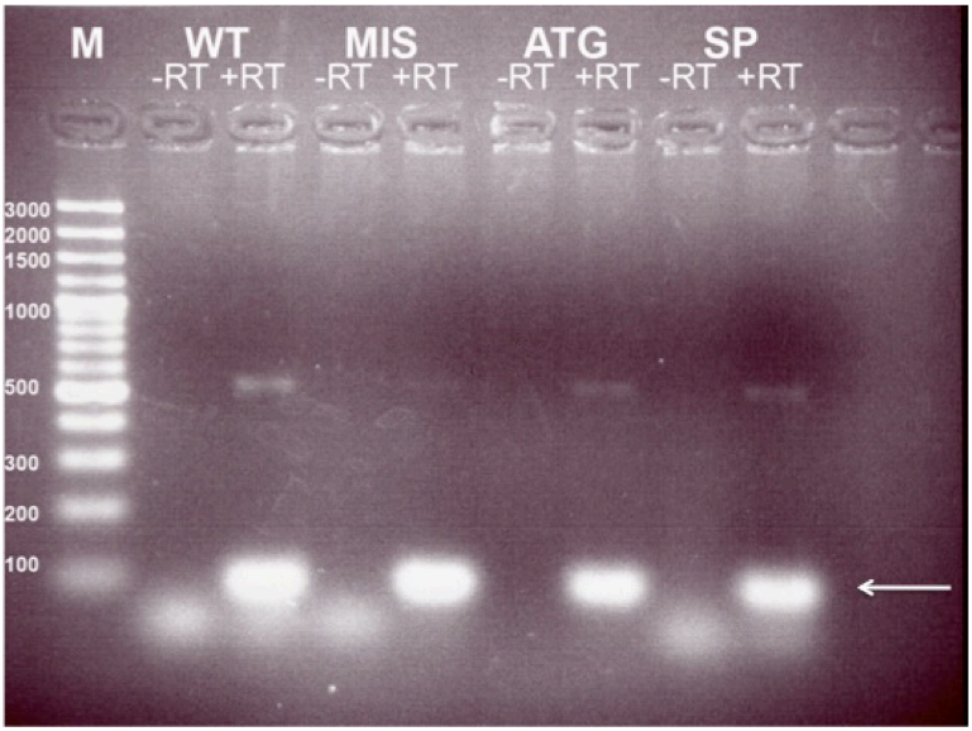


Fig S4

Figure S5. Depletion of *mec17* mRNA in zebrafish by SP MOs. Total cDNA was made using randomly-primed mRNA isolated from either control (WT) or embryos injected with 5 bp mismatch MO (MIS), ATG-MO (ATG), or splice-site MO (SP), and amplified using either primers corresponding to the coding sequence of the b-actin gene (top panel) or primers that correspond to the sequence of *mec17* between exon 1 and exon 5 (bottom panel). As controls, amplifications were performed on samples that lacked reverse transcriptase (-RT). Note that the b-actin gene primers gave a robust product of the expected size (100 bp) for all +RT samples (top). With the *mec17* primers, a product of the predicted size (345 bp) is amplified in +RT reactions, except for embryos treated with MEC-17-SP MOs, which do not show a product, consistent with downregulation of mec17 mRNA, possibly by the nonsense-mediated mRNA decay pathway.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100

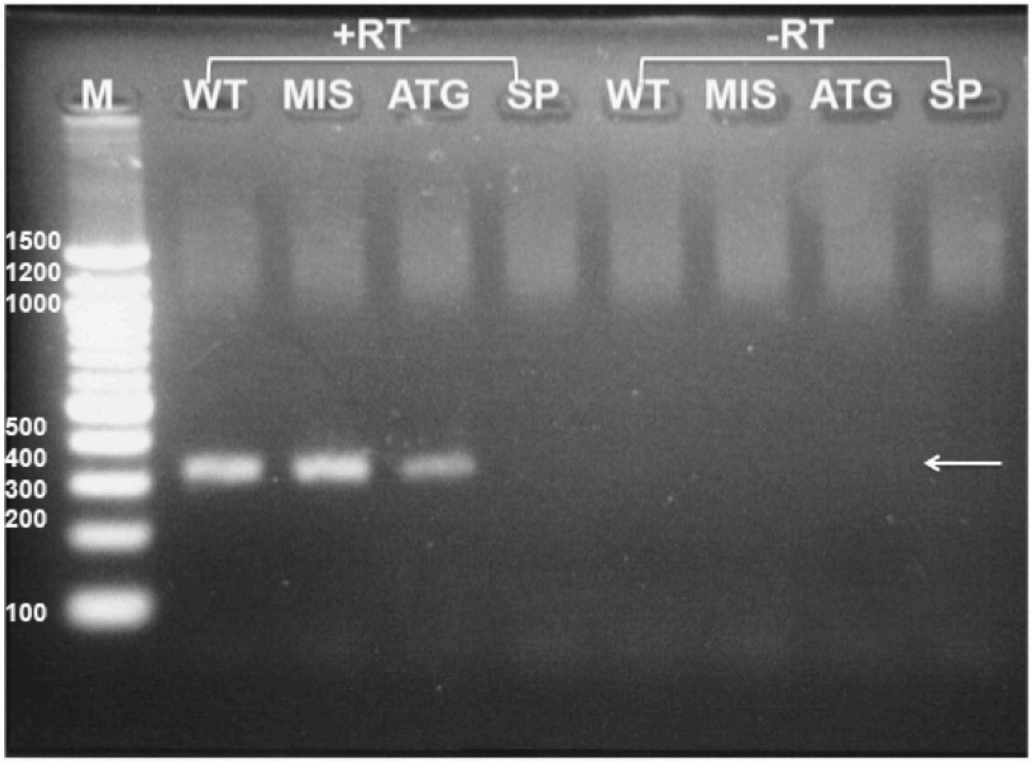
+RT
-RT
M
WT MIS ATG SP
WT MIS ATG SP
1500
1200
1000
500
400
300
200
100

Fig S5

Figure S6. A silver stained SDS-PAGE gel with proteins purified on a GST-bind column (Novagen) from bacteria expressing either GST or GST-Mm-MEC-17.

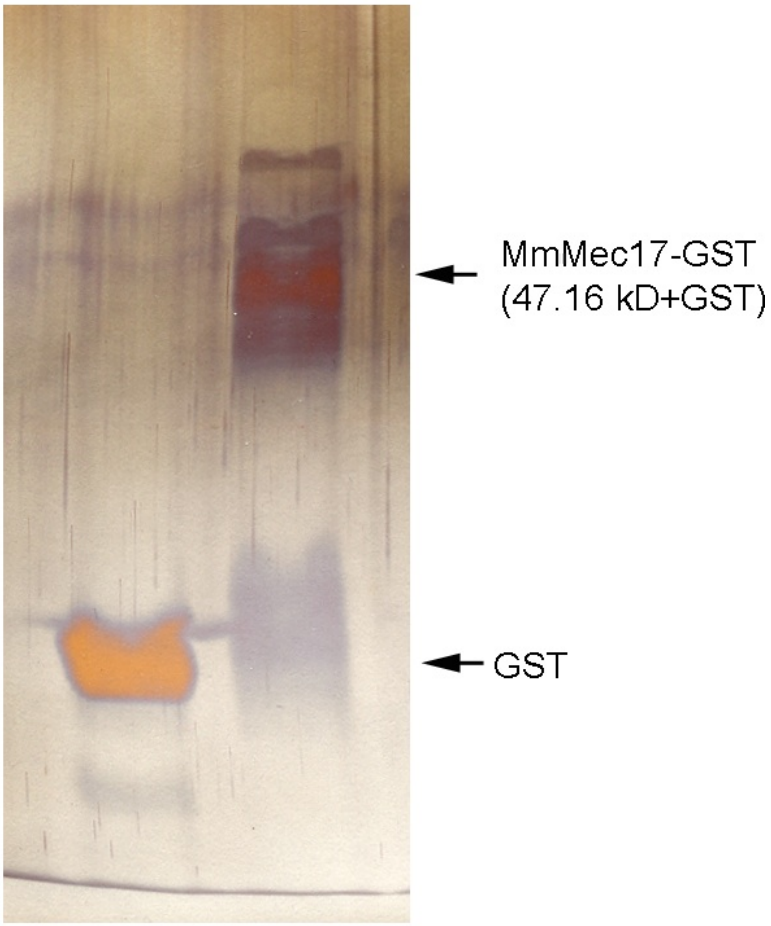

Fig S6

Figure S7. (A) MEC-17 activity tubulin acetylation activity on axonemes *in vitro* does not require a salt-labile axoneme-associated component. MEC17-KO axonemes were used without any treatment or exposed to solutions containing NaCl (0-1 M) for 30 min at 4^{o}C, washed and used for *in vitro* acetylation using recombinant GST-MmMEC-17. (B) MEC-17 prefers microtubules over unpolymerized tubulin as a substrate. *In vitro* acetylation assays were performed using either axonemes or tubulin purified from the MEC-17-KO strain. A comparison between lanes 3 and 4 (for reactions with purified tubulin) and lanes 7 and 8 and 9 and 10 (for reactions with axonemes) show that paclitaxel stimulates MEC-17 activity. Since paclitaxel promotes microtubule polymerization and stabilizes microtubules, these data suggest that MEC-17 prefers microtubules over dimeric tubulin as a substrate.

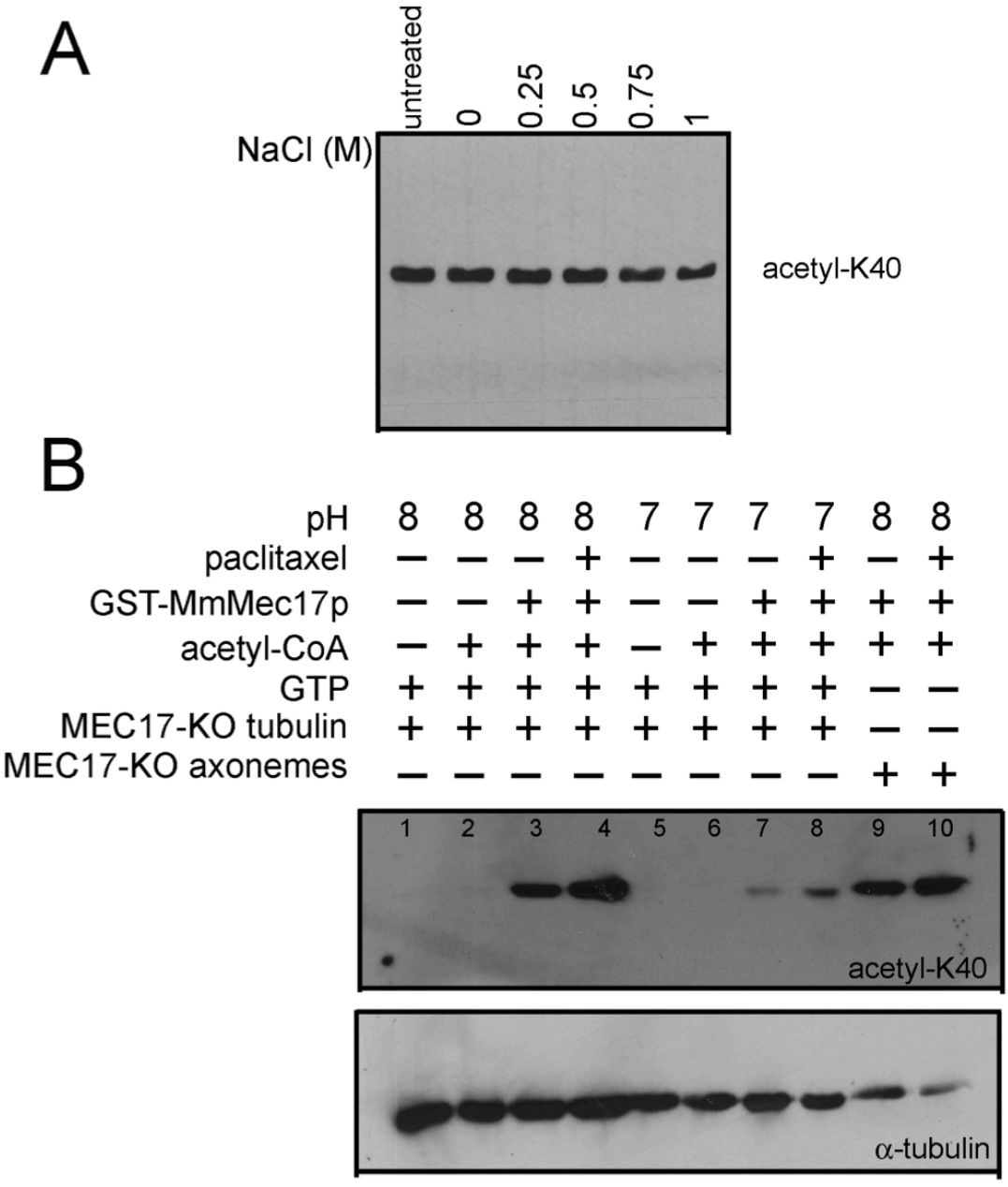
A
untreated
0
0.25
0.5
0.75
1
NaCl (M)
acetyl-K40
B
pH 8 8 8 8 7 7 7 7 8 8
paclitaxel − − − + − − − + − +
GST-MmMec17p − − + + − − + + + +
acetyl-CoA − + + + − + + + + +
GTP + + + + + + + + − −
MEC17-KO tubulin + + + + + + + + − −
MEC17-KO axonemes − − − − − − − − + +
1 2 3 4 5 6 7 8 9 10
acetyl-K40
α-tubulin

Fig S7

Figure S8. MEC-17 acetylates by entering the microtubule lumen from the end. Axonemes purified from the MEC-17-KO strain of *Tetrahymena* were subjected to an *in vitro* acetylation assay with either with recombinant GST (A-C) or GST-Mm-MEC-17 (D-L) and subjected to double immunofluorescence with the anti-a-tubulin 12G10 mAb (red) and pan acetyl-K antibodies (green). The green and red channel images were intentionally aligned with an offset to compare them side-by-side.

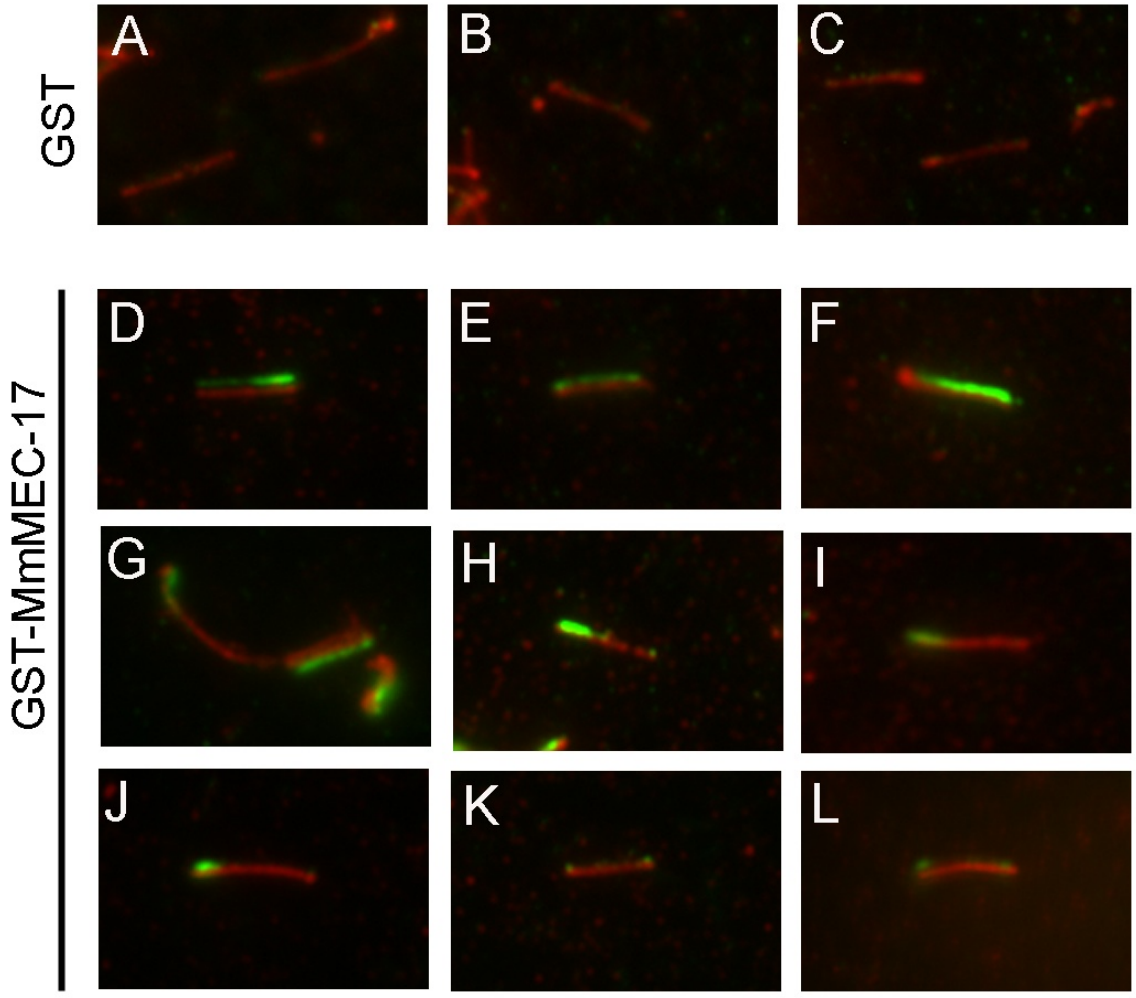

Fig S8

Videos S1-S3. Zebrafish morphants injected with 1 ng of: random sequence MOs (S1), MEC-17-ATG MO (S2) and MEC-17-SP (S3) were probed with a needle at 72 hpf. Embryos injected with 5bp mismatch MOs behave like those shown in video S1 (see Supplementary Videos).

CHAPTER 3

MICROTUBULES AND AXON ELONGATION IN THE TOUCH RECEPTOR NEURONS OF *C.ELEGANS**

Microtubules and axon elongation in the touch receptor neurons of *C.elegans.

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ABSTRACT

One key unresolved question in the field of *C. elegans* mechanosensation is the role of the 15 protofilament (pf) microtubules in touch sensation. Previous studies suggested that the 15 pf microtubules play a sensory role in touch sensation by delaying the adaptation of the mechanosensitive channel thus, keeping it open longer to aid the passage of a mechanoreceptor current (MRC) of optimal amplitude, which is required to elicit a response[1]. However, a recent study by Topalidou *et al* showed that mutants with severe microtubule defects respond to touch sensation bringing into question the contribution of 15 pf microtubules, and microtubules in general, to touch sensation[2]. We set out to explore the question about the role of microtubules in touch sensation in an unbiased way using a suppressor screen. We chose *mec-17(u265); Δatat-2(ok2415)* mutant as a starting point as in this genetic background microtubules are highly disorganized but still present[2]. Here, we identify *sma-2* as a suppressor of *mec-17(u265); Δatat-2(ok2415)* mediated touch insensitivity. We show that the mechanism of suppression is based on making the animal smaller, which leads to shortening of the axon of TRNs. We found that this suppression requires a functional mechanosensitive channel and possibly works through microtubules. Together, we propose two models for how shortening of the axon can bypass the need for 15 pf microtubules. According to the concentration model, a reduction in the axon length concentrates components such as microtubules and channels per unit length of the axon. Thus, more channels are open at a time and held open longer aiding the passage of a higher MRC. Alternatively, we propose a propagation model, where microtubules are important for long-range propagation of the touch signal.

Regardless of the model, our studies indicate that microtubules are important for touch response only when the axon elongates beyond a threshold value of length.

INTRODUCTION

One of the most unique features of the touch receptor neurons (TRNs) in *C. elegans* are their wide diameter microtubules with 15 protofilaments. Indeed, the TRNs were initially called "microtubule cells" even before the identification of their sensory role[3,4]. In spite of the identification of TRNs three decades ago, the specific contribution of the unique 15 pf microtubules to touch sensation remains unresolved. These 15 pf microtubules are mainly assembled from MEC-12 α-tubulin and MEC-7 β-tubulin[5,6](although there is a possibility that other tubulin isotypes could be expressed in TRNs and possibly co-polymerize with MEC-12 and MEC-7). All other cell types in *C. elegans* except cilia of sensory neurons are comprised of microtubules that are made up of 11 pfs[4]. The 15 pf microtubule organization seems to provide an adaptation for a specific function of microtubules in TRNs. In severe loss of function mutants of *mec-12* and *mec-7*, the 11 pf microtubules replace the 15 pf microtubules and such animals are touch-insensitive[4]. Although the 11 pf microtubules are sufficient for the proper growth and maintenance of TRNs, they are insufficient for touch response, suggesting a specialized sensory role that only the 15 pf microtubules can fulfill. Further validation of a sensory role for 15 pf microtubules came from studies on colchicine treated wild-type adults. Colchicine is a natural compound that strongly binds tubulin and causes microtubules to depolymerize. Treating wild-type adults with colchicine after the 15 pf microtubules have fulfilled their roles in growth and transport in the neuron causes depolymerization of the 15 pf

microtubules and leads to a loss of touch sensitivity[7]. Interestingly, for unclear reasons, microtubules outside of TRNs are not affected by colchicine (unless in extremely high concentrations), presumably due to small differences between the mec tubulins and non-mec tubulins that are expressed in other cell types[4]. Electrophysiological comparisons between touch insensitive alleles of *mec-12* that differ in the ability to support the assembly of the 15 pf microtubules suggested a specific sensory function of 15 pf microtubules. Specifically, the mechanosensory channel-mediated currents are detectable in the TRNs (using patch clamping on dissected animals). However, the current amplitude becomes smaller in mutants that have a more severe defect in the 15 pf microtubules and the adaptation time of the channel is reduced suggesting that microtubules prevent the channel from closing too early[7]. One possibility is that the physical properties of the plasma membrane that surrounds the channel are affected by the 15 pf microtubules. For example, it has been proposed that bundles of the 15 pf microtubules could act as a rigid platform that imposes mechanical force on the plasma membrane[7]. *mec-17* is among the genes required for touch sensation that is highly expressed in the touch receptor neurons[2]. We had previously identified MEC-17 as an α-tubulin acetyltransferase that acetylates MEC-12 at K40[8]. Surprisingly, we found that the touch response is only mildly reduced in worms that have K40R and K40Q substitutions of MEC-12, suggesting that MEC-17 has another function besides acetylating K40 [8]. Even more surprisingly, Topalidou *et al* have shown that the acetyltransferase activity of MEC-17 is not needed for touch response[2]. Moreover, *mec-17* mutants that have been rescued of touch insensitivity by non-acetylating versions of MEC-17 are severely defective in the microtubule number and pf organization[20]. These studies question the

importance of microtubules in the touch response or at the least indicate that TRNs can tolerate a major loss in microtubules as long as an inactive form of MEC-17 is present. It is not clear whether the inactive MEC-17 works through the residual microtubules that form in the absence of MEC-17 acetyltransferase activity. It is honest to say that right now the function and relationship between MEC-17 and 15 pf microtubules are a puzzle. The only experiment that addressed the question whether MEC-17 mediated acetylation of K40 MEC-12 contributes to microtubule organization was done in *C. elegans* TRNs[9]. Cueva *et al* showed that expressing a transgenic version of MEC-12 with K40R mutation (that is expected to not be acetylated) into *mec-12(e1607)* null mutants caused severe microtubule organization defects similar to the defects observed in the *mec-17* mutant animals[20]. However, these experiments suffer from the disadvantage that the transgenes were introduced in the form of extra chromosomal arrays and thus the gene product dosage could not be controlled tightly. We set out to bring more clarity to the puzzling role of microtubules and MEC-17 in TRNs by using a suppressor screen strategy to identify interactors of *mec-17*. Using genetic mapping and whole genome sequencing, we have identified *sma-2* as a suppressor of *mec-17* mediated touch insensitivity. We have shown that touch insensitive mutants with severe microtubule defects can be rescued by making the axon size smaller. We propose that the sensory roles 15 pf microtubules play in touch sensation could be either in making the membrane more sensitive to a touch stimulus or aiding in the long range propagation of the touch stimulus. This novel sensory function adds to the more recent unconventional roles microtubules have been identified to play including a role in the regulation of gene expression and in signaling.

RESULTS

sma-2 is a suppressor of _mec-17(u265); Δatat-2(ok2415)_

mec-17(u265); Δatat-2(ok2415) has two missense mutations in *mec-17* and a deletion in Δ*atat-2*. This strain lacks acetylation of K40 a-tubulin (Akella *et al;* unpublished observation) , is severely defective in microtubule and pf organization and is slightly better at responding to touch than the double deletion strain[2]. There are a few reasons we chose this strain for mutagenesis and not the double deletion strain. It is generally accepted that it is relatively easier to find suppressors of missense alleles than null alleles. This strain also has the advantage that it is slightly better at responding to touch than the double deletion. Moreover, the *mec-17(u265)* allele has more microtubules than the double deletion mutant *mec-17(ok2109); Δatat-2(ok2415)*. By using this strain, we could identify suppressors of the microtubule and protofilament defects in these mutants i.e. we could identify genes that interact with MEC-17 to fulfill its acetyltransferase function since microtubule organization seems to require the acetyltransferase function of MEC-17[2]. These suppressors could also provide us clues regarding other binding partners, which could even be substrates of MEC-17 acetyltransferase activity. Moreover, since we are using touch sensitivity for our screen and since touch sensitivity has been shown to not require the acetyltransferase function of MEC-17[2], we could obtain genes that could provide us insights into the non-acetyltransferase functions of MEC-17 as well. These suppressors of the non-acetyltransferase function of MEC-17 might also help us answer an important question: Is the non-acetyltransferase function of MEC-17 that contributes to touch sensation working through microtubules?

We performed ENU mutagenesis of touch insensitive *mec-17(u265); Δatat-2(ok2415)* worms and analyzed 40,000 F2s using a gentle touch assay to identify suppressors. Each of the mutagenized worms was individually evaluated for the ability to respond to 10 touches with an eyebrow hair probe. Two suppressors that have partially recovered touch response were isolated from a mutagenized population (Fig 1B). Due to the method of handling all or some of these animals could originate from the same mutagenized F2. Both suppressor strains (#1, #19) were noticed to be smaller than normal animals at the same developmental stage including the adults. When both suppressors were backcrossed to the non-mutagenized *mec-17(u265); Δatat-2(ok2415)* strain, the partial touch response co-segregated with a smaller animal size (Fig 1A). We utilized the small size phenotype to our advantage during mapping of the suppressor mutation. Using SNP mapping method, we mapped the suppressor #1 mutation to a gene between 0 and -1 map units on chromosome III (Fig s1)[10]. Next, using a cross, we determined that #1 and #19 mutations do not complement in an F1 double heterozygote, indicating that both mutations occurred in the same gene. Using whole genome sequencing, in both suppressor #1 and #19 we found 3 mutations in the mapped interval that are predicted to result in a change in the gene product. Among them there is a mutation in *sma-2*, a receptor activated SMAD in the TGFβ pathway[11]. sma mutations result in a small animal body. The suppressor had a mutation in a splice site donor of *sma-2* that results in altered splicing that introduces an early stop codon after exon 5 of *sma-2* (Fig 1C). By DNA sequencing, we determined that the above *sma-2* mutation was not present in the non-mutagenized parent strain. We tentatively assumed that the suppression is due to the *sma-2* mutation and we designated the new *sma-2* allele as *sma-*

2 (*ek32*). SMA-2 acts within the TGF-b pathway as a cytoplasmic SMAD mediator (R-smad)[11]. The suppression could be due to loss of TGF-b signaling or could represent an unknown side activity of *sma-2*. To distinguish between these two possibilities, we tested whether a similar suppression can be obtained by loss of another component in the TGF-b signaling pathway. *sma-4* encodes a co-SMAD in the TGF- β pathway[11]. A mutation in the *sma-4* gene rescued the touch insensitivity in the *mec-17(u265); Δatat-2(ok2415)* animals showing that the suppression is not specific to *sma-2* and that likely any defect in the TGF-β pathway can rescue touch insensitivity defects in the *mec-17(u265); Δatat-2(ok2415)* animals(Fig 2A).

2. Making the *mec-17(u265); Δatat-2(ok2415) animals smaller* rescues touch insensitivity

The above data link TGF-b signaling to MEC-17. Importantly, there is recent evidence linking microtubules with signal transduction and gene expression pathways specifically in the TRNs of *C. elegans*[12]. On the other hand, the observation that two different sma mutations can suppress touch insensitivity in *mec-17(u265); Δatat-2(ok2415)* led us to test whether a sma mutation outside of the TGF-b pathway could also suppress touch insensitivity. To this end, we introduced a mutation in *sma-1* into the *mec-17(u265); Δatat-2(ok2415)* animals. *sma-1* encodes a b-spectrin that plays a critical role in embryonic elongation by stabilizing the attachments between the circumferentially oriented actin fibers and the apical membrane of the embryo[13,14]. Defects in *sma-1* cause a weakening of these attachments and lead to improper embryonic elongation and thus, these animals are smaller at hatching unlike the TGF-b sma mutants that become smaller

after hatching. Touch assays on the *sma-1(e30); mec-17(u265); Δatat-2(ok2415)* showed that these animals are touch responsive at the level similar to the *sma-2; mec-17(u265); Δatat-2(ok2415)* and *sma-4(CB502); mec-17(u265);Δatat-2(ok2415)* animals (Fig 3A). We conclude that sma mutations in two distinct pathways rescue the touch insensitivity phenotype of *mec-17(u265); Δatat-2(ok2415)*. The simplest explanation is that the rescue of touch insensitivity occurs from making the animal smaller. Furthermore, TRNs in sma animals have shorter axons (Fig 2B, 2C). Thus, possibly the mechanism of suppression is based on axon shortening, suggesting that *MEC-17* plays a role during axon elongation, or that its function is only important in an elongated axon. This was unexpected due to the fact that we obtained only two suppressor strains, both of which were mutated in the same gene whereas a general-size based suppression would be expected to result in more suppressors after ENU mutagenesis as there are 21 sma genes in *C. elegans.*

3. Small animal size rescues touch insensitivity in mutants entirely lacking the 15 pf microtubules

mec-17(u265); Δatat-2(ok2415) animals are known to have defects in the organization of microtubules in TRNs. More precisely, these animals have fewer and shorter microtubules that fail to keep a normal 15pf organization (hence, have a variable pf number)[2,9]. Thus, the rescue could somehow be correcting the organization of microtubules. To further explore this possibility, we considered that if the suppression is based on the correction of microtubule organization defects, this suppression might not be possible in a more severe mec microtubule mutant that entirely lacks the 15 pf microtubules. To test this possibility, we introduced a *sma-1* mutation into a *mec-*

12(e1607) background. *mec-12(e1607)* is a likely null allele of *MEC-12* a-tubulin that was recently reported to entirely lack microtubules in the mature touch receptor neurons[9]. Touch assays on the *sma-1(e30); mec-17(u265); atat2 (ok2415)* strain led to the surprising finding that touch insensitivity in these animals is rescued at the level similar to the rescue of the *sma-1(e30); mec-17(u265); Δatat-2(ok2415)* mutants (Fig 3B). Thus, we generalize that mutations that disrupt the15 pf microtubules and even completely eliminate them from the mature TRNs can be rescued by reducing animal body size. This opens up the possibility that the mutation is a bypass of microtubules. Note however, that we cannot rule out a possibility that the suppression is a modifier of microtubule organization. Although the *mec-12(e1607)* animals were reported to not possess any 15 pf microtubules in the touch receptor neuron, it is possible that they have some 11 pf microtubules that were missed by transmission electron microscopy. Moreover, the *mec-12(e1607)* animals extend a TRN process on the basis of immunostaining against MEC-18, a touch receptor neuron protein[7]. It is known that *C. elegans* TRNs fail to extend a process without any microtubules[4]. Thus, it is likely that the suppression might work through the remaining 11pf microtubules in these mutants.

4. The suppression of touch insensitivity by reducing the size of the neuron requires a functional mechanosensitive channel

The above data show that sma can suppress a severe defect in the organization of 15 pf microtubules, either by bypassing the requirement for microtubules or by correcting the microtubule organization. If the sma mutation bypasses microtubules, this opens up the possibility that reducing the size of the axons in TRNs can bypass mutations in other

components of the touch response pathway or even that the suppressed animals sense touch using neurons different from TRNs. To determine whether the sma suppression is working through the touch receptor neuron pathway, we introduced a *sma-1* mutation in a touch insensitive *mec-4 (u253)* strain. MEC-4 encodes the mechanosensitive channel[1]. Reducing the animal body size failed to rescue the touch insensitivity in the *mec-4 (u253)* animals showing that the suppression in the sma *mec-17(u265); Δatat-2(ok2415)* animals requires a functional mechanosensory channel to be present in the TRNs (Fig 4).

DISCUSSION

Models

To explain our findings, we put forth two models: a concentration model and a propagation model. According to our concentration model, the rescue in touch sensitivity in mutants with severe microtubule defects occurs due to a concentration of factors such as microtubules and channel components that occurs in a smaller volume of a shorter axon (Fig 5a). The *mec-17(u265); Δatat-2(ok2415)* mutants have fewer microtubules according to transmission electron microscopy data[20]. Assuming that there is no feedback to the cell body about the length of the axon, the same amount of material could be sent into the axon regardless of the length. This material includes the components of the mechanosensitive channel and the tubulins that make the microtubules in touch receptor neurons. Thus, this could lead to an increase in the number of microtubules and channels per unit length of the axon. A suppression of touch insensitivity could occur the following way: for an appropriate response to be elicited to touch, in wild-type animals, 14-25 channels are activated at a time[1]. Thus, by concentrating the number of channels

in the smaller axons, more channels can be opened (and presumably activated) by touch at the same time. Moreover, on the basis of recordings of the channel adaptation time in *mec-12* mutants with varying levels of defects in the 15 pf microtubules, O' Hagan *et al* have proposed a role for the 15 pf microtubules in keeping the mechanosensitive channels open longer[1]. Thus, in a smaller axon, not only could more channels be opened at one time, but also more channels could be held open by the microtubules. Concentrated microtubules could help in keeping the channels open by making the membrane more rigid; therefore, even a small deformation in the membrane could lead to a response. Moreover, microtubules make several attachments to the membrane; therefore, a deformation in the membrane could be passed on to other regions on the axon through microtubules, thus activating more channels at a single touch. Thus, one can imagine a loop where more channels open up, and are held open for a longer period, letting a MRC of a higher amplitude pass through. This MRC might be of enough amplitude for the sub-optimal response to touch observed in the sma suppressor strains.

Alternatively, we propose a propagation model where microtubules become important for the long-range propagation of a signal (Fig 5b). Touch assays on weaker alleles of *mec-7* b-tubulin suggested that these mutants vary in their touch responsiveness based on the position they are touched at. These *mec-7* mutants are more responsive to touch when touched closer to the synapse[6]. So, it is possible that in a smaller axon, the distance the signal has to travel to the synapse is reduced and that is why the suppressed animals respond to touch. If this is the case, microtubules are important for the propagation of the signal along the axon. The shortening of the axons bypasses this function of microtubules in signal propagation.

Whatever the role microtubules may play in touch sensation, whether it is a more physical role in keeping the channel open longer or for propagation of the touch signal, it seems to be less important when the size of the axon is reduced. This provides us insights about how important microtubules are for touch sensation in an animal that is longer in size.

CONCLUSIONS AND FUTURE EXPERIMENTS

Microtubules have long been known to be required for the growth of a neuron and for transport of components in the neuron. More recently, the 15 pf microtubules in the TRNs of *C. elegans* have been shown to perform non-conventional functions in the regulation of gene expression via a p38 MAP kinase signaling pathway[12]. Moreover, 15 pf microtubules have a sensory role that cannot be fulfilled by the 11pf microtubules that substitute in their absence[7]. This sensory role came into question when it was observed that mutants with severe defects in microtubule and pf organization could still respond to touch. We show here, to our knowledge for the first time, a role for microtubules for touch sensation that is dependent on the length of the axon of the TRN. We show that microtubules are important for touch sensation in wild-type animals with a long axonal process. Whether this role that microtubules play is physical or whether it is a role in signal propagation is unknown.

Although it is known that defects in cytoskeleton associated proteins lead to slight defects in body size, we show for the first time, that reducing the body and thus, neuron size can bypass the function of microtubules. A *C. elegans* mutant of doublecortin had fewer and shorter microtubules in the TRNs and is touch insensitive and these animals

are slightly smaller in size than wild-type[15]. It is puzzling that the simultaneous reduction of body size and number of microtubules has not rescued the touch insensitivity defect in these mutants. The finding that the *zyg-8* mutants are touch insensitive although they have a smaller body size but, have fewer microtubules supports the idea that microtubules in a *sma-1(e30); mec-17(u265); Δatat-2(ok2415)* could be concentrated and thus, the animals are more responsive. However, why is it that the *zyg-8* mutants have fewer microtubules in spite of being smaller is unknown. Could it be that the presence of a remaining few15 protofilament microtubules with MEC-17 provide the cell body with a feedback regarding the length of the axon and this feedback is lost in mutants of *mec-17* or the genes encoding the components of the 15 protofilament microtubules?

We are testing our concentration model using immunostaining against the channel component MEC-4 and also studying the microtubule density. We believe the suppression could work through microtubules. Although the *sma-1(e30); mec-12(e1607)* could be seen as pointing otherwise, it is unlikely that the *mec-12(e1607)* strain has no microtubules in the TRN. It is known that the *mec-12(e1607)* animals make an axon. It is also known that depolymerizing the 11 pf microtubules (that substitute for 15 pf microtubules) in mutants of *mec-7* b-tubulin using benomyl (a microtubule-depolymerizing agent) prevented a TRN process to be extended[4]. Thus, it is likely that the suppression of touch insensitivity by reducing the size of the axon still works through microtubules.

We are developing an immunofluorescence-based assay to examine microtubule density in the TRNs to test the hypothesis that smaller *mec-17(u265); Δatat-2(ok2415)* animals have an increased microtubule density. Transmission electron microscopy

showed that the *mec-17(u265); Δatat-2(ok2415)* animals have fewer microtubules in the TRN[20]. Thus, we are working on finding conditions under which we can detect the deficiency in the microtubule density in this mutant without resorting to TEM which by the nature of the method gives a very limited view. Unfortunately, so far we have not been able to visualize the difference in microtubule density between wild-type, *mec-17(u265); Δatat-2(ok2415)* and sma- *mec-17(u265); Δatat-2(ok2415)* animals by immunostaining using an antibody against a- tubulin and GFP-MEC-4 marker to light up the TRNs. Getting the antibodies into worms and labeling the neurons uniformly while keeping the soluble GFP-MEC4 marker inside neurons has been very challenging. Alternatively, we are testing for the involvement of microtubules in this suppression by depolymerizing microtubules in the TRNs of *sma-1 (e30)* animals. We will use colchicine to depolymerize the 15pf microtubules in the TRNs of adult *sma-1 (e30)* and wild-type animals after the microtubules have fulfilled their role in neuron growth. We will use the *bus-17* background to promote the drug entry into adult animals[7]. Treating adults with colchicine has been shown to lead to a selective loss of touch sensitivity[7]. Touch assays on *sma-1(e30)* animals with disrupted microtubules should give us an answer as to whether a smaller axon can respond while having no microtubules at all. If colchicine prevents touch response both in *sma-1* mutants and wild-type, we will conclude that the suppression requires microtubules. Intuitively, it seems that microtubules are required for touch sensation perhaps by playing a physical role. There seems to be a certain microtubule density that is required per unit length of the axon. Indeed, there is an increase in the number and length of microtubules as the axon gets longer during various stages of development[3]. Alternatively, the *sma-1* mutant worms

but not wild-types could respond to touch when microtubules are depolymerized by colchicine. If this is the case, we will need to use TEM to confirm that colchicine indeed depolymerizes microtubules in the *sma-1* background. One could imagine that the drug sensitivity changes in the sma background, for example because the shorter axon has a different concentration of tubulin, or that a smaller animals is more drug resistance by an unrelated cause. If TEM confirms loss of microtubules by colchicine, this will indicate that all microtubules are needed for touch sensation only when the axon elongates to a threshold size.

Savage *et al* identified that touch insensitive animals of weaker mutant alleles of *mec-7* b-tubulin do respond to touch when touched closer to the synapse. We had considered the possibility that by making an axon shorter, the distance the signal needs to travel is reduced, and thus, reducing the dependence on microtubules for touch sensation. This would suggest a role for microtubules in the propagation of the signal along the axon. Preliminary data suggests that *mec-17(u265); Δatat-2(ok2415)* do not have positional dependence of touch sensitivity and argues against the propagation model (our unpublished data). We had also considered a possibility that by reducing axon size, the touch stimulus at the middle of the body could also be sensed by other neurons in the head and the nose. Although this possibility is far-fetched considering that the point of touch is far away from the head, it is also unlikely because *atat-2* is required for nose touch[16]. Moreover, we also foresaw the unlikely possibility that this size based suppression was working through a pathway that didn't require TRN function. However, the lack of suppression of touch insensitivity in the *mec-4* mutants by making their axon shorter eliminated this possibility. Taken together, our results suggest that microtubules

play important roles in touch sensation and these roles become less important in the context of a shorter axon.

MATERIALS AND METHODS

Strains

The *wild-type* strain N2, Hawaiian CB456, *mec-17(u265)* have been previously described.

Δatat-2(ok2415), sma-1(e30), sma-2(CB502), sma-4(e729), mec-4(u253); zdls5 expressing GFP from a *mec-4* promoter were obtained from the *C. elegans* Genetic Center which is funded by the National Institutes of Health. *mec-12(e1607)* was a kind gift from Dr. Martin Chalfie at Columbia University. All animals were grown and maintained at 20°c using standard procedures as described previously[17]. Double and triple homozygotes were obtained using standard genetic crosses and were verified either phenotypically or genotypically by PCR when necessary.

Suppressor Screen and Genetic Mapping

L4 animals of *mec-17(u265); Δatat-2(ok2415)* strain were treated with 0.4mM ENU for four hours. Roughly 40,000 F2 progeny from the mutagenized animals were screened for a rescue of touch insensitivity. Any animal that responded 5 or more times to touch was separated for further analysis. Suppressors were backcrossed to the non-mutagenized strain *mec-17(u265); Δatat-2(ok2415*) four times. The rescue of touch insensitivity segregated with a smaller animal size. However, the suppressor mutation was recessive in nature and slightly low in penetrance. Genetic mapping to identify the location of the suppressor mutation was done using standard SNP mapping methods as described

previously[10]. Briefly, for chromosome mapping, worms were crossed to Hawaiian animals (CB456) and sorted out on the basis of their body size. Two sets of lysates were made, small and non-small, representing the suppressed and non-suppressed populations. The mutation was identified to be on Chromosome III on the basis of linkage to N2 DNA, the background the mutagenized strain was in. Interval mapping on individual F2s suggested that the mutation was between -1 and 0 Map Units on Chromosome III. For Whole Genome Sequencing to identify the location of the suppressor mutation, the suppressor strains were outcrossed to N2 four times. Genomic DNA was purified from Sup#1 and Sup #19 using standard techniques. Sequencing data was analyzed using online Galaxy software using the published workflow[18]. PCR on the non-mutagenized *mec-17(u265); Δatat-2(ok2415*) strain confirmed the lack of this mutation in the parent non-mutagenized strain.

Touch assays

Standard touch assays were performed as described before. Data was typically generated from three experiments with 20 animals each being touched 10 times (therefore from an average of 600 touches). The *sma-4* touch assay data however, was from only one experiment due to technical difficulties. Statistical analysis was performed using Student's T-test. Error bars represent Standard Error of the Mean. * represents a significant difference between the touch responses of strains being compared.

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FIGURES AND FIGURE LEGENDS

Figure 1. A mutation in *sma-2* suppresses touch insensitivity in *mec-17(u265); Δatat-2(ok2415)* animals

A. Suppressed animals are smaller than non-mutagenized. Scale bar represents 0.1mm.

B. Suppressed animals respond twice more often than *mec-17(u265); Δatat-2(ok2415)* when touched. Error bars represent Standard Error of Mean.

C. Suppressors are mutated in *sma-2*. Suppressors have a G1108A mutation. G1108 is a splice donor. The mutation is predicted to lead to improper splicing leading to an early termination. Wild-type SMA-2 is 418 amino acids. Mutated version of SMA-2 is 61 amino acids long.

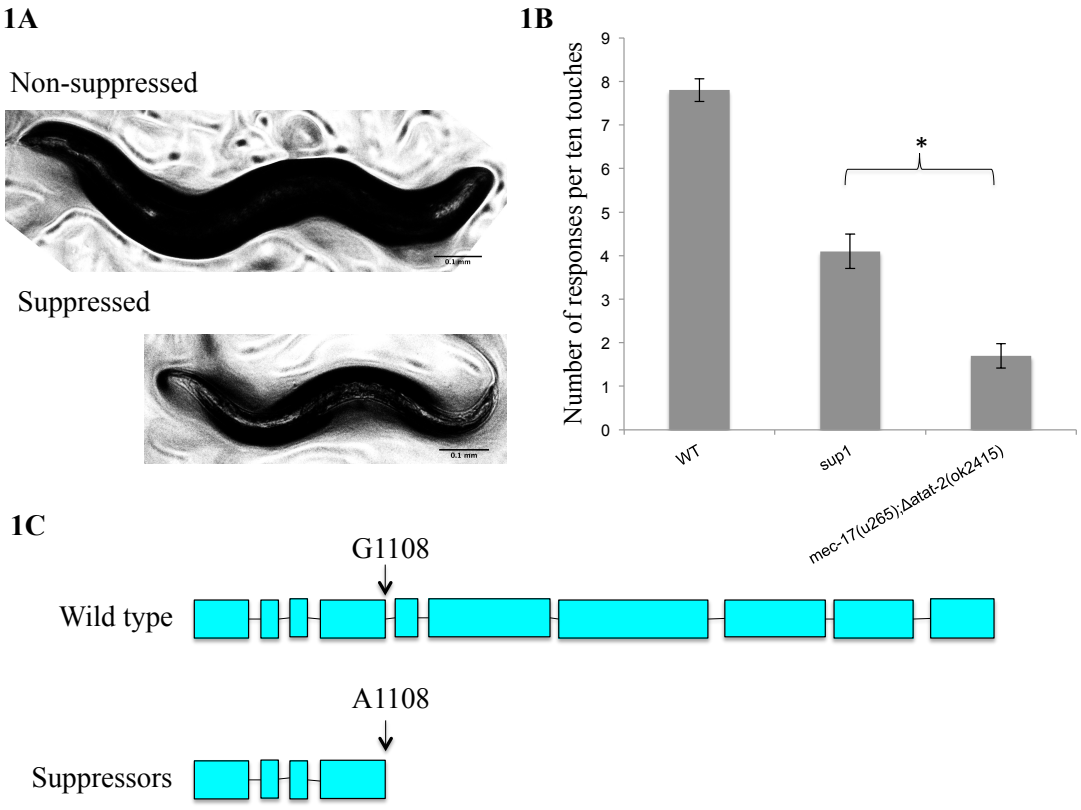

1A
Non-suppressed
Suppressed
1B
Number of responses per ten touches
0
1
2
3
4
5
6
7
8
9
*
WT
sup1
mec-17(u265);Δatat-2(ok2415)
1C
G1108
Wild type
A1108
Suppressors

Fig 1

Figure 2. Defects in TGF-b signaling components rescue touch insensitivity in *mec-17(u265); Δatat-2(ok2415)* animals

A. Touch assays on *sma-4(e729); mec-17(u265); Δatat-2(ok2415)* and *sma-2(ek32); mec-17(u265); Δatat-2(ok2415)* Error bars represent S.E.M. p*mec-4* GFP is the genetic background they are in. This was to visualize the TRNs in these strains.

B. Touch receptor neurons are smaller in size in *sma-2(ek32); mec-17(u265); Δatat-2(ok2415)* than non-suppressed *mec-17(u265); Δatat-2(ok2415)*. Both strains were crossed to *zdls5* to visualize TRNs. Both images were taken at the same magnification. White line represents the distance from the cell body to the point of termination of the TRN.

C. TRNs in suppressed animals are ~70-80% of *wild-type* length. Ovals represent the cell body from which the TRN process emerges. The white line from 2B was copied on to the cell body in this image to show the difference in length.

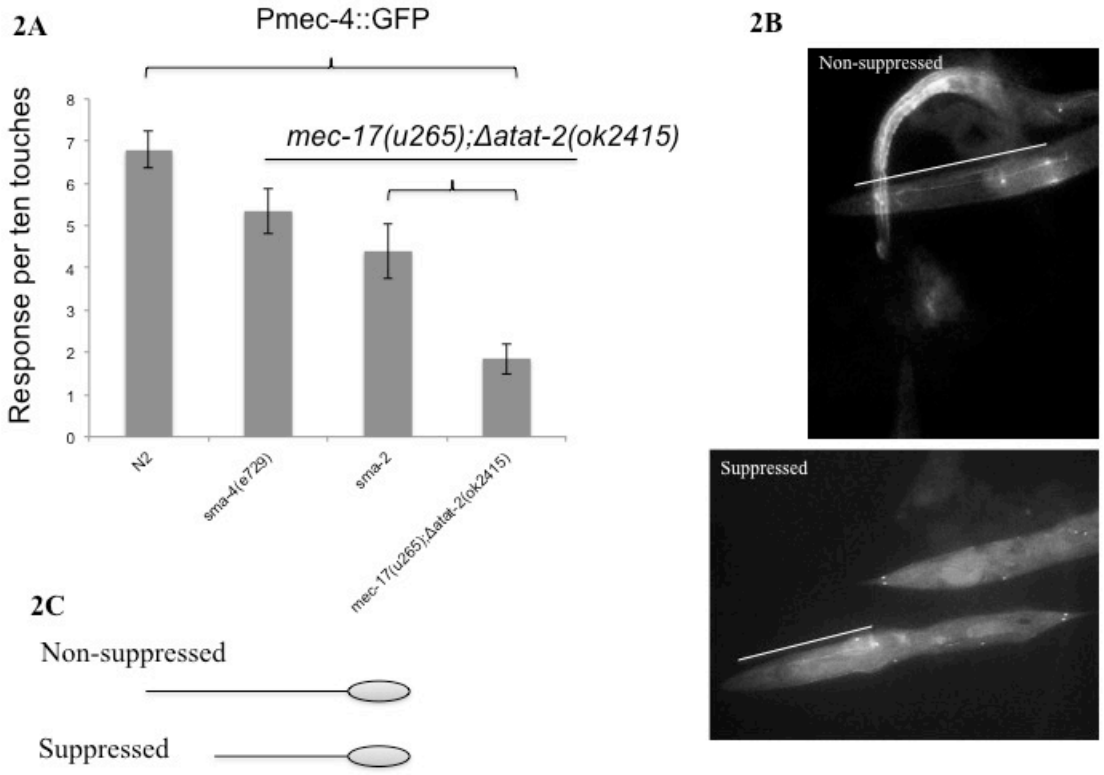

2A
Pmec-4::GFP
mec-17(u265);Δatat-2(ok2415)
Response per ten touches
0
1
2
3
4
5
6
7
8
N2
sma-4(e729)
sma-2
mec-17(u265);Δatat-2(ok2415)
2B
Non-suppressed
Suppressed
2C
Non-suppressed
Suppressed

Fig 2

Figure 3. Reducing the size of the axon of TRNs suppresses touch insensitivity in mutants with severe microtubule defects.

A. Touch assays on *sma-1(e30); mec-17(u265); Δatat-2(ok2415)* and *sma-2(ek32); mec-17(u265); Δatat-2(ok2415)*. Error bars represent S.E.M. (Although *sma-2* (CB502) control is missing here, the touch response of this strain is as good as *wild-type*)

B. Touch assays on *sma-1(e30); mec-12(e1607)*. Error bars represent S.E.M.

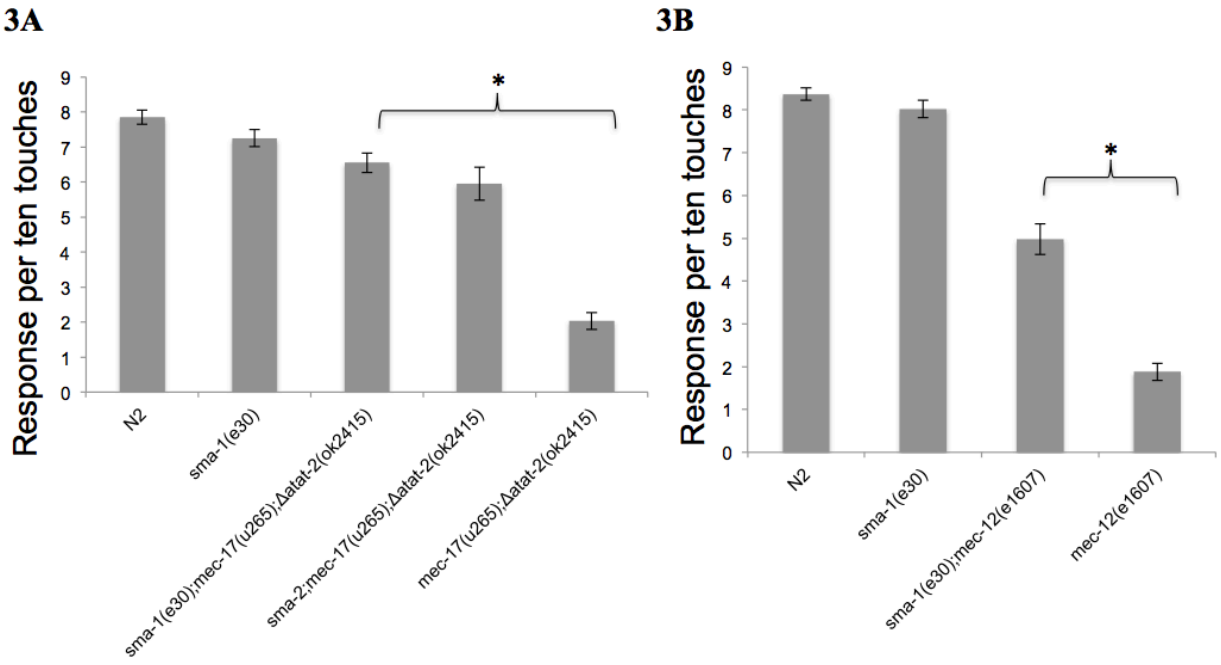


Fig 3

Figure 4. Suppression of touch insensitivity of *mec-17(u265); Δatat-2(ok2415*) by reducing the size of the axon requires a functional mechanosensitive channel.

Touch assays on *mec-4 (u253); sma-1(e30).* Error bars represent S.E.M.

4

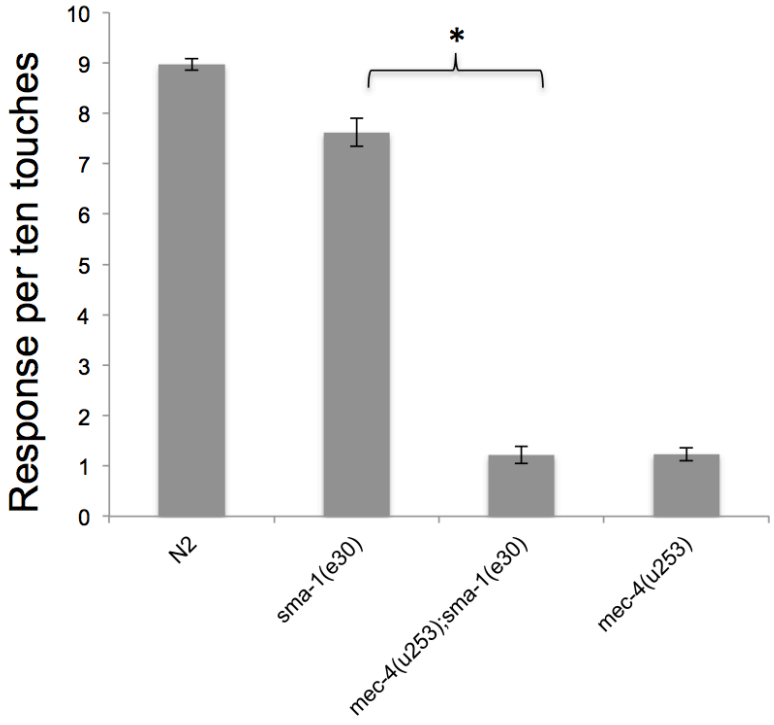


Fig 4

Figure 5. Models

5a. Concentration model: In a smaller touch receptor neuron as in the *sma-1(e30); mec-17(u265); Δatat-2(ok2415)* animals, factors such as microtubules and channel components could get concentrated. Black bars denote microtubules. Channels are not shown. Circles represent the cell body, ovals represent the TRN process. In a *wild-type* sized *mec-17(u265); Δatat-2(ok2415),* these factors could be diluted and thus, the contribution of these factors to touch sensation cannot be bypassed.

5A

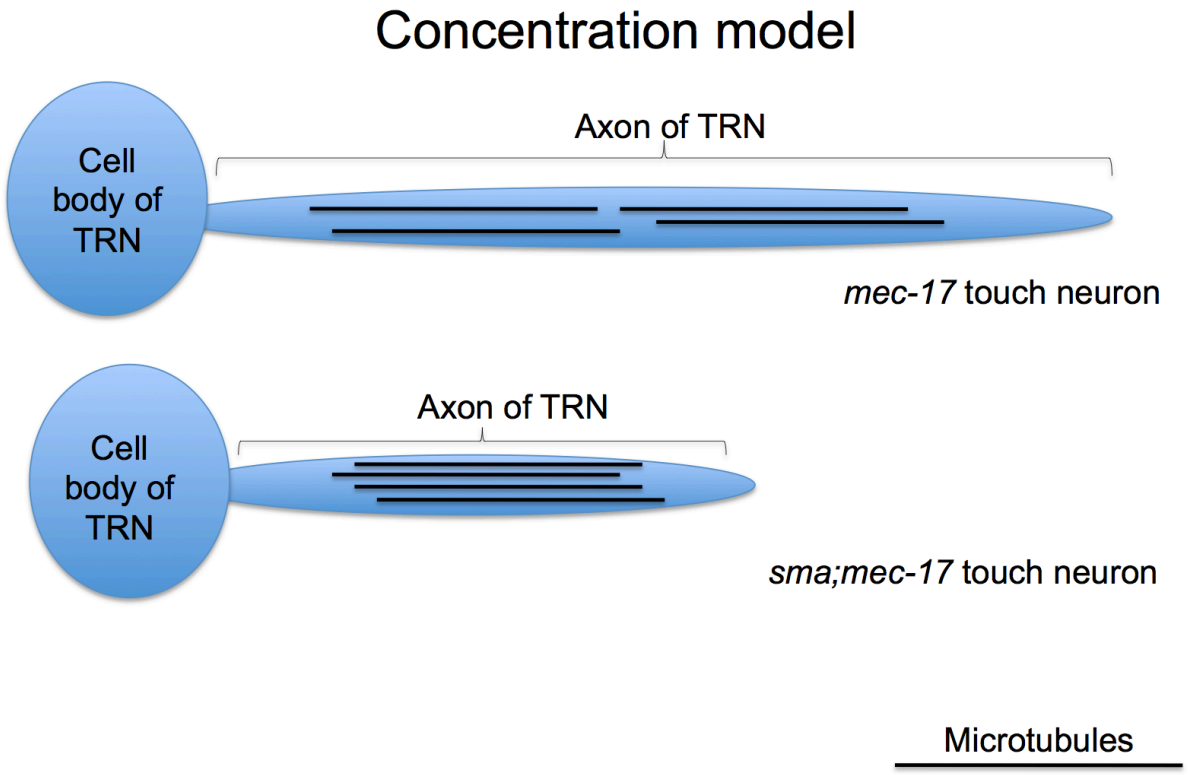
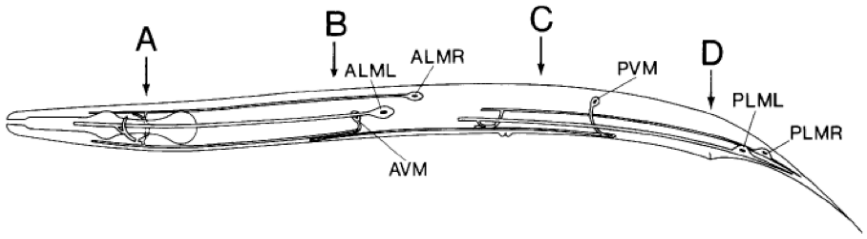


Fig 5a

Figure 5b. Propagation model: In the longer TRN process, the distance that the touch signal has to travel to reach the synapse is lessened in a smaller animal suggesting microtubules are important for the propagation of the stimulus along the axon. Points A and D represent the synapse and B and C are farther from the cell body. Adapted from[6]

5B



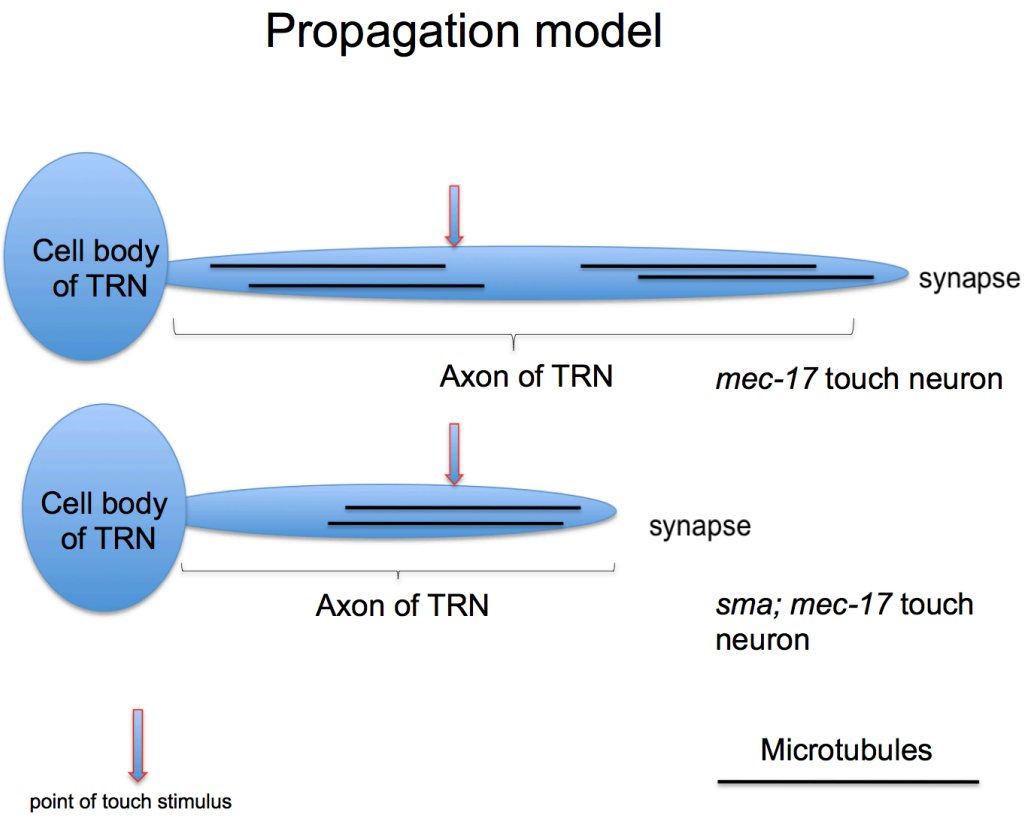


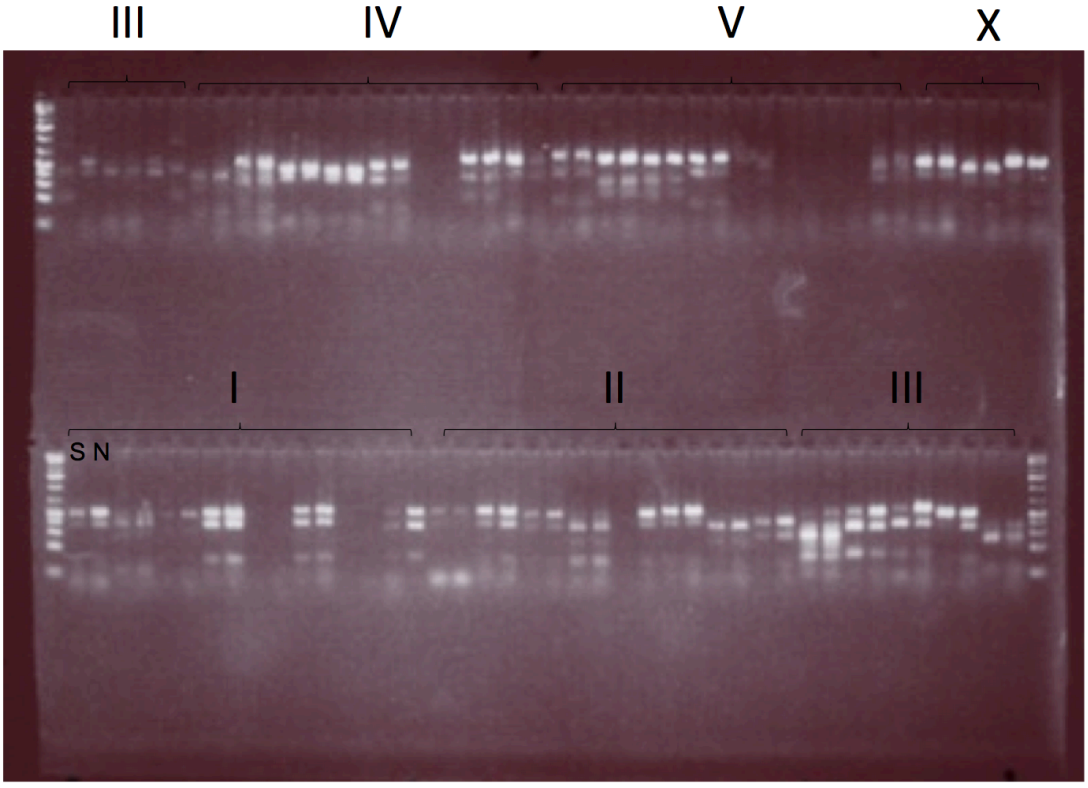
Fig 5b

SUPPLEMENTAL INFORMATION

Supplemental figures

Figure S1. Suppressor 1 is mutated in a gene on chromosome III.

Genotyping gel showing results of chromosome mapping using the methods described in [10]. DNA was extracted from pools of small and non-small F2 worms from a suppressor*Hawaiian cross. Each alternating lane contains DNA from a small or non-small worm pool. Regions of the chromosome showing linkage to N2 DNA (the genetic background the suppressor was in) represent the location of the mutation.



S- Suppressed

N-Non- suppressed

Fig S1

Fig S2. Suppression of touch insensitivity not through suppression of altered morphology of *mec-17(u265); Δatat-2(ok2415)* animals.

TRNs from *mec-17(u265); Δatat-2(ok2415)* have altered morphology with features such as a long ectopic process emerging from the cell body and branches stemming off the main process. Suppressor strains show the same defects. Genotypes are indicated on the image. All strains are in a *zdls5* background to visualize the TRNs.

Top panels show the whole body of the animal. Bottom panels show a magnified view of the morphological defects in *mec-17(u265); Δatat-2(ok2415)* and suppressor strain *sma-2(ek32); mec-17(u265); Δatat-2(ok2415)*

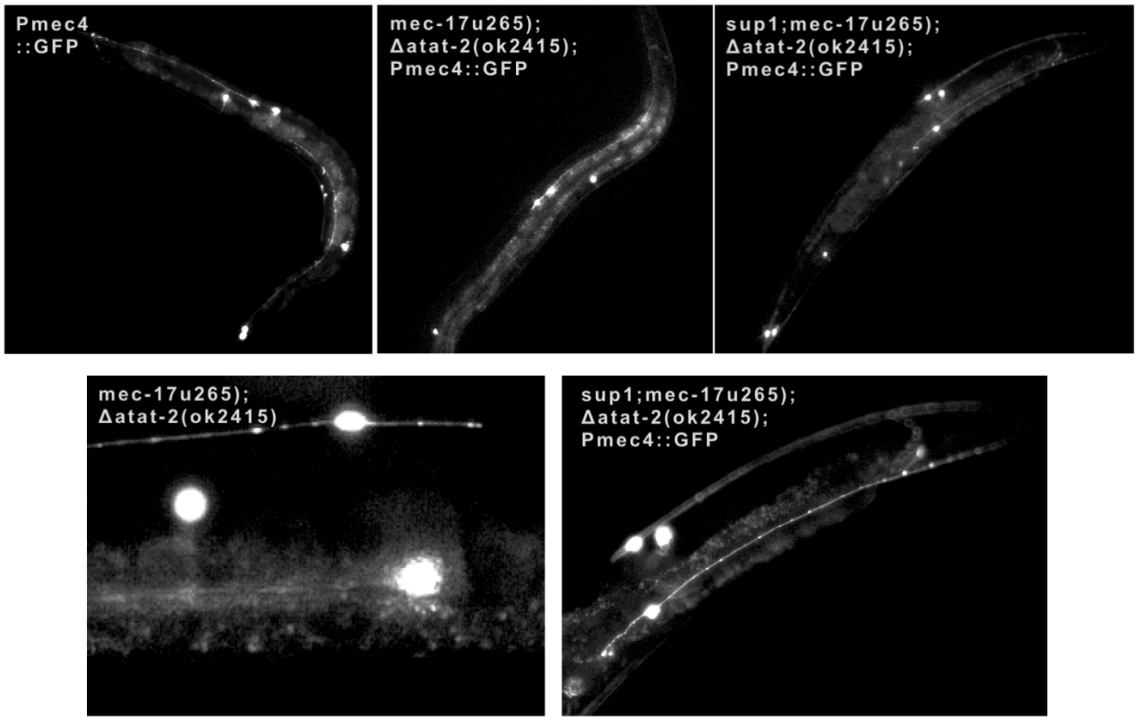


Fig S2

Figure S3. *mec-17(u265); Δatat-2(ok2415)* animals display an "accelerated aging" phenotype.

TRNs in *mec-17(u265); Δatat-2(ok2415)* show altered morphology by L4 stage. These changes in wild-type animals emerge as older adults. L1, L2 and L4 are larval stages of *C. elegans* development.

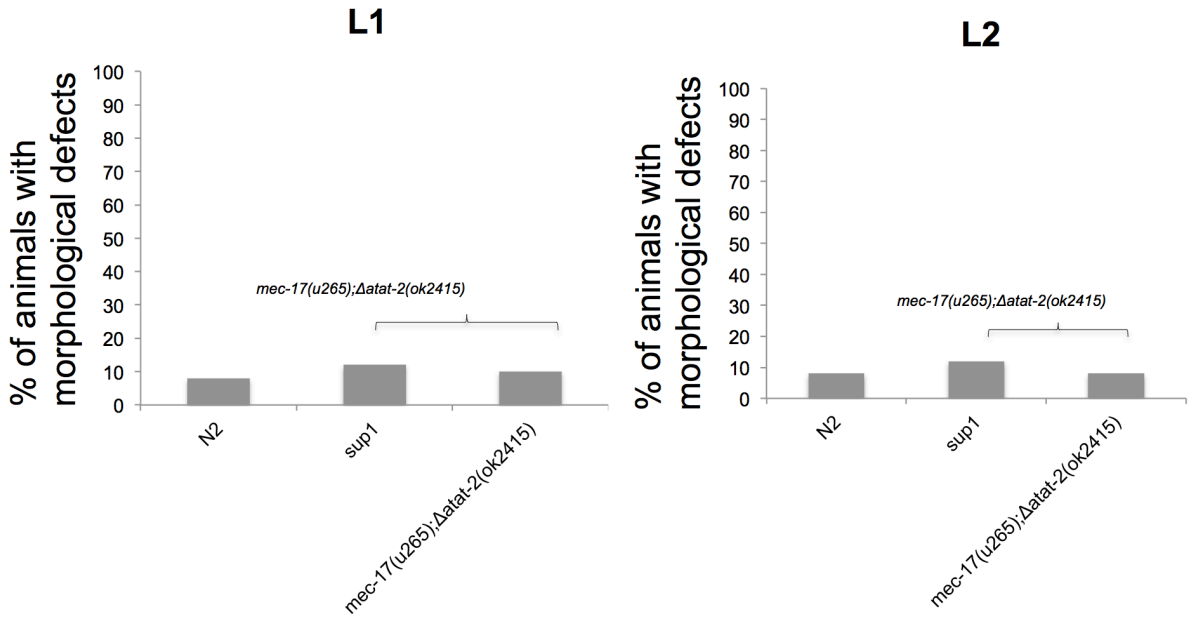


Fig S3 continued on next page

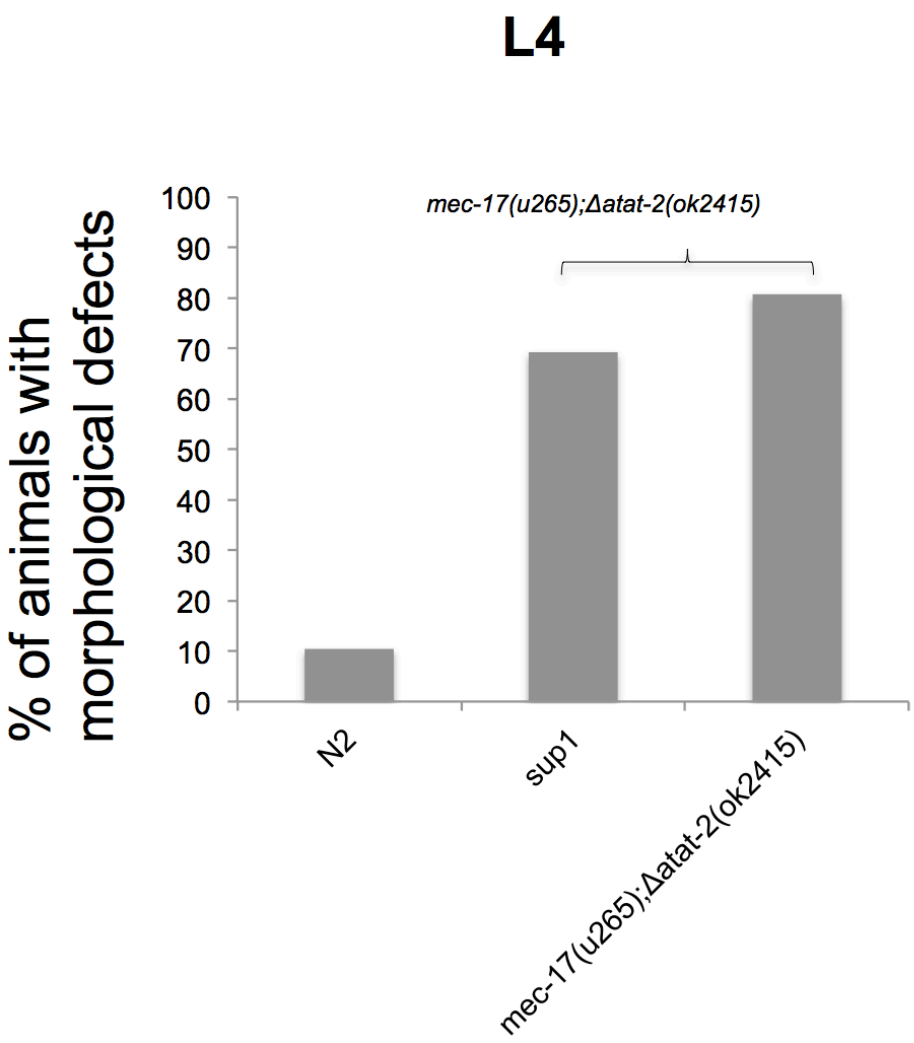

L4
% of animals with morphological defects
100
90
80
70
60
50
40
30
20
10
0
mec-17(u265);Δatat-2(ok2415)
N2
sup1
mec-17(u265);Δatat-2(ok2415)

Fig S3

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

DISCUSSION

***In vivo* validation that MEC-17 is the α-tubulin acetyltransferase.**

Although lysine acetylation was one of the earliest identified post-translational modifications of tubulin, it is one of the least understood. For a long time, the progress made on understanding of the *in vivo* functions of this modification was limited by a lack of the knowledge about the enzyme that acetylates tubulin. Through a combination of genetic and biochemical approaches in three different model organisms, we have identified MEC-17 as the a-tubulin acetyltransferase[1]. Independently, the Nachury group has also identified MEC-17 as the major tubulin acetyltransferase[2]. Interestingly, the Nachury group found that MEC-17 evolved in strict association with cilia. In fact, MEC-17 is a better predictor of a ciliated organism as compared to classical markers such as IFT proteins[2]. In the light of this finding, it is puzzling that *Tetrahymena* lacking *MEC-17* assembles motile cilia. Furthermore, *mec-17* knockout mice lack microtubule acetylation in most tissues suggesting that MEC-17 is the predominant alpha tubulin acetyltransferase in mice as well[3,4]. This eliminates the possibility that the other candidates put forth as a-tubulin acetyltransferases- ELP3 REF and ARD1/NAT1 are important for microtubule acetylation[5,6]. The loss of microtubule acetylation observed in the absence of ELP-3 and ARD-1/NAT-1 could be an indirect effect of loss of these enzymes. Thus, MEC-17 is the primary a-tubulin acetyltransferase whose catalytic activity is conserved from ciliates to mammals.

Structural validation that MEC-17 is the α-tubulin acetyltransferase

Following our lab's and Nachury lab's discoveries, four laboratories produced crystals of MEC-17, confirmed its specificity for a-tubulin and addressed the structural features of MEC-17[7-10]. MEC-17 is a monomer in solution but can form dimers and oligomers when not bound to acetyl-coA and tubulin. All four studies observed that the shape of MEC-17 is very similar that of GCN5 (the histone acetyltransferase that has been studied extensively) despite extremely weak sequence homology. However, MEC-17 has unique structural motifs that play important roles in the binding and acetylation of the K40 of a-tubulin. MEC-17 has a conserved basic region that is well suited to bind the luminal loop containing the K40 residue of a-tubulin that is flanked by acidic residues. MEC-17 binds both acetyl-coA and a-tubulin. The active site (with the residue that mediates catalysis) of MEC-17 is present in a cleft into which the K40 loop is inserted. In MEC-17, the residue that acts as the base for catalysis has been predicted to be different by different groups. One study suggested that a very conserved residue D157 acts as the base for catalysis[7]. This residue is very well placed near the K40 residue to initiate the catalytic reaction. A D157N mutant of human MEC-17 failed to acetylate tubulin from Ptk2 cells *in vitro* suggesting that this residue is very important for microtubule acetylation[7]. However, in another study, a D157A mutant of MEC-17 did acetylate microtubules although with efficiency that was 10% of wild-type MEC-17[8]. Moreover, this mutant enzyme was shown to form monomers and dimers in solution suggesting that this residue is not required for catalysis and when mutated, causes changes in the structure that lead to dimerization. This is consistent with mutating another residue D117 of zebrafish MEC-17[9]. D117 and D151 (a residue homologous to D157 of humans) were candidates for the

catalytic residue in zebrafish[9]. D117 when mutated in zebrafish leads to the formation of dimers. D117 and D151 seem to be important for tubulin binding similar to the D157 of humans. There is an intriguing possibility that D157 has two functions: catalysis and oligomerization, and that the state of oligomerization could be dependent on the binding of acetyl-coA[9]. Possibly the oligomerized forms of MEC17 could be inactive. Alternatively, the oligomerization of mutant forms of the protein represent an artifact of a mutant protein *in vitro*. However, the former explanation could be possible since there is now evidence that suggests that MEC-17 uses autoacetylation to regulate its own enzymatic activity[11]. Mass spectrometric analysis revealed four conserved lysines that were acetylated by MEC-17 on MEC-17 *in vitro* and mutating all four of these lysines to arginine led to a dramatic reduction of microtubule acetylation activity when compared to a wild-type MEC-17 expressed in NIH3T3 cells[11].

Another residue that is a good candidate as the base for catalysis is Q58 (based on the numbering of the human MEC-17 enzyme). Mutating this residue led to a complete failure of MEC-17 to acetylate tubulin[8]. This residue is also positioned close to the acetyl group of acetyl-coA suggesting that the residue is important to hold acetyl-coA. Moreover, this residue is also in close proximity to a water molecule near the lysine K40 binding cleft[8]. In some cases, a water molecule could play a role in catalysis by deprotonation of the lysine residue of the substrate. The Q58A mutants still bind to acetyl-coA suggesting that this residue is important either for catalysis or stabilization of a reaction intermediate such as the acetylated lysine still bound to coA. The latter suggestion seems to be more likely since in *C. elegans,* Q58 is occupied by a leucine. It is unlikely that a leucine acts as a base for catalysis. This is because leucine is an

uncharged amino acid. So, it is hard to imagine how it can remove a proton from the lysine of the substrate. More importantly, Q58 is mutated to a phenylalanine in the *mec-17(u265)* mutant[12]. This mutant lacks detectable acetylation of microtubules consistent with the importance of Q58 (L58) in holding the acetyl-coA so as to let the acetylation reaction reach completion[8].

To conclude, both extensive *in vivo* studies as well as structural approaches have identified MEC-17 as the K40 a-tubulin acetyltransferase.

Significance of microtubule acetylation *in vivo*

We aimed at studying the *in vivo* functions of microtubule acetylation using knockouts of *mec-17* in *Tetrahymena thermophila* and zebrafish. A complete loss of MEC-17 led to a loss of microtubule acetylation in *Tetrahymena thermophila* but the mutant phenotype was nearly normal except for a mild change in resistance to anti-microtubule drugs such as taxol. While a morpholino-based knockdown of *mec-17* in zebrafish led to a variety of phenotypes (including a smaller head, smaller body, smaller eyes, hydrocephalus and an impaired response to being poked gently with a probe), a null mutation in *mec-17* introduced by a TALEN site specific nuclease resulted in the F2 generation homozygotes that are superficially normal (Tseng W., Akella *et al* unpublished observations, see below). Thus, we focused our functional studies on the *C. elegans* model. *C. elegans* mutants of *mec-17* have greatly reduced microtubule acetylation in the touch receptor neurons and an easily observable phenotype that is based on the function of TRNs: a loss of touch response[13].

Surprisingly, in two independent studies, the MEC-17 knockout mice lacking microtubule acetylation in most tissues are mostly normal except for mild sperm motility

defects and a slight deformation in the dentate gyrus, a structure in the brain that plays a role in learning and memory [3,4]. The MEC-17 knockout mice are slightly anxious according to a behavioral test. This phenotype is exactly opposite to the behavioral phenotypes observed in the HDAC6 knockout mice, which were less anxious and showed anti-depressant like behavior according to behavioral tests which compared how the HDAC6 knockout mice respond to stressful and non-stressful situations[14]. Therefore, it seems that in a mammalian model microtubule acetylation plays subtle functions and that its function while subtle, in the brain is important enough to contribute to behavior. It would be interesting to see if the mouse MEC-17 phenotypes worsen with age. It is possible that a subtle defect could become more serious over time. Although farfetched, a reduction of microtubule acetylation was suggested to be an early event in Alzheimer's disease where an increase in neurofibrillary tangles, one of the signs of pathology associated with Alzheimer's disease correlated with a decrease in the levels of microtubule acetylation[15].

Although *mec-17* knockout mice have sperm motility defects, it was surprising that the flagellar 9+2 axoneme structure seen in an electron microscope was not affected[3]. This again is similar to the nearly normal phenotype of HDAC6 knockout mice, which had hyperacetylation of tubulin but were viable and had no phenotypes except for a slight reduction in immune response when challenged. Thus, through direct and indirect approaches, it has been found that cells can tolerate a great degree of variation in the level of tubulin acetylation and that this modification is quite dispensable in spite of its ubiquitous nature.

Although microtubule acetylation has been suggested to play a role in several processes including neuron migration, regulation of binding of motors, regulation of intracellular motility and neuron polarization via indirect methods such as replacement of K40 of α-tubulin or HDAC6 knockout or overexpression[5,32-34], so far, there is evidence only for a role for microtubule acetylation in the migration of cortical projection neurons. RNAi on MEC-17 in mice led to a migratory defect in the cortical projection neurons similar to those observed in mouse e17.5 embryos, which overexpressed an A40 a-tubulin instead of a K40[35]. Moreover, the migration defects in the *mec-17* RNAi mice were shown to be rescued by the overexpression of a K40Q a-tubulin that is expected to mimic acetylated a-tubulin. This result suggests that microtubule acetylation by MEC-17 is important for neuron migration.

Initially we reported that zebrafish embryos in which we depleted MEC-17 with morpholinos have developmental defects such as smaller body size, smaller heads, smaller eyes, hydrocephalus and impaired response to gentle poking[1]. To address the specificity of the knockdown phenotypes, we used control morpholinos that had a similar sequence but were predicted not to target MEC-17 mRNA due to a point mutation. However, we were not successful in performing a gold standard for the morpholinos experiments: showing that injection of a targeted mRNA rescues the morpholino phenotype. This experiment was difficult because injections of MEC-17 mRNA alone led to overexpression phenotypes (our unpublished data).

To determine whether the phenotypes observed in the *mec-17* knockdown zebrafish were specific or a side effect of morpholino toxicity, in collaboration with Wei- Chia Tseng and Dr. Scott Dougan we generated a *mec-17* knockout zebrafish. We used

TALEN technology to generate these knockouts. TALENs are artificial DNA binding proteins fused to a restriction enzyme such as FokI[16]. Two TALENs that bind on either side of a DNA target sequence form FokI heterodimers that cleave the DNA sequence target. This results in the activation of the non-homologous end joining repair pathway, which leads to insertions and deletions at the target site. Preliminary data on the *mec-17* knockout fish suggests that the morpholino phenotypes might be side effects of injection of morpholinos. The *mec-17* homozygous knockout F2 fish lack microtubule acetylation but do not display obvious morphological defects except for a low frequency defects in embryonic development. However, to eliminate the possibility because of a maternal mRNA contribution, we are generating an F3 generation of *mec-17* knockout zebrafish. It is not very surprising that *mec-17* knockout F2 fish lack any phenotype because *mec-17* knockouts in mice do not show any gross morphological defects. The *mec-17* knockout zebrafish can be observed for a longer period to see if they develop any defects over time, for example with aging. Recently, tubulin PTMs have been shown to play critical roles in axon regeneration. It was observed that post-axonal injury, the levels of tubulin PTMs such as deglutamylation increase to sustain growth of the remodeling microtubule network in the injured axon[17]. Moreover, in mice, tubulin is deacetylated after injury and this injury-mediated deacetylation of tubulin occurs only in peripheral neurons and not neurons of the central nervous system[18]. It is important to mention that in mice, only peripheral neurons regenerate post-injury while the neurons of the central nervous system cannot suggesting that there might be a new role for microtubule acetylation in regulating the regenerative capacity of microtubule based structures such as neurons post-injury. Mice sciatic nerves (peripheral nerves) when injured showed a reduction in the levels of

tubulin acetylation whereas optic nerve (Central Nervous System) did not show any changes in the level of acetylation. This microtubule deacetylation close to the injury site is mediated by HDAC5 and this deacetylation seemed to be important for control of growth cone dynamics in axon regeneration[18]. It is known that injury triggers a local destabilization of microtubules so, it is not certain whether this reduction in microtubule acetylation level is a direct effect of HDAC5 deacetylating microtubules or whether dynamic microtubules don't live long enough to be acetylated. Whichever it is, zebrafish are a great model for studies aimed at identifying the role of microtubule acetylation and in general any tubulin PTM in axon regeneration. It is remarkable that in zebrafish, unlike other vertebrates such as mice, neurons of the Central Nervous System are also capable of regeneration[19]. Not only can zebrafish be used to study the role of individual proteins in axon regeneration but, zebrafish can also be used to identify why only peripheral nerves can regenerate in mice and other vertebrates. It is known that myelin plays an inhibitory role in the regeneration of neurons of the CNS post injury. One question we could answer using zebrafish is whether there is injury-triggered deacetylation of microtubules in the central nervous system of zebrafish. If yes, then this would further support a role for microtubule acetylation in the regulation of regenerative capacity of neurons. Zebrafish could be explored to identify further more factors that contribute to promoting regeneration.

The understanding of the function of MEC-17 *in vivo* is complicated by multiple observations that indicate that in addition to tubulin acetylation MEC-17 acts non-enzymatically. Key observations came from studies in *C. elegans*. Unlike other models in which MEC-17 was studied, the *C. elegans mec-17* mutants have a robust phenotype: a

lack of microtubule acetylation and lack of touch response mediated by the six touch receptor neurons. We asked whether loss of microtubule (tubulin) acetylation in *mec-17* mutants is what led to touch insensitivity in these animals. To our surprise, replacing the only K40 residue of MEC-12 (the only a-tubulin in C. elegans that has K40, and the a-tubulin required for touch sensation) with R or Q reduced touch response only slightly[1]. Our observation was the first indication that MEC-17 function *in vivo* could be more complex, and not solely based on generating acetyl-K40 on a-tubulin. This finding was later extended by others who found that in fact, MEC-17 contributes to touch sensation acting in a way that does not involve catalysis of lysine acetylation (see below)[20]. On the other side, one recent study showed that *C. elegans* with the mutated K40 on MEC-12 a-tubulin (to R) has touch receptor neurons with disorganized microtubules[21]. TEM studies on these strains showed severe disruption of the organization of 15 pf microtubules including a reduction in the microtubule number and length and increased variation in the protofilament number consistent with the protofilament defects observed in *mec-17* null and missense mutant animals, suggesting that MEC-17 mediated acetylation of MEC-12 at K40 contributes to microtubule stability and regulation of microtubule protofilament number[20,21]. Furthermore, based on molecular modeling simulations, K40 microtubule acetylation could promote interprotofilament interactions by aiding the formation of an interdimer salt bridge[21]. However, the weakness of this study is that these data were generated using extra chromosomal arrays expressing transgenic versions of MEC-12. We are aiming at addressing this ambiguity by using TEM to study our strains expressing an integrated single copy of K40/ R40 MEC-12 in a *mec-12(e1607)* background. A key observation that resolves the discrepancies between our and the Goodman lab study was

made by the Chalfie lab found that the touch response defect of the *mec-17* null mutant can be rescued by expression of a version of MEC-17 that lacks the acetylation activity[20]. This experiment also eliminated a possibility that MEC-17 contributes to touch sensation by acetylating a non-K40 residue on MEC-12 or even another protein. However, the MEC-17 mutants with an acetylation-inactive form of MEC-17 continue to have severely disorganized microtubules similar to those seen in the null MEC-17 mutant[20]. This surprisingly indicates that microtubules are less important for touch sensation than originally thought, and that the 15 pf microtubules can be greatly reduced and replaced by other diameter microtubules and the animals still respond to touch. At this point of time, we can definitively say that the catalytic function of MEC-17 is via microtubules since acetylation incapable versions of MEC-17 do not rescue microtubule defects observed in *mec-17* mutants[20]. Whether MEC-17 acts via microtubules to fulfill its non-enzymatic function is still unclear but our suppressor studies suggest that this is the case (see below).

Non-enzymatic function of MEC-17

The non-acetylating function of MEC-17 was recently suggested to be a destabilization of microtubules. Fibroblasts isolated from the *mec-17* knockout mouse have microtubules that are more resistant to depolymerization by nocodazole[11]. This result is in contrast to our data on *Tetrahymena thermophila* that showed increased sensitivity of MEC-17 knockout cells to oryzalin (another microtubule depolymerizing drug) suggesting that MEC-17 could play a role in microtubule stabilization. Also, an earlier paper showed that acetylated microtubules are more resistant to depolymerizing drugs[22]. Moreover, it is difficult to predict how to tie the microtubule destabilizing effect of MEC-17 observed in

mec-17 knockout mice cells to the low microtubule number in the TRNs of the *mec-17* mutants in *C. elegans*[20]. However, the mouse study correlating MEC-17 with a microtubule destabilization activity should not be dismissed as an odd result. One possibility is that MEC-17 actually stabilizes microtubules (by acting inside them) but that destabilization of a subset of microtubules creates an excess of soluble tubulin dimers that can polymerize into abnormal microtubules in other locations in the same cell. For example, while the *mec-17* mutants of C. elegans have fewer microtubules in the axon, the same cells have extra branches and ectopic process emerging off the main axon or off the cell body (Akella *et al*; unpublished observations). It is important to remember that in neurons, microtubules can be nucleated acentrosomally[23,24]. It is possible that a and b-tubulin localized at the branch could nucleate some microtubules later. Microtubules can be polymerized and fill the empty this space similar to how microtubules invade a space available upon actin depolymerization during neuron growth[25]. It is likely that MEC-17 not only generates more seeds for more number of microtubules to be nucleated but, it could be that MEC-17 is required for the structural stability (via promoting interprotofilament interactions through its enzymatic activity and perhaps the luminal material in the presence of MEC-17 makes the microtubule more stable from the inside as well) and without MEC-17, the microtubule falls apart, adopts different protofilament configurations and is very unstable and more seeds are available from the depolymerizing microtubules that permits formation of branches. It would be interesting to look at EB-1 dynamics in the presence and absence of MEC-17. EB dynamics should tell us whether there is an increase in the number of growing microtubules in the mutants of *mec-17*.

The TRNs of mec17 mutants display certain morphological features such as a long ectopic process that emerges from the cell body and branches that sprout off the main process. It is not clear if these are defects or just signs of a prematurely old axon because these features are also observed in older wild-type animals. However, the remarkable feature of the *mec-17* mutants is that these phenotypes emerge much earlier in these animals by L4 (late larval stage) in contrast to older wild-type adults[26]. It is safe to say that these animals show an "accelerated aging" phenotype now increasingly common among mutants of genes that encode components of the cytoskeleton including MEC-7 b-tubulin and the tubulin deglutamylase enzyme CCPP-6[17,27,28]. It would be interesting to know how microtubules and microtubule based processes such as transport change with age.

Role of microtubules in the TRNs of *C.elegans*

It was very puzzling to us that the contribution of the microtubule acetylation function and in general, the acetylation function of MEC-17 was minimal to touch sensation. We wondered about how MEC-17 contributes to touch sensation non-enzymatically. Moreover, the finding that mutants with severely disorganized microtubules can sense touch did not fit with previous observations emphasizing the importance of the 15 protofilament microtubules for touch sensation. To probe this further, we devised a suppressor screen strategy to identify genetic interactors of *mec-17* that would provide us insights into how MEC-17 is required for proper microtubule organization in the TRNs and also to identify how MEC-17 contributes non-enzymatically to touch sensation. More importantly we wanted to know if the non-acetylation function of MEC-17 was through microtubules. We identified *sma-2* that encodes a receptor SMAD in the TGF-

β signaling pathway as a suppressor of *mec-17* mediated touch insensitivity. TGF-b signaling pathway plays a major role in the regulation of body size in *C. elegans*[29]. The biggest surprise was that making the *mec-17* animals smaller and thus, reducing the size of the TRN could rescue touch sensation. What was even more surprising was that a mutant suggested to have no 15pf microtubules in the TRN could be rescued of touch insensitivity by reducing the size of the neuron. Although it is known that mutations in cytoskeleton associated genes lead to a slightly smaller body size, it was unexpected that reducing body size could bypass microtubule function. It is important to mention that another protein ZYG-8, the *C. elegans* homolog of doublecortin, that selectively stabilizes and promotes the formation of 13 protofilament microtubules in vertebrates when mutated, causes a reduction in the number and length of microtubules in TRNs[30]. However, microtubule architecture is not altered in zyg-8 mutants[30] suggesting that in *C. elegans* TRNs, MEC-17 could be the major organizer of microtubules. It is interesting that mutations in *zyg-8* cause touch insensitivity with a small reduction in body size. It is puzzling why these mutants are touch insensitive although they have a smaller body size. The finding that these *zyg-8* mutants have fewer microtubules supports our concentration model. Is it possible that the presence of MEC-17 or the remaining 15 pf microtubules in these animals prevents a bypass of microtubule function by reducing body size? Perhaps a double mutant of *mec-17* and *zyg-8* or double mutants of *zyg-8* and the genes encoding the tubulins that make 15pf microtubules could help answer this question.

Because the acetyltransferase inactive MEC-17 also seemed to rescue the morphological defects observed in the TRNs of *mec-17* animals along with touch insensitivity[20], we wondered whether the morphological defects in the *mec-17* mutants

contribute to touch insensitivity. However, there seems to be no correlation between morphological defects and touch insensitivity because, all of our suppressed strains display morphological defects yet are partially touch sensitive. Whether these morphological defects arise due to an increase in TRN gene expression is not known. It is unlikely that the *mec-17* mutants have increased TRN gene expression since it was observed that microtubule depolymerization using colchicine led to a reduction in gene expression in touch receptor neurons[31]. It is noteworthy that this non-conventional role of microtubules seems to be just the beginning of an identification of novel roles for microtubules in events other than transport and growth. Whether the shortening of the axon rescues touch insensitivity by forming synapses with some other neurons is currently unknown.

MEC-17 as a marker of the microtubule lumen

Acetylation is fast emerging as one of the most prominent post-translational modifications with the number of substrates rivaling those that are phosphorylated and ubiquitinated[36]. One of the remarkable features of microtubule acetylation is its luminal location of the K40 residue that atubulin is acetylated on[37]. Although there are more residues in atubulin that could be acetylated, K40 is highly preferred for microtubule acetylation *in vitro.* Indeed, MEC-17 across several species has evolved from a conserved core similar to histone acetyltransferases into a structure that could accommodate the K40 containing loop[7-10]. Moreover, the structural data gives us the dimensions of the catalytic domain of MEC-17, which is 3-6nm[7,8]. How MEC-17 gains access to the lumen of the microtubule is currently unknown. Our previous work indicated that the enzyme could gain access to the lumen via diffusion using the open microtubule ends[1]. Others have

suggested that the enzyme could also access the lumen through the openings between the protofilaments or through lattice defects[2,21]. The former suggestion is unlikely because the dimensions of these openings are ~1.7nm which is too small for the enzyme to fit in. Moreover, it was observed that MEC-17 acetylates microtubules with much more efficiency than tubulin dimers[1]. This could be because once the enzyme gains access to the lumen, it can have a very high concentration because the inner diameter of the lumen is 15nm. TEM study of TRNs in *mec-17* mutant showed that MEC-17 is required for the presence of an electron dense material in the lumen of microtubules[20,38]. Whether the luminal structure is *MEC-17* or something else that requires MEC-17 is unknown yet. It is important to mention that the lumen of the microtubule has been an enigma with many "sightings" of luminal staining across olfactory axons of frog to the TRNs of *C. elegans*[39,40]. More recently, cryo-electron tomography on microtubules in hippocampal neurons suggested that luminal particles inside microtubules occur in increments of 4nm[38]. These luminal particles were observed to be in different conformations. Some particles were bound to both sides of the microtubule wall and some bound only on one side and some unbound[38]. It is not known whether these particles are in transit or stably attached to the lumen. The 4nm spacing of the particles suggests they are not directly dependent on K40 acetylation (if it were the case, the spacing would be expected to be 8 nm, the size of the tubulin dimer). At present tubulin K40 acetylation is the only known molecular event that occurs in the lumen and MEC-17 is the first luminal marker.

Role of the 15 protofilament microtubules in touch sensation

The 15 protofilament microtubules of the TRNs have been an enigma. Despite being one of the most unique structural characteristics of these neurons, their function has not been

identified yet. Why is it that 15 protofilament microtubules are selectively acetylated over 11 protofilament microtubules? Is it possible that the lumen of the 11 nm microtubule is not large enough for the enzyme to gain access to the luminal side? While in other species 13 pf microtubules undergo acetylation by MEC-17, in *C. elegans* the reduction to the size of 11 pf could exclude MEC-17. The 15 protofilament microtubules seem to play a specialized role perhaps by making the membrane stiffer and thus more responsive. How a smaller TRN axon can get away with severely defective microtubules is not yet known. Is the smaller size of the TRN axon similar to earlier developmental stages where these axons have fewer microtubules that the later stages of development? Certainly the low microtubule number seems to be sufficient since the earliest larval stage animals in *C. elegans* seem to respond to touch[41]. Our experiments to test both our models should give us an answer as to what roles microtubules play in longer animals that contributes to touch sensation. Whether in smaller animals, transport is efficient enough and thus, components of mechanosensitive channels are well placed and if in a longer axon, this transport is disrupted is not known. Our immunostaining using an antibody against MEC-4 should give an answer to this question as well. Moreover, our immunostaining for MEC-4 and colchicine experiment should help us answer an important question: Is there any feedback to the cell body about how long the axon is? All in all, our experiments might help provide insights into the sensory role microtubules play in the TRNs of *C. elegans.* This would add to the new list of non-conventional roles microtubules have been identified to play including regulation of gene expression.

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