Chapter 7: Conclusions and Discussion

Intracellular trafficking is important in neurons for its development, structure, and function. Long distance transport is mediated through microtubules that are involved in regulating multiple processes such as structure, transport, and signaling pathways. The sequence of both α and β tubulins is conserved across organisms (Cleveland and Sullivan, 1985; Little and Seehaus, 1988; Burns, 1991). In this work, I found novel SNPs in mec-7, a β tubulin gene, which could give TRN specific mitochondrial and vesicle trafficking and neuron morphology defects in C. elegans. This study gives additional residues that are crucial in β tubulin for TRN morphology, trafficking, and mechanosensation. mec-7 is not essential for other neurons and growth thus provides more opportunities for modifications and understanding the interactions and tubulin biology in the cell.

7.1) Isolated mutants are alleles of mec-7

Mutants isolated from the screen, designed to target molecules affecting the mitochondrial distribution, belong to the same complementation group with the allelic strength: \( tb118 > tb132 > tb133 \). SNPs annotation from Whole genome sequencing gave novel SNPs in two candidate genes: mec-7 and a non-annotated protein-coding gene \( K09F5.6 \). Considering the trafficking and neuron morphology defects exclusively in touch receptor neurons, mec-7 that is essential for specific large diameter microtubules in TRNs and mechanosensation appeared to be the major candidate which could give the mutant phenotype. Though we could not rule out the possibility of the contribution of other candidate gene \( K09F5.6 \) in giving the TRN phenotype. Consistent with the sequencing results, non-complementation with mec-7(e1343) and mec-7(e1506) confirmed that the mutants are alleles of mec-7 as discussed in chapter 4. Comparison of \( tb118/+ \) and mec-7(e1506)/\( tb118 \) showed that the presence of wild-type mec-7 copy could rescue the mitochondrial distribution phenotype. Heterozygotes \( tb118/+ \) showed mitochondria distributed throughout the neuronal process without branching phenotype, though showed occasional curved neuronal process ending.
7.2) Contribution of K09F5.6 is not known

K09F5.6 encodes for a non-annotated protein and expresses in body wall muscles and neurons (Watson et al., 2008; Fox et al., 2005; Smith et al., 2010; Von Stetina., 2007; Spencer et al., 2011). It shows homology to mediator MED15 that is involved in transcription with RNA polymerase II. K09F5.6 might be involved in transcription in cells and signaling by an unexplored mechanism. Though the role of each mediator in transcription is not well defined, in vitro and in vivo study of various mediators show that they possibly interact with different transcription activation domains of different transcription activators which bind to DNA (Kato et al.,2002). Genetic studies have demonstrated the role of Mediator in activation as well as repression (Song et al., 1996; Li et al., 1995). Biochemical experiments suggest that mediators could increase the rate of transcription initiation complex assembly thus increase the efficiency of transcription activation. Med15 (Gal11) has shown direct interaction with general transcription factors including, TFIIE and TFIIH and an absence of MED15 (GAL11), MED2 or MED3 (PGD1) lead to synthetic lethality along with mutations in the large subunit of TFIIE (Sakurai and Fukasawa, 2000). Considering K09F5.6 expression in multiple cells and neurons, might also express in TRNs at low levels or absent, as the defect in mutants are restricted to TRNs, thus, it is highly unlikely that K09F5.6 would have a large contribution to the phenotype.

7.3) mec-7 mutants showed TRN specific defects

The mutants tb118, tb132, and tb133 showed multiple defects exclusively in touch receptor neurons viz. mitochondrial and vesicle accumulation near cell soma and neuron morphology as ectopic branching along the neuronal processes. MEC-7 is enriched in TRNs and expressed in low levels in PVD and head neurons. mec-7 show 93% homology with Drosophila β tubulin and other β tubulins. In addition to highly variable C- terminal domain among β tubulins, MEC-7 is distinguished from other isoforms by seven amino acid residue positions as residues 35 (Gln), 127 (Thr), 198 (Ser), 278 (Ash), 293 (Cys), 343 (Asp), and 429 (Ala) (Sulston et al., 1989).

mec-7 is crucial for the formation of 15 protofilament microtubules in TRNs which are arranged in bundles and essential for mechanosensation as in mec-7 null mutants, where 15 protofilament microtubules are replaced by 11 protofilaments like in other neurons, do not respond to gentle
touch by eyelash (Chalfie and Sulston, 1981; Chalfie and Thomson 1979, 1982). Mec behavior of the mapped mutants suggests that the TRNs are not functional. Though we have not looked at microtubule structure, it is possible that in mutants the mec-7 might have been replaced by other tubulin genes and thus the ratio of 11 protofilaments to 15 protofilament microtubules have increased. C. elegans genome show nine α- and six β-tubulin genes (Consortium, 1998; Gogonea et al., 1999). Recently four tubulins (tba-1, tba-2, tbb-1, and tbb-2) have been shown to play some redundant role in TRNs (Fukushige et al., 1993, 1995; Lockhead et al., 2016). We cannot say if the trafficking and branching phenotype could be separable or linked. Dominant and semi-dominant mec-7 mutants have been observed to show ectopic branching phenotypes, but null mutants are mec without any branch morphology defects. In this study, we have isolated novel recessive mutants which show severe neuron morphology defects in addition to trafficking defects.

In mutants, the defects are severe in PLM compared to ALM and the extent of the neuron morphology defects show an increase with the development. During the differentiation, PLM, and ALM show difference in few molecules which are involved in the maintenance of the neurons and their specific expression in either ALM or PLM (Toker et al., 2003; Chen and Chalfie, 2015). Thus, the difference in the extent of phenotype in PLM and ALM could be due to the involvement of additional neuron-specific molecules.

Initially, it was believed that mec-7 β tubulin is not important for neuron development (Chalfie and Sulston 1981), but later studies showed that like other β tubulins mec-7 is also involved in determining structural, functional and physiological properties of TRNs (Savage et al., 1989; Savage et al. 1994; Chen et al., 2014; Lockhead et al., 2016). Defects in mec-7 and mec-12 have been shown to cause the major defects in TRNs outgrowth and positioning. The strong mutants also showed short neuronal processes as shown some dominant alleles of mec-7 and in recent mec-7 and mec-12 null (Savage et al. 1994; Chen et al., 2014; Lockhead et al., 2016). The isolated mutants also showed defects in microtubules stability and dynamics in TRNs.

Mutations in mec-7 do not affect microtubules in other neurons (Chalfie and Thompson, 1982) which could be the reason why the mutants did not show any gross defects in neuron morphology and cargo distribution in non TRNs. Though mutants do not show any morphological defects in other neurons, they are sensitive to aldicarb which could be due to very
weak signal in TRNs synapses as it transmits signal by graded potential i.e., gets accumulated and transmitted (Hobert, 2005; Lockery and Goodman, 2009).

7.4) Touch Receptor Neurons in mutants did not change fate to sister sensory neurons, PVD or FLP

In addition to the simple morphology of touch receptor neurons (TRNs), the mechanosensory circuit also shows neurons with elaborate branching. Transcription factors play important role in fate determination and arrangement of sensory neurons, their downstream targets play important role in differentiation and maintenance of the diverse fate (Jinushi-Nakao et al., 2007). Mechanosensory neurons have been categorized and differentiated further in subgroups that respond to specific stimuli depending on their origins. The presence of MEC-3 is crucial for differentiation and function of set of sensory neurons as the absence of *mec-3* results in defective gentle as well as harsh touch and neuron morphology (Way and Chalfie, 1988; Smith et al., 2010 and Tsalik et al., 2003). Mutants of AHR-1 and ZAG-1 showed the conversion of AVM and PVM fate into PVD like neuron showing extra branching as present in PVD dendrites at AVM and PVM positions respectively (Smith et al., 2013). FLP neurons in the head also show an elaborately branched structure and respond to harsh mechanical stimuli (Albeg et al., 2011; Smith et al., 2010; Chatzigeorgiou and Schafer, 2011). FLP and TRN neurons fate restriction is maintained by few molecules such as *alr-1* and *egl-44* (Topalidou et al., 2011; Topalidou and Chalfie, 2011). TRNs in mutants *tb118*, *tb132* and *tb133* do not show defects in the fate determination path as did not express markers specific to the PVD and FLP neurons.

7.5) Mutants have defects in microtubule dynamics and stability in TRNs

The isolated mutants of *mec-7* show defects in microtubule dynamics as observed with the help of EBP-1 and EBP-2 dynamics and have less stable microtubules as large gaps in acetylation of TRNs observed. Moreover, mutants also showed interaction with acetyltransferases *atat-2* (Topalidou et al., 2012), the observations together suggest that the morphology defects are more due to defective microtubules. The importance of microtubules dynamics in neuron development, maintaining structure and function are well reviewed (Dubey et al., 2015). The ratio of the stable
and dynamic population could be important for the structure, function, and physiology of the neuron.

The length of touch receptor neurons in adult animals is ~500µm formed by overlapping microtubules of 10-20µm in length. In isolated novel alleles of mec-7, there might be a reduction in the number of 15 protofilament microtubules and increase in 11 protofilament microtubules as have been observed in some mec-7 loss of function or null mutants (Chalfie & Thomson, 1982). MEC-7 β tubulin has three domains N-terminal, intermediate filament and C-terminal domain. N-terminal had GTP binding site and the intermediate domain has dimerization domain. the ‘hinge’ region between aa250 and aa300 has been implicated in the interactions between protofilaments (fig.a). Residues of the N-terminal domain are important for protein folding and conformation and contain the guanine nucleotide binding region (Downing and Nogales, 1998; Löwe et al., 2001). The residues in the C-terminal domain bind both MAPs and motor proteins (Littauer et al., 1986; Löwe et al., 2001). The isolated mutants show homozygous missense mutation as E110K, N256D, and S364S as shown in figure a. E110K is novel SNP whereas residue N256S and 364 has been correlated with diseases where they affect microtubule stability.

The defects in microtubule dynamics could be the primary reason for the defects in trafficking in mutants. As shown in Yogev et al., 2016, vesicles pause at microtubule ends and their movement is affected by the microtubule length, my hypothesis is that microtubule defects in the mutants might have affected motors interaction with the microtubule and unstable and short microtubules could have lead to the cargo clustering in the proximal region and in between the neuronal process. Both EBP-1:: CFP and EBP-2:: GFP tracking data showed less growth and more shrinking of microtubules in mutants compared to wild-type indicating the presence of a large fraction of short and unstable microtubules in the mec-7 mutants viz. tb118 and tb132.
**Figure 1: SNPs location in MEC-7 Protein**

The mec-7 amino acid sequence with highlighted changed residues. Changed amino acid and corresponding allele mentioned parallel to Mec-7 sequence. Residues affected, **N256D** present in *tb118* and *tb132* and **S364S** in *tb132*, in the alpha- beta dimerization zone. N-terminal, Intermediate, and C-terminal domains are marked with ( ).

**E110K in *tb133* in the provisional chain could affect the conformation**

E110K is a novel SNP which is present in the GTP-binding region of the N-terminal domain. This substitution of Glutamic acid to lysine, residues often involved in making salt bridges, lead to a change in minus to plus charge on the residue, thus could affect interaction with the corresponding residues. Semidominant allele *mec-7(u222)* show the G109E change that affects GTPase activity (Savage et al., 1994). This residue is just adjacent to E110K thus possibly could affect GTP hydrolysis or confirmation of the dimer. Heterodimers, α-β tubulins, undergo GTP hydrolysis at the β chain and form protofilaments which interact together to form microtubule. The mutation viz. E110K present in the provisional region of the protein possibly affects...
polymerization of dimers formed by mec-7 β and mec-12 α chains but show weak trafficking defects possibly due to reduced 15 protofilament and increased ratio of 11 protofilament by other β tubulin isomers with mec-12 α tubulin in TRNs.

**N256D mutation in strong mutants could lead to defects in α-β heterodimerization**

Residue 256 is highly conserved in the intermediate domain at H8 helix, SNP in which has been correlated with diseases such as complex cortical dysplasia, polymicrogyria as N256S substitution (Guerrini et al., 2012). The brain of a patient with N256S substitution showed pachygyria with a thick cortex and thin corpus callosum. The patient had hypotonia, microcephaly and delayed psychomotor development. N256D mutation in β-chain is present the alpha-β interaction domain thus, could cause defects in dimerization in turn leading to defects in microtubule formation and stability. This region lies between amino acid residues 250 to 300 which has also been shown to be important for protofilament interactions to form microtubule (Rudolph et al., 1987; Savage et al., 1989; Driscoll et al., 1989). This substitution leads to a change of Asparagine to Aspartic acid, thus changing the charge of the residue which could severely affect the interaction between the α and β chain, which could be the possible reason of severe phenotype in both strong mutants with this substitution change.

**S364S mutation could cause unstable microtubule**

LRRK2 directly interacts with specific β tubulin TUBB, TUBB4, and TUBB6. The specific binding affinity is shown by lysine 362 and alanine 364 of β tubulin (Law et al., 2014). This residue has been predicted to be poorly accessible in stabilized microtubules but exposed in dynamic microtubules. Consistent with this prediction, endogenous LRRK2 shows preferential localization to dynamic microtubules in growth cones compared to adjacent microtubule bundles in the neuronal process. The residue in β tubulin has been mapped in a luminal region closer to lysine 40 of α tubulin in α-β dimer by Molecular modeling. The interaction of LRRK2 with β tubulin could be relevant to microtubule dynamics, as embryonic fibroblasts from LRRK2 knockout mice show enhanced microtubule acetylation (Law et al., 2014).
Thus, S364S in *C. elegans* β-tubulin, which is a silent mutation in C-terminal, do not change amino acid Serine, might also be important for similar interaction and microtubule dynamics. This residue being in the C-terminal domain might be important for interactions with MAPs or motor proteins and residues in C-terminal are also important for microtubule assembly (Huffaker et al., 1988; Savage et al., 1994; Shojania et al., 2015).

Though acetylation of a microtubule is known to occur at alpha tubulin, MS study shows that beta tubulin also has residues which undergo modification acetylation (Choudhary et al., 2009). The gaps in acetylation staining suggest either the microtubules are not continuous, or the pool of dynamic microtubule has increased in the mutants. Therefore, as there are defects in beta-tubulin, it might have resulted in a reduction of microtubule stability which in turn affected kinesin-1 binding, thus transport in the neuron.

### 7.6) The Ratio of anterograde motors might have altered in mutants

The microtubule in mutants show defects in the stability in TRNs of strong mutants. *Kinesin-1* (KIF5) shows a preference for acetylated microtubules in cultured non-neuronal cells, whereas other anterograde *Kinesin-3* (KIF1A) family motors do not show such microtubule selectivity (Reed et al., 2006; Cai et al., 2009). The possibility of Kinesin-1 hypomorphs not showing a further reduction in mitochondrial number in the mutants could be due to the presence of alternate motors which are involved in mitochondrial transport, possibly *kinesin-3* which can walk on both dynamic and stable microtubules, acetylated or deacetylated microtubules (Cai et al., 2009). The absence of cargo could also severe the neuronal morphology defects in the mutants as observed in strong mutants as weak allele *tb133* showed cargo distributed throughout the neuronal process and long neuronal process with minimal branching defects. This idea was supported by over-expression of *unc-104:: GFP* in the strong mutant *tb118; tbIs2222*, where the neuron length was rescued, and extent of branching decreased. It did not rescue the phenotype completely indicating that in microtubule mutants the defects are not only through motor pathway but through additional pathways which affect trafficking and neuronal process morphology.
7.7) Mitochondrial dynamics contributes to the formation of stationary mitochondrial positions

Mitochondria in TRNs of *C.elegans* are uniformly distributed maintaining constant density across development. Mitochondrial transport is known to be coordinated with axon growth (Morris and Hollenbeck, 1993). We used the microfluidic device for a long-time interval to understand mitochondrial distribution pattern in the same neuron in an animal during the development. With the development mitochondrial positions move apart yet maintaining a constant density (chapter 6). The mitochondrial dynamics give rise to the new stationary mitochondrial positions by fission and fusion. In addition, this dynamic pool of mitochondria contributes to the turnover of stationary mitochondrial positions by such mitochondrial interactions and with the possible involvement of additional fission-fusion molecules. Our study showed preliminary results in the field of understanding mitochondrial distribution in a growing neuron that the distribution and placing of docked mitochondrial positions are regulated. This method of long-term imaging of single animal in the microfluidic device would be very useful for studying developmental processes, aging and cell biological processes in the single animal for a long time interval.

7.8) Hypothesis for *mec-7* mechanism for causing defects in mutants

*mec-7* is crucial for the formation of 15 protofilament microtubule structure in TRNs and presence of the large diameter microtubule is crucial for mechanosensation (Savage et al., 1989; Bounoutas et al., 2009). Structure of tubulins are highly conserved among eukaryotes with differences mostly at C-terminal (Hsu et al., 2014; Popodi et al., 2008). *C. elegans* genome has nine α- and six β-tubulin genes. In PLM transcripts detected are *mec-7, mec-12, tbb-6, tba-1, tbb-1, tba-2, tba-4*, and *tbb-2* in decreasing order (Consortium, 1998; Gogonea et al., 1999). 15 protofilament in TRNs are specifically formed by *mec-7* and *mec-12*. The mechanism of involvement of the specific wide and bundled microtubule for mechanosensation is not well understood. In addition to forming microtubules, *mec-7* β tubulin is involved in signaling pathways. Therefore, considering the role of *mec-7* and
tubulin structures homology, the trafficking and neuron morphology defects in mapped mutants could be due to following possibilities (fig.2):

1) Due to microtubules dynamics and unstable structure
2) Through transcription effect of mec-7, might be through DLK or MAPK pathway (Bounoutas et al., 2011)
3) Combination of microtubule dynamics and signaling pathways

Microtubule stability and dynamics are important for binding of motors and growth of the neurons. The unstable microtubule could be regions of improper binding of motors along the tracks which in turn could affect the number of cargos along the neuronal process. The other possibility is that since less cargo enter the process in mutant animals, the microtubules are unable to receive stabilizing factors and thus deform with development.

mec-7 also involved in regulating gene expression levels of multiple proteins through MAPK pathways, thus would affect the functioning of the neuron. Multiple microtubule binding proteins also involved in signaling processes that could lead to the defects in trafficking in the neuron. Multiple mutations in β tubulin is known to cause severe neuropathies (Niwa et al., 2013). This work provides novel residues in β-tubulin molecules which could be important for such interactions and regulation of microtubule structure and cell function.
Model showing possible mechanisms of MEC-7 giving mutant phenotype

- Microtubule structure
- Transcription effect
- Both

Genetic interaction with MEC-12

DLK pathway
Motor levels down?

Model for development of TRNs

TRN fate determination

alt-1

unc-86 \(\rightarrow\) mec-3 \(\rightarrow\) mec-4 \& mec-7

Model for regulation of gene expression in TRNs

TRN Specific phenotype

Duggan et al, 1998

Bounoutas et al, 2011

- Asparagine to Aspartic acid
- Aβ interaction domain (H8 helix, β Intermediate domain)

Could affect:
- MT formation
- Protocilament interactions
- Stabilization of microtubules
- Correlated with diseases

\(tb\textit{118}\) residue 256=N/D

\(tb\textit{132}\) residue 364=S/S, 256N/D

- Serine silent mutation
- C-terminal

Could affect:
- Interaction to MAPs, Motors and stabilizing factors
- LRRK interaction

- Glutamic acid (−) to Lysine (+)
- GTP Binding region (N-terminal)

Could affect:
- Conformation
- Dimerization
- Protofilament formation
- Interactions with signaling molecules (MAPK/DLK pathways)

Alpha- Beta tubulin dimer: PDB id 1TUB

\(tb\textit{133}\) allele residue 110=E/K

b)
c) **Figure 2– Proposed model for the mechanism through which MEC-7 could be giving mutant phenotype**

a) processes and mechanisms that could be affected to give phenotype, b) processes of involvement of mutated residues, c) Schematic showing possible microtubule structure and affected motor binding and stabilizing proteins binding in wild type and mutant (scale is hypothetical for presentation)