

UNIVERSITY OF CALIFORNIA SAN DIEGO

DLK-1 signaling utilizes isotype-specific interaction of tubulins to protect neurons

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

Chihin Feng

Committee in charge:

Professor Yishi Jin, Chair
Professor Andrew Chisholm
Professor Andreas Ernst
Professor Andres Leschziner

2024

Copyright

Chihin Feng, 2024

All rights reserved.

The thesis of Chihin Feng is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2024

TABLE OF CONTENTS

Thesis Approval Page	iii
Table of Contents	iv
List of Figures	vi
List of Tables	vii
Acknowledgements	viii
Abstract of the Thesis	ix
Introduction	1
Chapter 1: TBA-2 heterodimerizes with BEN-1(L246F) to form stable MT patches mainly through amino acids 216, 218, and 287	10
Background	10
Materials and Methods	12
Results	17
Discussion	19
Figures	21
Tables	26
Chapter 2: Multiple bZIP transcription factors facilitate the formation of the MT patches	33
Background	33
Materials and Methods	35
Results	38
Discussion	40
Figures	42
Tables	47
Chapter 3: DLK-1 is required at the late larval stage to promote the MT patch formation	55
Background	55
Materials and Methods	56

Results	60
Discussion	61
Figures	64
Tables	67
References	71

LIST OF FIGURES

Figure 0.1. BEN-1(L246F) forms stable patch regions within microtubules	7
Figure 0.2. TBA-2 is required for the MT patch formation	8
Figure 0.3. BEN-1(L246F) and TBA-2 form the stable MT patches through a DLK-1-dependent upregulation of TBA-2 expression	9
Figure 1.1. TBA-2 and TBA-1 differ at amino acids 216, 218, and 287 located at the tubulin interfaces	21
Figure 1.2. Utilizing CRISPR-Ca9 genome editing to introduce the amino acid changes D216S, E218D, and S287N in TBA-2	22
Figure 1.3. TBA-2 forms the stable MT patches with BEN-1(L246F) mainly through amino acids 216, 218, and 287 of TBA-2	23
Figure 1.4. <i>tba-2(D216S; E218D; S287N)</i> in combination with <i>tba-1(0)</i> does not cause significant defects in embryonic development, locomotion, and growth	24
Figure 1.5. Representative gel images for the PCR genotyping of each allele	25
Figure 2.1. Protein sequence alignment of CEBP-1, ZIP-4, and ZIP-2	42
Figure 2.2 Gene illustrations for <i>cebp-1</i> , <i>zip-4</i> , and <i>zip-2</i> show the knockout alleles and genotyping primers	43
Figure 2.3. Multiple bZIP transcription factors of the C/EBP family redundantly facilitate the formation of the BEN-1(L246F) patches	44
Figure 2.4. CEBP-1 overexpression (OE) significantly increases the formation of the BEN-1(L246F) patches across neurons, but ZIP-4 OE and CEBP-2 OE do not	45
Figure 2.5. Representative gel images for the PCR genotyping of each allele	46
Figure 3.1. 1 hour of 35°C heat shock is sufficient to induce a high level of <i>Phsp-16.2-GFP::DLK-1</i> expression in 1 hour	64
Figure 3.2. Acute expression of DLK-1 at the L4 stage is sufficient to rescue BEN-1(L246F) patch formation	65
Figure 3.3. Representative gel images for the PCR genotyping of each allele	66

LIST OF TABLES

Table 1.1. <i>C. elegans</i> strain information	26
Table 1.2. Allele information and genotyping primers	30
Table 1.3. CRISPR reagents	32
Table 2.1. <i>C. elegans</i> strain information	47
Table 2.2. Allele information and genotyping primers	54
Table 3.1. <i>C. elegans</i> strain information	67
Table 3.2. Allele information and genotyping primers	70

ACKNOWLEDGMENTS

I want to share my sincere appreciation to all members of the Jin and Chisholm labs for their immense support and inspiration. During my three years of training in the Jin lab working alongside everyone, there have been so many fun and precious memories that I will remember for the rest of my life.

I want to give special thanks to my research mentor Dr. Junxiang Zhou and my thesis advisor Professor Yishi Jin for their constant and meticulous guidance and mentorship during my scientific journey. They taught me a tremendous amount of knowledge and skills and inspired me to always think critically and creatively about my sciences. The impacts of their mentorship go beyond sciences and will continue to inspire me beyond my master's training.

Most materials in Figure 0 were provided by Dr. Junxiang Zhou with permission to serve as the introductory and background information for this thesis.

ABSTRACT OF THE THESIS

DLK-1 signaling utilizes isotype-specific interaction of tubulins to protect neurons

by

Chihin Feng

Master of Science in Biology

University of California, San Diego 2024

Professor Yishi Jin, Chair

Microtubules are dynamic cytoskeletons composed of tubulins and play important roles in neuronal development, maintenance, signaling, and functions. Dysfunction of tubulins leads to the perturbation of microtubules which has been linked with many neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. To maintain homeostasis, neurons are able to detect and respond to internal stresses through different signaling pathways. Here, I utilized a genetic model of *C. elegans* to investigate the neuronal responses to microtubule perturbation. The Dual Leucine zipper Kinase (DLK) is a neuronal stress-sensing MAPKKK. A gain-of-function mutation in the neuronal β -tubulin BEN-1 (BEN-

1(L246F)) activates the *C. elegans* homolog of DLK, DLK-1, and its downstream P38 MAPK pathway. The activated DLK-1 MAPK pathway promotes the expression of the α -tubulin TBA-2, which preferentially heterodimerizes with BEN-1(L246F) to form stable regions within microtubules. This DLK-1-dependent regulation of microtubule properties protects neuron morphology, synapse formation, and synaptic vesicle trafficking from microtubule perturbation. During my master's research, I investigated the mechanistic details of this stress-response pathway. I identified key residues in TBA-2 that facilitate its specific interaction with BEN-1(L246F). I also uncovered multiple bZIP transcription factors of the C/EBP family that likely act downstream of DLK-1 to regulate microtubule properties. Lastly, I revealed the age-dependent involvement of DLK-1 in regulating microtubule properties. Together, my findings further elucidated the DLK-1-dependent regulation of tubulin isotypes, which protects neurons from microtubule perturbation.

Introduction

α - and β -tubulins are the components of microtubules (MTs)

Alpha (α)- and beta (β)-tubulins are small GTPases that are conserved across eukaryotes. Most eukaryotes express multiple α - and β -tubulin isotypes, which heterodimerize into tubulin dimers. The dimers subsequently polymerize into protofilaments and get assembled into microtubules (MTs) (Desai & Mitchison, 1997). Microtubules are dynamic cytoskeletons with diverse functions, such as supporting cell structure, cargo trafficking, cell division, and cell movement (Mitchison & Kirschner, 1984; Janke & Magiera, 2020). While both α - and β -tubulins are bound by GTP, α -tubulins contain a GTP that cannot be hydrolyzed because it is buried within the heterodimer (Desai & Mitchison, 1997; Nogales, 2000). β -tubulins, however, contain an exchangeable GTP that can be hydrolyzed into GDP after the tubulin heterodimers are incorporated into microtubules (Desai & Mitchison, 1997; Nogales, 2000). This change in the guanine nucleotide state of β -tubulins plays an important role in the dynamic nature of microtubules because GTP hydrolysis within β -tubulins causes a structural change to the protofilaments and destabilizes microtubules (Erickson & O'Brien, 1992; Wang & Nogales, 2005).

Microtubules support neuron development, maintenance, signaling, and functions

This dynamic nature of microtubules allows them to serve critical functions in neurons. After the initial stage of neurogenesis, microtubules in a particular neurite become hyperstable to determine the putative axon and neuronal polarity (Witte et al., 2008). Subsequently, dynamic microtubules enable the progression of the growth cone at the tip of a growing neurite and steer growth in the proper direction (Lowery and Van Vactor, 2009). Once neurons mature,

microtubules continue to serve as the structure backbones that maintain the integrity and shapes of neurons. Various cargos, such as organelles, synaptic vesicles, and signaling proteins, are transported along microtubules by motor proteins. Consistent with their critical roles in neurons, perturbation of microtubules can lead to a wide range of neuropathies (Dubey et al., 2015). However, little is known about how neurons respond to microtubule perturbation in order to maintain homeostasis.

Tubulins in *Caenorhabditis elegans*

To study neuronal responses to microtubule perturbation *in vivo*, I used the model organism *Caenorhabditis elegans*, which has a transparent body ideal for fluorescent imaging of cellular dynamics (Chalfie et al., 1994). Additionally, these tiny worms possess a fully mapped genome that can be easily manipulated to test the function of specific genes or even residues in neuronal stress responses, making them an excellent model for my study.

C. elegans expresses nine α -tubulins and six β -tubulins most of which share over 80% amino acid similarity to the most broadly-expressed human α - and β -tubulins (TUBA1A and TUBB, respectively) (Hurd, 2018; O'Hagan et al., 2022). These different α - and β -tubulin isoforms likely arise from gene duplications, and they have unique and, for some, overlapping expression patterns and functional profiles across various tissues and neurons (Nishida et al., 2021; Moore et al., 2023). The different tubulin isoforms mosaically form microtubules, and the specific tubulin composition further enables the diverse functions and structures of microtubules. For example, in the touch receptor neurons of *C. elegans*, the α -tubulin MEC-12 and β -tubulin MEC-7 enable the formation of 15-protofilament microtubules needed for touch sensation, while the rest of *C. elegans*' neurons contain mainly 11-protofilament microtubules (Chalfie &

Thomson, 1982; Chalfie & Au, 1989; Fukushige et al., 1999). Additionally, the α -tubulin TBA-6 is expressed in a set of ciliary neurons where it plays an important role in the formation of the doublet MTs and the regulation of sensory functions (Hurd et al., 2010; Silva et al., 2017). That being said, many tubulin isotypes also have overlapping or even redundant functions. The redundancy between tubulins, along with the heterogeneity of tubulin isotypes expressed, made *in vivo* investigations of specific tubulin isotypes challenging.

The Dual-Leucine zipper Kinase (DLK) regulates neuronal development and functions

The Dual-Leucine zipper Kinase (DLK) is a conserved mitogen-activated protein kinase kinase kinase (MAPKKK) that acts upstream of a P38 MAPK pathway in *C. elegans*. The *C. elegans* homolog of DLK, DLK-1, plays important roles in synaptogenesis and axon regeneration. DLK-1 is a substrate of the E3 ubiquitin ligase RPM-1 that negatively regulates and maintains DLK-1 at its proper levels. Loss of RPM-1 leads to overexpression of DLK-1 and activation of its downstream MAPK pathway, which disrupts synaptic development and architectures (Zhen et al., 2000; Nakata et al., 2005). In neurons injured by laser axotomy, DLK-1 activates its downstream P38 MAPK cascade involving MKK-4/PMK-3/MAK-2 to stabilize the mRNA of the bZIP transcription factor CEBP-1 which modulates the expression of various genes needed for the regrowth (Yan et al., 2009). DLK-1 has also been shown to promote neuronal microtubule dynamics needed for neurite outgrowth or neuron remodeling by downregulating the kinesin-13-like protein KLP-7 and spastin SPAS-1 or by promoting $\Delta 2$ post-translational modification and tyrosination of α -tubulins (Ghosh-Roy et al., 2012; Kurup & Jin, 2015). In the touch receptor neurons of *C. elegans*, the loss of the α -tubulin MEC-12 and β -tubulin MEC-7 disrupts microtubules and results in a general reduction of protein levels. The

reduction is dependent on DLK-1 and its downstream P38 MAPK cascade, suggesting that DLK-1 senses and responds to microtubule perturbation (Bounoutas et al., 2011).

A gain-of-function mutation in the neuronal β -tubulin BEN-1 activates the DLK-1 MAPK pathway and increases benzimidazole-sensitivity

From a forward genetic screen done by Dr. Yue Sun to identify novel regulators of DLK-1, over 30 mutants were isolated that caused the pan-neuronal upregulation of DLK-1. Through whole genome sequencing and mutation mapping, Dr. Junxiang Zhou identified one of the mutations to be in the neuronal β -tubulin gene *ben-1*. The mutation changes a cytosine to thymine at the 1860 nucleotide position (the first nucleotide (A) in the *ben-1* ORF is 1; the numbering includes the intron sequences), resulting in the amino acid change from a leucine (L) to phenylalanine (F) at the 246 location (from now referred to as *ben-1(L246F)* (gene) or BEN-1(L246F) (protein)). Structurally, the BEN-1(L246F) mutation is located at the T7 loop of the protein which is involved in GTP binding and the longitudinal intradimer-interaction between BEN-1 and its α -tubulin binding partners (Nogales et al., 1999). *ben-1*, named for benzimidazole-sensitivity, is the only identified gene in *C. elegans* that confers sensitivity to benzimidazole, which is a common anti-microtubule chemical used to target parasitic nematodes (Driscoll et al., 1989; Davidse, 1986; Gupta et al., 2004). While the loss of function of *ben-1* confers resistance to benzimidazole, the *ben-1(L246F)* mutation makes the animal hypersensitive to benzimidazole, indicating that *ben-1(L246F)* is a gain-of-function mutation.

BEN-1(L246F) forms stable “patch” regions within the microtubules of adult animals

To investigate the effect of the *ben-1(L246F)* mutation on the expression levels and patterns of BEN-1, Dr. Junxiang Zhou tagged both BEN-1(WT) and BEN-1(L246F) through a split-GFP system, generating stains that express reconstitutedGFP::BEN-1(WT) and reconstitutedGFP::BEN-1(L246F). While the overall expression levels of BEN-1 were not significantly affected by the L246F mutation, the *ben-1(L246F)* mutants showed strong enrichments of reconstitutedGFP::BEN-1(L246F) in fiber-like patches along the neurites across different regions of neurons (now referred to as MT patches or BEN-1(L246F) patches) (Figure 0.1 A-B; Figures 0.1-0.3 were adapted from Dr. Junxiang Zhou with permission). The MT patches were approximately 0.5 μm in width and vary greatly in length from 1 μm to over 20 μm . The MT patches were observed in the ventral and dorsal nerve cord (bundle of neurites) as well as in the single-neurite commissures of the reconstitutedGFP::BEN-1(L246F) mutants (Figure 0.1 A-C). The location of the MT patches in these regions appeared to be mostly random. In addition, the MT patches only started to form after the late larval stage (L4) of the animals and continued to increase in number as the animals aged (Figure 0.1 C). Through additional experiments done by Dr. Junxiang Zhou, the MT patches have the following properties: (1) the MT patches are bound by the End Binding Protein, EBP-2, that specifically binds to GTP-bound, stable MTs; (2) once the MT patches form, they remain stable for at least 1.5 hours. Together, these data suggest that the MT patches are stable regions of MTs that are enriched with GTP-bound BEN-1(L246F) proteins.

In my master’s thesis research, I investigated the mechanistic details that underline the MT patch formation in the *ben-1(L246F)* mutants. From my research study, I identified key residues in the α -tubulin TBA-2 that enable its specific interaction with BEN-1(L246F) in

forming the MT patches. I also identified multiple bZIP transcription factors that redundantly act downstream of DLK-1 to facilitate the MT patch formation. Lastly, I uncovered the age-dependent regulation of the MT patch formation by DLK-1.

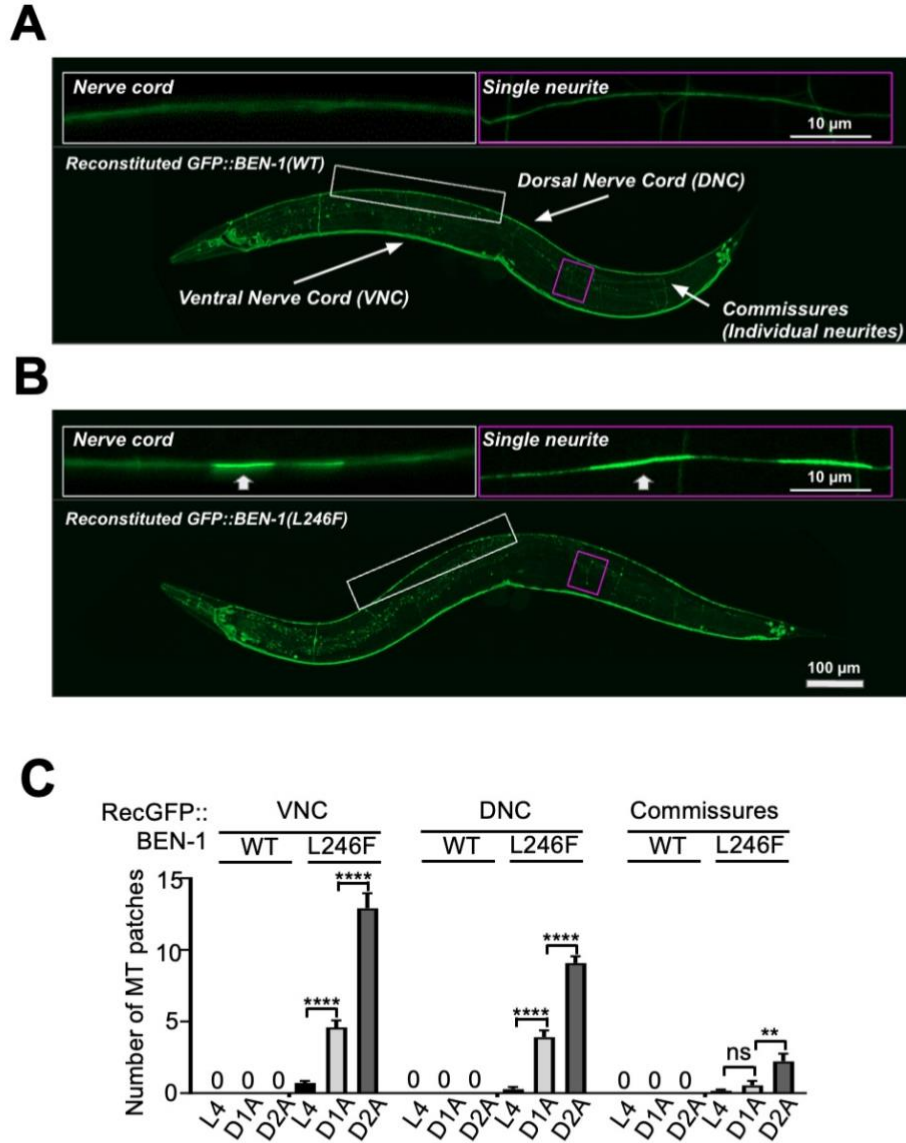


Figure 0.1. BEN-1(L246F) forms stable patch regions within microtubules (figures/data adapted from Dr. Junxiang Zhou with permission). (A) Confocal images of reconstituted GFP-tagged BEN-1(WT) (recGFP::BEN-1(WT)). (B) Confocal images of reconstituted GFP-tagged BEN-1(L246F) (recGFP::BEN-1(L246F)). MT/BEN-1(L246F) patches are indicated with white arrows in the zoomed-in images. (C) MT patch quantification at the late larval stage (L4), day 1 adult stage (D1A), and day 2 adult stage (D2A) in three different regions of neurons. VNC = ventral nerve cord; DNC = dorsal nerve cord; Commissures = single neurite processes. Quantification was conducted in the recGFP::BEN-1(WT) and recGFP::BEN-1(L246F) backgrounds.

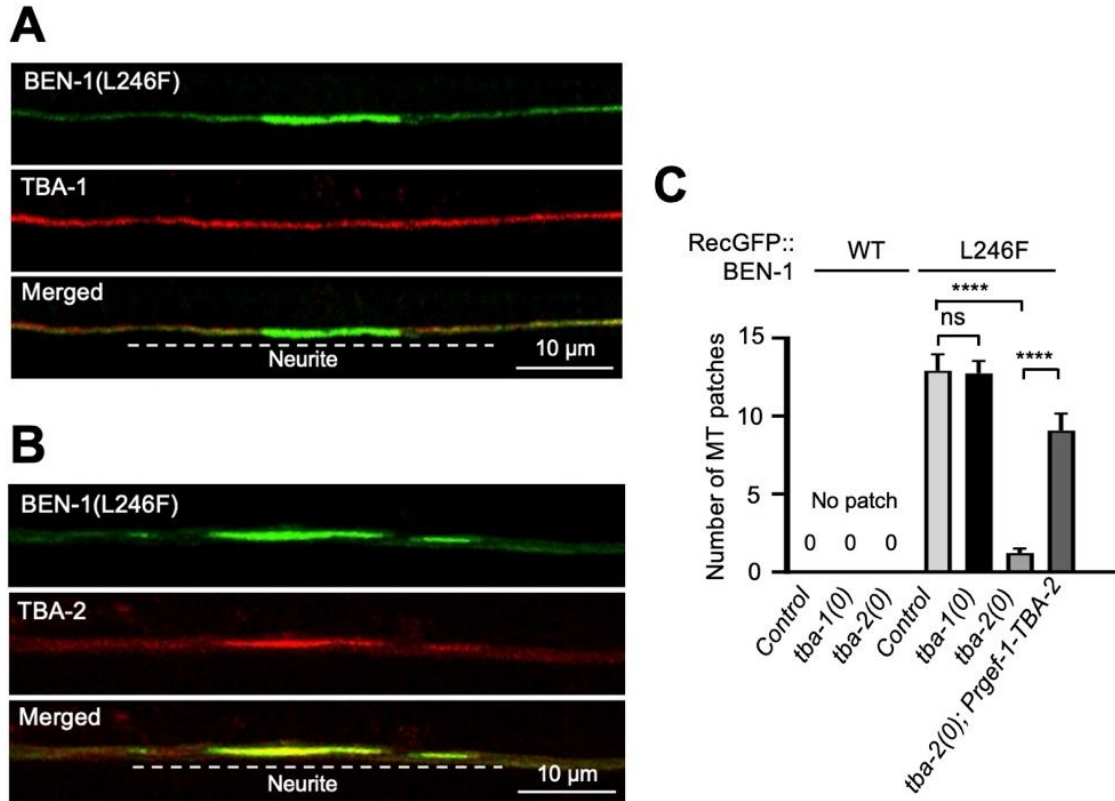


Figure 0.2. TBA-2 is required for the MT patch formation (figures/data adapted from Dr. Junxiang Zhou with permission). (A) Confocal images of recGFP::BEN-1(L246F) and mKate2::TBA-1 co-labeling. (B) Confocal images of recGFP::BEN-1(L246F) and mKate2::TBA-2 co-labeling, showing co-enrichment of BEN-1(L246F) and TBA-2 at the MT patches. (C) MT patch quantification of day 2 adult animals for *tba-1* or *tba-2* knockout (0) in the recGFP::BEN-1(WT) and recGFP::BEN-1(L246F) backgrounds. *Prgef-1-TBA-2* is a transgenic gene construct of *tba-2* driven by a pan-neuronal promoter and is used to rescue *tba-2(0)*. The MT patch data shown here were collected from the ventral nerve cord.

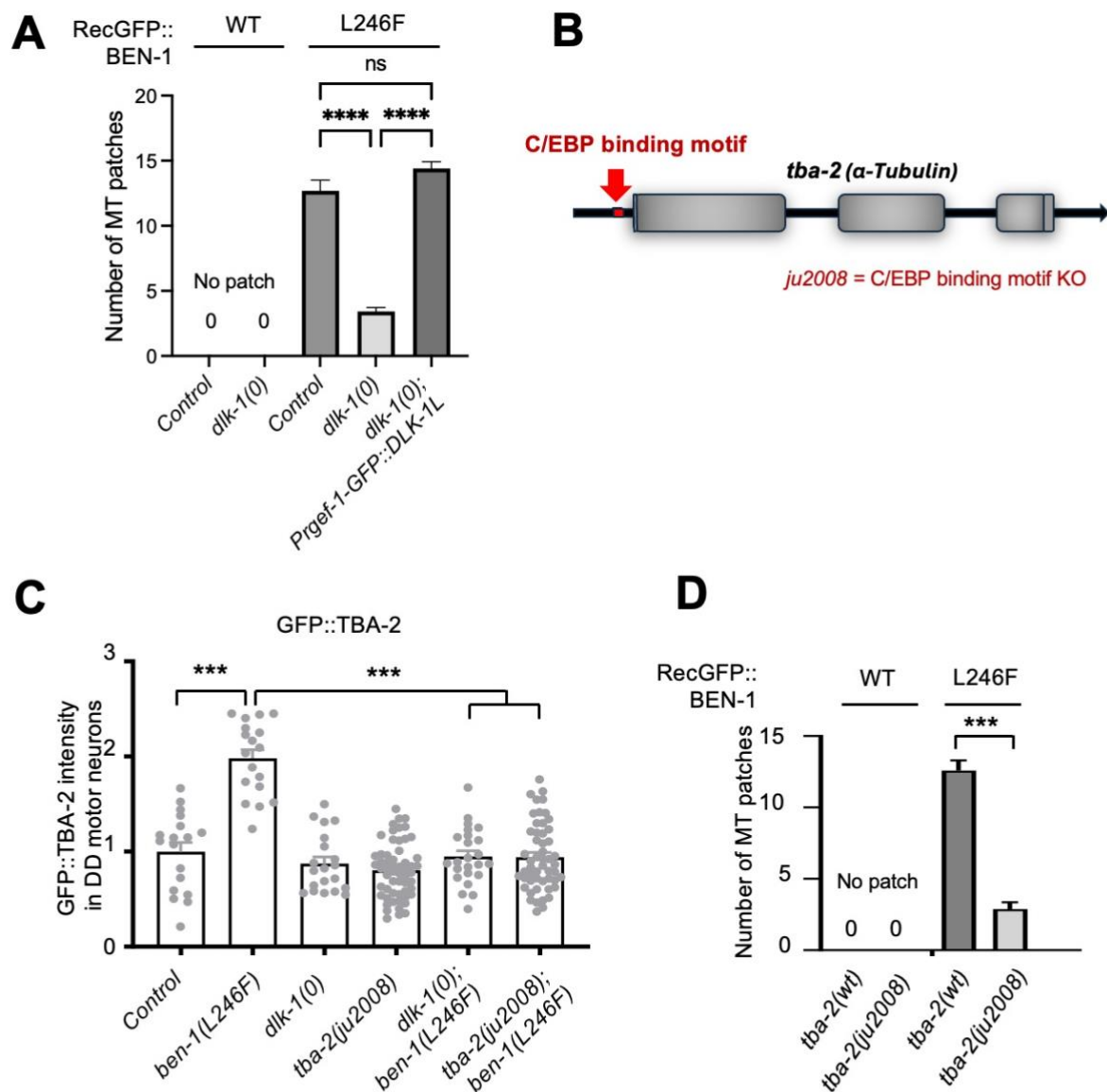


Figure 0.3. BEN-1(L246F) and TBA-2 form the stable MT patches through a DLK-1-dependent upregulation of TBA-2 expression (figures/data adapted from Dr. Junxiang Zhou with permission). (A) MT patch quantification of day 2 adult animals with *dlk-1* knockout in the recGFP::BEN-1(WT) and recGFP::BEN-1(L246F) backgrounds, showing that DLK-1 facilitates the MT patch formation. *Prgef-1-GFP::DLK-1L* is a single-copy insertion of a pan-neuronal-promotor-driven *dlk-1* used to rescue *dlk-1(0)*. (B) Simplified illustration of the *tba-2* gene, showing the C/EBP binding motif. (C) GFP::TBA-2 intensity quantification in the DD motor neurons, showing that the upregulation of TBA-2 in the *ben-1(L246F)* mutants relies on *dlk-1* and the C/EBP binding motif of *tba-2*. (D) MT patch quantification of day 2 adult animals in the recGFP::BEN-1(WT) and recGFP::BEN-1(L246F) backgrounds with or without the removal of the C/EBP binding motif of *tba-2*, showing that the MT patch formation in the *ben-1(L246F)* mutants relies on the C/EBP binding motif and the upregulation of TBA-2. The MT patch data shown here were collected from the ventral nerve cord.

Chapter 1: TBA-2 heterodimerizes with BEN-1(L246F) to form stable MT patches mainly through amino acids 216, 218, and 287

Background

According to the preliminary data from Dr. Junxiang Zhou, TBA-2, a broadly expressed α -tubulin in neurons, also enriches in the BEN-1(L246F) patches, suggesting that the BEN-1(L246F) patches are comprised of BEN-1(L246F)/TBA-2 heterodimers (Figure 0.2 A-B). Genetic null of *tba-2* abolishes the MT patch formation in the reconstituted GFP::BEN-1(L246F) mutants, further confirming the involvement of *tba-2* in the MT patches (Figure 0.2 C). Additionally, the DLK-1 MAPK pathway is not only activated by the *ben-1(L246F)* mutation, but DLK-1 and its downstream MAPK PMK-3 are also required for the MT patch formation (Figure 0.3 A). Mechanistically, in the *ben-1(L246F)* mutants, the DLK-1 pathway upregulates the expression of TBA-2 through a C/EBP (CCAAT/enhancer-binding protein) binding motif in the *tba-2* promoter (the motif was initially identified by Kim et al. (2016)) (Figure 0.3 B). Removing DLK-1 or the C/EBP binding motif of *tba-2* abolishes the upregulation of TBA-2 in the *ben-1(L246F)* mutants and abolishes the formation of the MT patches (Figure 0.3 C-D). These data suggest that a high level of TBA-2 and its specific interaction with BEN-1(L246F) are required for the MT/BEN-1(L246F) patch formation.

Although the α -tubulin TBA-1 is also broadly expressed in neurons and is 97.32% identical in primary sequence to TBA-2, TBA-1 does not co-enrich in the BEN-1(L246F) patches and is not required for the patch formation (Figure 0.2 A, C). Based on the previous results, I hypothesized that the unique amino acids of TBA-2 contribute to its specific interaction with BEN-1(L246F) in forming the MT patches.

In my thesis research, I utilized CRISPR-Cas9 genome editing to change specific residues of TBA-2 to TBA-1 in the reconstituted GFP::BEN-1(L246F) mutants. I found that amino acids 216, 218, and 287 at the tubulin interfaces of TBA-2 facilitate the specific interaction between TBA-2 and BEN-1(L246F) in forming the MT patches.

Materials and Methods

C. elegans strains and genetics

All strains were maintained on nematode growth media (NGM) plates seeded with the *E. coli* strain OP50 (Brenner, 1974). Strains were maintained at 20°C unless otherwise specified. The Bristol (N2) strain was used as the wild type. Strains used in this chapter are described in Table 1.1. The genotyping methods for the CRISPR-edited strains are described in Table 1.2. Alleles were either genotyped using standard PCR or using a fluorescence compound microscope (see Tables 1.1 and 1.2 for details). Figure 1.5 shows representative gel images of the PCR genotyping results.

DNA sequence and protein analysis

DNA sequence analysis was performed using ApE and SnapGene. Protein structure predictions were generated using ColabFold and analyzed using UCSF Chimera (Jumper et al., 2021; Mirdita et al., 2022). Protein alignment was performed using T-Coffee (Tcoffee.org.eu) and formatted using Boxshade (Notredame et al., 2000).

CRISPR-Cas9 genome editing

To generate *tba-2(D216S; E218D)*, the following crRNA was designed and utilized 5'-GCUCAACAUCCAAGUUUCUG-3' (PAM: CGG). A 139 bp HDR template was designed to introduce the three nucleotide changes (G646A, A647G, G654T) that result in the amino acid changes D216S and E218D (see Table 1.3 for sequence information). One additional nucleotide change (C642T; results in a silent mutation) three base pairs downstream of the cut site was also included to, first, further prevent recutting of the Cas9-gRNA complex, and, second, to generate

an ApoI restriction enzyme digestion site for easier genotyping (Figure 1.2). The *tba-2(D216S; E218D)* edit was done in N2 animals and then introduced into the reconstitutedGFP::BEN-1(L246F) mutants through genetic crosses (Table 1.1).

To generate *tba-2(S287N)* and *tba-2(D216S; E218D; S287N)*, the following crRNA was designed and utilized 5'-GCUAUUGGUGAUGUCGCUGA-3' (PAM: CGG). A 132 bp HDR template was designed to introduce the two nucleotide changes (G910A, C911T) that result in the amino acid change S287N (see Table 1.3 for sequence information). Two additional nucleotide changes C905A and C908T were incorporated; C905A was to mutate the PAM site and C908T was to generate the MseI restriction enzyme digestion site for easier genotyping (C905A and C908T both result in silent mutations) (Figure 1.2). The S287N edit was done in the reconstitutedGFP::BEN-1(L246F) background with either *tba-2(wt)* (CZ29879) or *tba-2(D216S; E218D)* (CZ30409) to generate *tba-2(S287N)* and *tba-2(D216S; E218D; S287N)*, respectively.

Both co-injection-marker and co-CRISPR methods were used in my study which differ in the content of the injection mix. First, for the co-injection method, it was used for generating *tba-2(D216S; E218D)* and *tba-2(D216S; E218D; S287N)*. To prepare the mix for injection, 1 µl of tracrRNA (40 µM) was combined with 1 µl of the crRNA of interest. The 2 µl mix then underwent a “melting” process (95°C for 30 seconds; 90°C for 30 seconds... 30 seconds for every 5°C decreased until the 30 seconds for 30°C completed). After the melting process, 0.8 µl of Cas-9 (40 µM) and 2.2 µl of ddH₂O were added to the melted mix. After at least 10 minutes of incubation at room temperature (~22.5°C), the 5 µl of RNP mix was made. The final mix for injection included 1 µl of the RNP complex, 1 µl of HDR template of interest (10 µM), co-injection markers (1.3 µl of 18 ng/µl *Pmyo-2::mCherry* for *ju2031* or 1 µl of 630 ng/µl *rol-6(su1006; in pRF4 plasmid)* for *ju2033*), and ddH₂O topped to 10 µl. The 10 µl injection mix

was centrifuged at 14000 rpm for 5 minutes and injected into the P0 worms at the day 1 adult stage.

The *dpy-10* co-CRISPR method was adapted to generate *tba-2(S287N)* (Arribere et al., 2014). To prepare the mix for injection, 1 μ l of tracrRNA (40 μ M) was combined with 0.5 μ l of the crRNA of interest (40 μ M) and 0.5 μ l of the *dpy-10* crRNA (40 μ M). The 2 μ l mix then underwent a “melting” process (95°C for 30 seconds; 90°C for 30 seconds... 30 seconds for every 5°C decreased until the 30 seconds for 30°C completed). After the melting process, 0.8 μ l of Cas-9 (40 μ M) and 2.2 μ l of ddH₂O were added to the melted mix. After at least 10 minutes of incubation at room temperature (~22.5°C), the 5 μ l of RNP mix was made. The final mix for injection included 1.5 μ l of the RNP complex, 1 μ l of HDR template of interest (10 μ M), 1 μ l of *dpy-10* HDR template (10 μ M), and ddH₂O topped to 10 μ l. The 10 μ l injection mix was centrifuged at 14000 rpm for 5 minutes and injected into the P0 worms at the day 1 adult stage.

For both methods, at least 16 P0 animals were injected. The injected P0 worms were kept at 25°C. After 3-4 days, the F1 progenies were visually screened for the expression of the co-injection markers or, for the co-CRISPR method, dumpy and roller phenotypes. The F1 progenies with the co-injection marker expression or co-CRISPR *dpy-10* phenotypes were isolated to self-reproduce and genotyped for the desired edits through PCR and the corresponding restriction enzyme digestion (Table 1.2 and Figure 1.2). The edits were verified through Sanger sequencing. The edited animals were then outcrossed to remove potential background mutations (Table 1.1).

MT patch quantification

A MT patch is a fiber-like structure enriched with reconstitutedGFP::BEN-1. A MT patch has an obviously higher reconstitutedGFP::BEN-1 intensity than its surroundings and is at least 1 μm in length. The number of patches on the ventral nerve cord (before the tail soma), dorsal nerve cord, and, for some, single-neurite commissures connecting the ventral and dorsal nerve cords were counted manually using a Zeiss Axio Imager M2 fluorescence compound microscope with a 63x objective oil immersion lens. Patch qualification experiments were performed in day 2 adult animals unless otherwise specified. To prepare day 2 adult animals for the analysis, animals at the L4 stage (easily distinguishable based on the white half-moon vulva) were picked and transferred to a new NGM plate. The L4 animals were left to grow at 20°C for 48 hours to reach the day 2 adult stage (a different temperature was used for the heat shock-induced DLK-1 experiments; see Chapter 3 for more details). The worms were then immobilized using 10 mM levamisole (in M9 buffer) on a 4% agar gel pad for the quantification.

Egg-laying and egg-viability analysis

To assess the egg-laying ability of the animals with a specific genotype, 3 L4 hermaphrodite animals were transferred to a new NGM plate. The animals were then left to grow and reproduce for 24 hours at 20°C. After 24 hours, the 3 parent animals were removed from the plate, and the number of eggs was counted manually under a Leica dissecting microscope. The number of eggs laid within the 24-hour period was recorded to represent the egg-laying ability.

After recording the number of eggs, the same plate was kept for another 24 hours at 20°C allowing the eggs to hatch. After 24 hours, the number of unhatched eggs was counted manually under a Leica dissecting microscope and documented. The percent unhatched was calculated by

dividing the number of unhatched eggs on one plate by the total number of eggs laid on that same plate. The experiment was repeated at least three times for each of the genotypes.

Capture of worm images on NGM plates

Images of worms were captured using the iPhone 13 built-in camera. The phone is attached to a Leica dissecting microscope through a phone adaptor. The following magnifications were used: (1) iPhone camera 1x; (2) eye lens 10x; (3) objective lens 1.6x. A short video (20-60 seconds) for each plate was taken. Screenshots were taken from the videos to generate the images.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9 and Google Sheets. The statistical tests were specified in the figure captions. ns: $P > 0.05$; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$

Results

Based on primary sequence alignments, TBA-1 and TBA-2 differ in 12 amino acids, each of which could contribute to the functional differences between TBA-1 and TBA-2 (Figure 1.1 A). Out of the 12 different amino acids, amino acids 216, 218, and 287 are the prominent candidates due to their locations on the tubulin surfaces, which can impact the interaction between tubulins and, thus, the stability and functions of microtubules (Figure 1.1 B). Amino acids 216 and 218 are between the H6 and H7 helices which, according to an electron crystallography study using bovine brain tubulins, were shown to be important for both longitudinal interactions between tubulins within the heterodimer and lateral interaction between tubulins from directly adjacent protofilaments (Nogales et al., 1999). Amino acid 287 is in the H9 helix which is involved in the lateral contact between tubulins from different protofilaments (Nogales et al., 1999).

To investigate the roles of these three residues in specifying TBA-2's heterodimerization with BEN-1(L246F) and functions in the MT patch formation, I utilized CRISPR-Cas9 genome editing to change the amino acid identity at the 216, 218, and 287 locations of TBA-2 to that of TBA-1 (Figure 1.2). Due to the proximity of amino acids 216 and 218, I first edited the 216 and 218 locations together and introduced TBA-2(*ju2031* D216S, E218D) into the reconstitutedGFP::BEN-1(L246F) mutants. TBA-2(D216S, E218D) resulted in a statistically significant decrease in the number of MT patches (Figure 1.3). I then edited amino acid 287 of TBA-2 in TBA-2(D216S, E218D) and generated TBA-2(*ju2033* D216S, E218D, S287N) in the reconstitutedGFP::BEN-1(L246F) background (216: aspartic acid (-) to serine (polar); 218: glutamic acid (-) to aspartic acid (-); 287: serine (polar) to asparagine (polar)). The addition of the S287N edit further decreased the number of MT patches (Figure 1.3). These data together

suggest that amino acids 216, 218, and 287 of TBA-2 contribute to its specific interaction with BEN-1(L246F) and the formation of the MT patches.

Importantly, changing the amino acid identity at the three residues of TBA-2 does not abolish the function of TBA-2. TBA-2 and TBA-1 are the two most broadly expressed α -tubulins that have important but redundant roles in diverse processes. Single null mutants of *tba-2* or *tba-1* are relatively healthy, but knockdown of both *tba-1* and *tba-2* through RNAi results in complete embryonic lethality (100% of eggs unhatched) (Phillips et al., 2004; Lu & Mains, 2005). With this in mind, I utilized a verified null allele of *tba-1*, *tba-1(ok1135)* (Baran et al., 2010). I then combined the null allele of *tba-1* (*tba-1(0)*) with *tba-2(D216S, E218D)* or *tba-2(D216S, E218D, S287N)* through genetic crosses. I found that the *tba-1(0) tba-2(D216S, E218D)* and *tba-1(0) tba-2(D216S, E218D, S287N)* mutants have wild-type egg-laying ability and nearly no embryonic lethality (0.238% and 2.701% of eggs unhatched, respectively), suggesting that *tba-2(D216S, E218D)* and *tba-2(D216S, E218D, S287N)* are not null alleles of *tba-2* (Figure 1.4 A-B). The *tba-1(0) tba-2(D216S, E218D)* and *tba-1(0) tba-2(D216S, E218D, S287N)* mutants also look superficially wild-type in terms of locomotion and growth, further suggesting that TBA-2(D216S, E218D) and TBA-2(D216S, E218D, S287N) likely do not affect other functions of TBA-2 other than interaction with BEN-1(L246F) (Figure 1.4 C).

Discussion

Specific residues facilitate the interaction and functions of tubulin isotypes

Most eukaryotes express a large family of tubulin isotypes. Due to the redundancy and heterogeneous expression of the isotypes in cells, it has been very challenging to study the interactions of specific tubulin isotypes *in vivo*. TBA-2 and BEN-1(L246F) specifically form heterodimers that enrich in the MT patches, and this phenotype serves as an excellent model for me to investigate the specific amino acids that dictate the interaction of tubulin isotypes. Amino acids 216, 218, and 287 of TBA-2 are three of the residues that facilitate the interaction between TBA-2 with BEN-1(L246F). Since amino acids 216, 218, and 287 are located at the interfaces between tubulin contacts, changing the three amino acids of TBA-2 to TBA-1 could impact the interaction between TBA-2 and BEN-1(L246F) by, first of all, altering the residue-to-residue interactions, and, second, changing the molecular structure of TBA-2. Both possibilities were supported by previously published studies. In the seminal works done by Nogales et al. (1999), specific residues at the tubulin interfaces were pinpointed to facilitate the lateral or/and longitudinal interactions of tubulins. A later *in vitro* study using the human β -tubulins β 2B and β 3 revealed that amino acid differences confer different molecular structures at the tubulin interfaces, which alter the architecture as well as the stability of MTs, both of which are important in determining MT functions (Ti et al., 2018). Through ColabFold prediction, I visualized and overlapped BEN-1(L246F)/ TBA-2(WT) dimer with BEN-1(L246F)/ TBA-2(D216S, E218D, S287N) dimer. Interestingly, while I did not observe any structural difference near the 216, 218, and 287 locations, a very slight structural difference was observed at amino acids 43-47 (near the H1-S2 loop) of TBA-2. This difference in the H1-S2 loop could be a mere artifact of the structural prediction, or if the difference indeed arose from TBA-2(D216S, E218D,

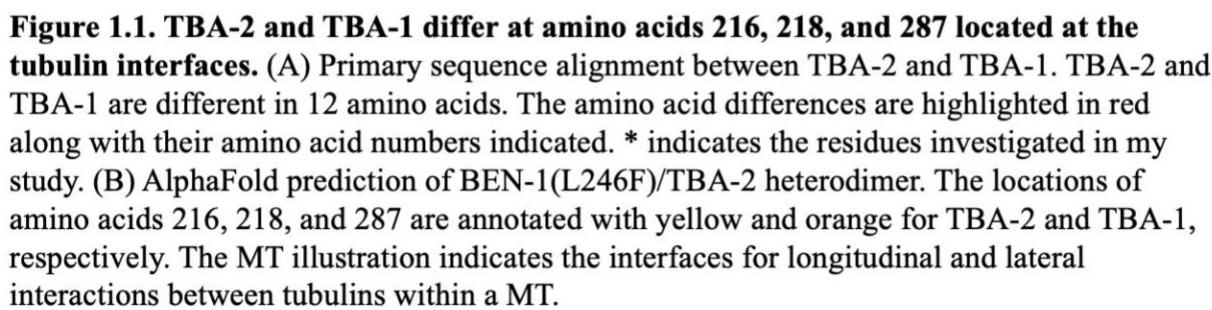
S287N), it could partly explain, structurally, how the three residues dictate interaction between TBA-2 and BEN-1(L246F).

There are still 9 amino acids that are different between TBA-2 and TBA-1, and their roles in mediating the interaction between TBA-2 and BEN-1(L246F) remain to be explored.

Prominently, TBA-2 has one less amino acid than TBA-1 in the C-terminal tail, which can impact MT functions by interacting with microtubule-associated proteins (MAP). It would be greatly interesting to investigate this residue difference in the future, as it may provide insights into whether interaction with MAPs would also facilitate the MT patch formation.

A

B



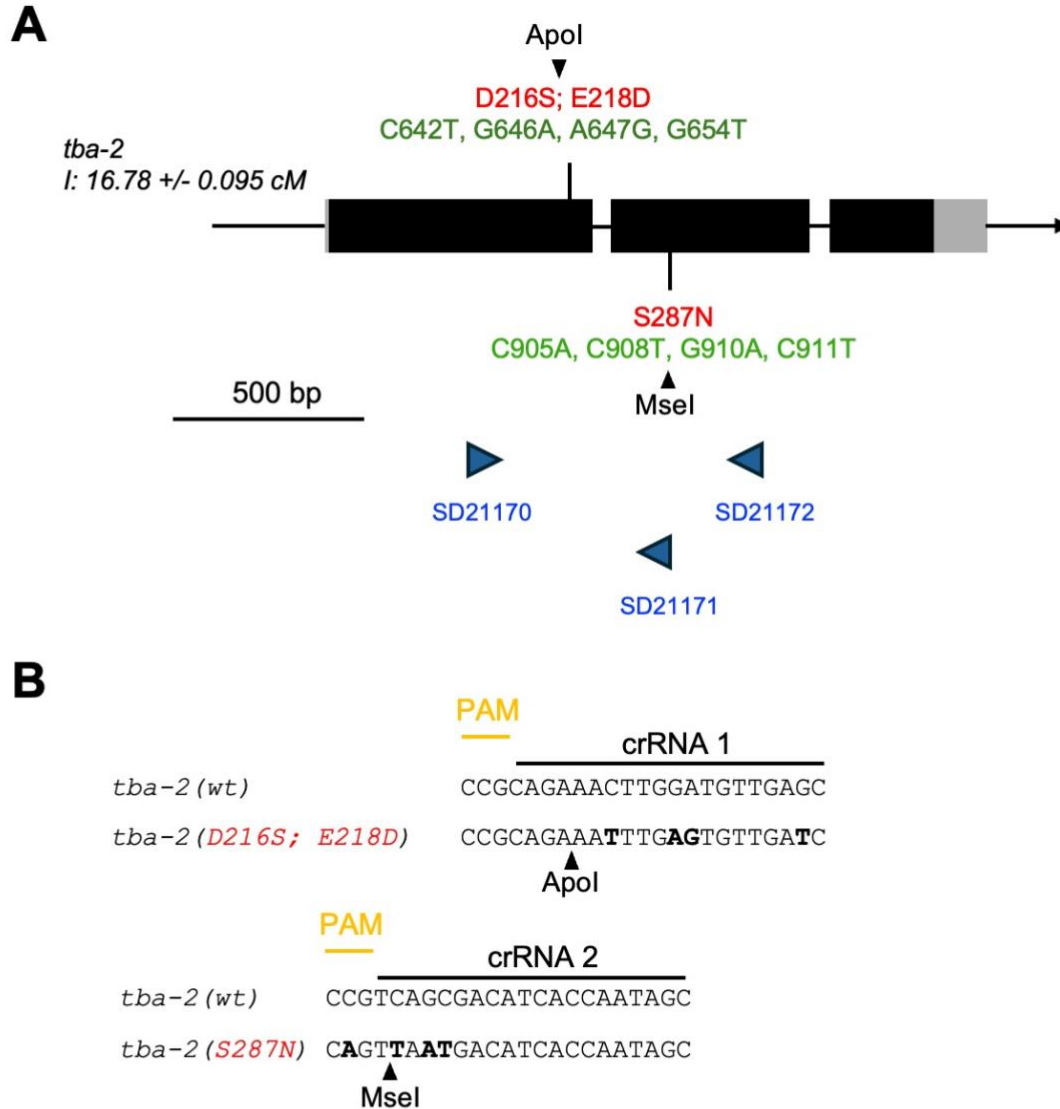


Figure 1.2. Utilizing CRISPR-Cas9 genome editing to introduce the amino acid changes D216S, E218D, and S287N in TBA-2. (A) The gene structure of *tba-2* shows the detailed nucleotide changes that result in the amino acid changes D216S, E218D, and S287N. Primers and restriction enzyme digestion sites utilized to genotype for successful edits are also shown. Black rectangles indicate the exons. Grey rectangles indicate the UTRs. Black lines between the rectangles indicate the introns. The amino acid changes D216S, E218D, and S287N are annotated in red. The nucleotide changes are annotated in green (see the specific roles of each nucleotide change in the “CRISPR-Cas9 genome editing” section of Materials and Methods). The Apol and Msel restriction enzyme digestion sites generated from the edits are indicated with black arrowheads. Primers used to genotype the D216S, E218D, and S287N edits are indicated with blue arrowheads. (B) Detailed sequences at the Cas9 cut sites. Each crRNA binding location is indicated with a black line. The location of each PAM on the complementary strand is indicated with a yellow line. The Apol (for *tba-2*(D216S; E218D)) and Msel (for *tba-2*(S287N)) cut sites are indicated with black arrowheads.

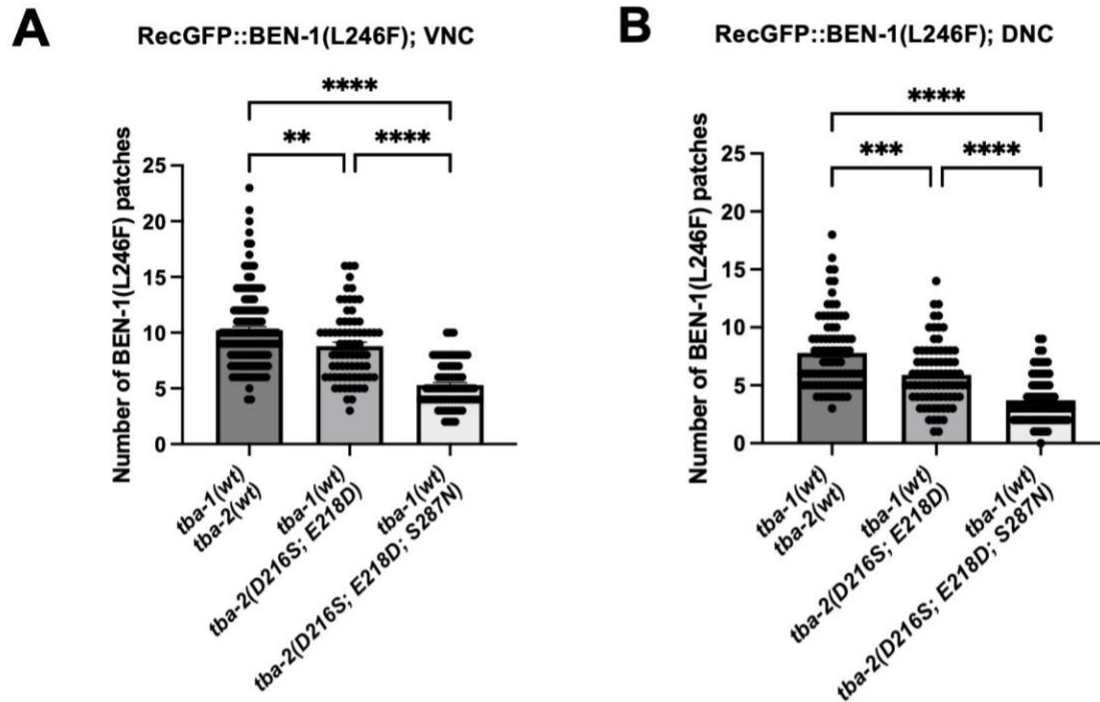


Figure 1.3. TBA-2 forms the stable MT patches with BEN-1(L246F) mainly through amino acids 216, 218, and 287 of TBA-2. (A-B) BEN-1(L246F) patch quantification in the TBA-2(D216S; E218D) and TBA-2(D216S; E218D; S287N) backgrounds. The CRISPR edits at amino acids 216, 218, and 287 significantly decrease the number of BEN-1(L246F) patches in both the ventral nerve cord (VNC) (A) and the dorsal nerve cord (DNC) (B). Each dot indicates the patch quantification from 1 animal. n = (A) 126; 73; 73. (B) 82; 79; 72. Error bar indicates mean $\pm$ SEM. Statistical analysis was performed using Welch's ANOVA with Dunnett T3 multiple comparisons test.

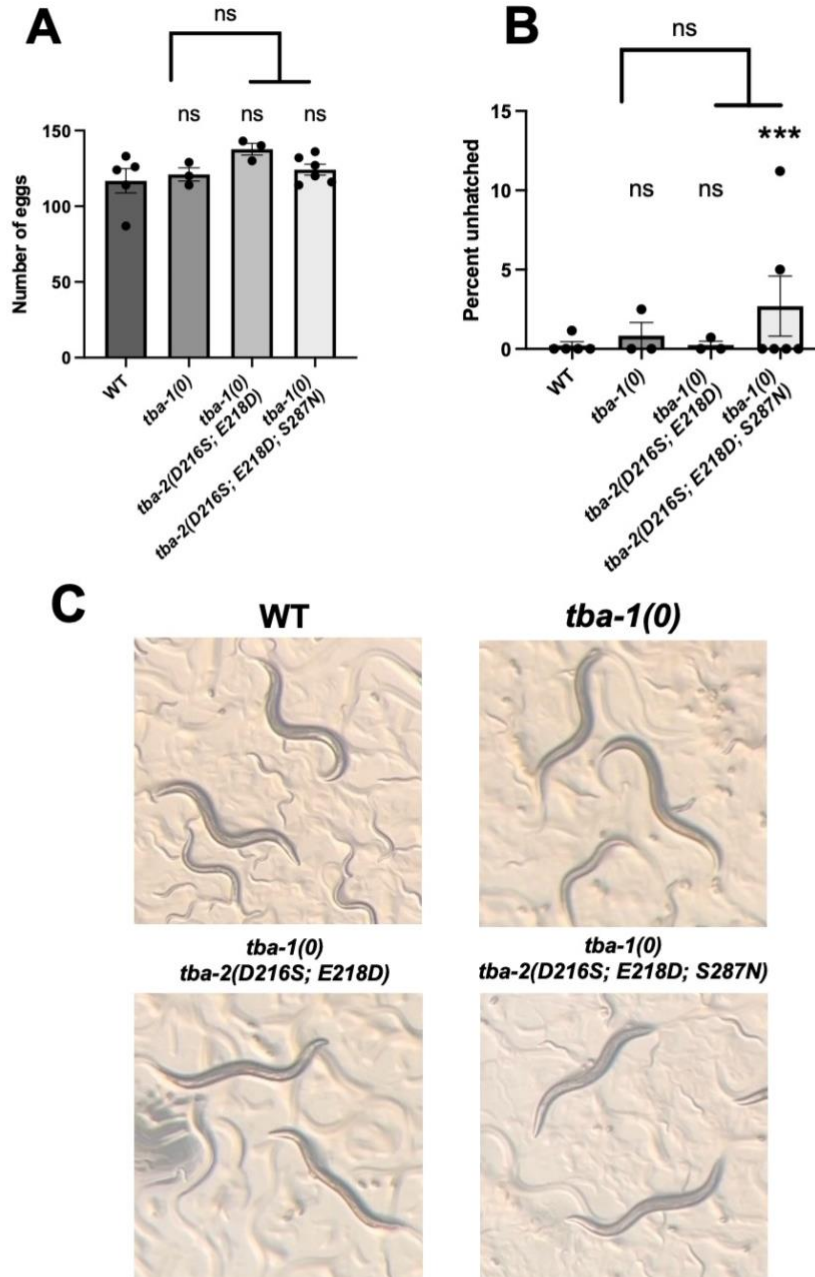


Figure 1.4. *tba-2(D216S; E218D; S287N)* in combination with *tba-1(0)* does not cause significant defects in embryonic development, locomotion, and growth. (A) The number of eggs laid by 3 L4 animals in 24 hours at 20°C. (B) The percentage of eggs that remain unhatched after 24 hours at 20°C. Percent unhatched = number of unhatched eggs after 24 hours / total number of eggs laid. Each dot represents the number of eggs or the percentage of unhatched eggs from 1 experiment for a total of at least 3 independent experiments per genotype. Error bar indicates mean $\pm$ SEM. Statistical analysis was performed using (A) Welch's ANOVA with Dunnett T3 multiple comparisons test and (B) Fisher's exact test. (C) Images of mixed-stage animals for the different genotypes, showing no obvious locomotive and morphological difference between the mutants and the wild-type control.

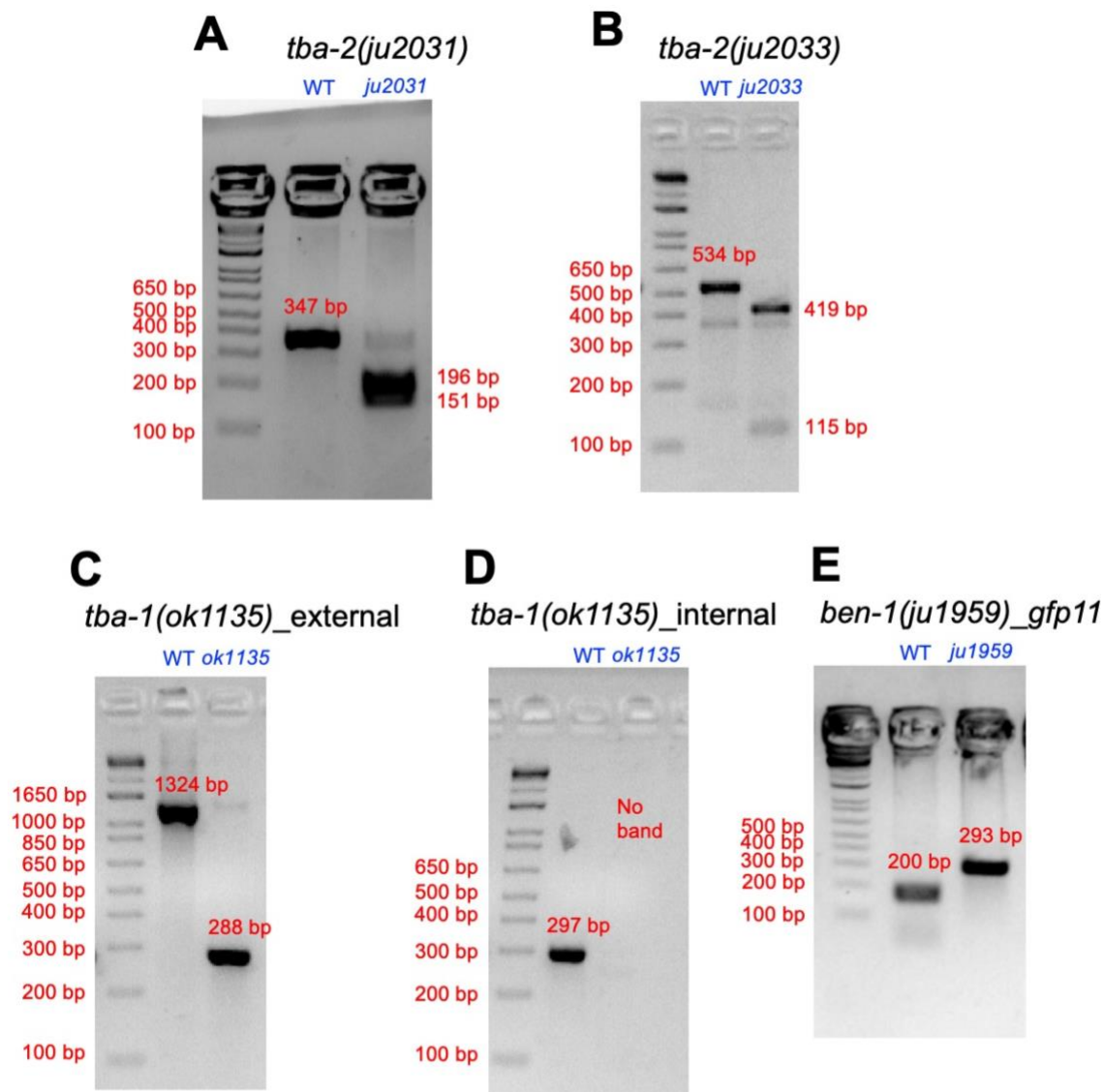


Figure 1.5. Representative gel images for the PCR genotyping of each allele. (A) Representative PCR results for WT and *tba-2(ju2031)* run on ~3% agarose gel. (B) Representative PCR results for WT and *tba-2(ju2033)* run on ~3% agarose gel. (C) Representative PCR results for WT and *tba-1(ok1135)* run on ~3% agarose gel. External primers flanking the deletion were used for the reaction. (D) Representative PCR results for WT and *tba-1(ok1135)* run on ~3% agarose gel. Internal primers within the deletion were used for the reaction. (E) Representative PCR results for WT and *ben-1(ju1959)* run on ~3% agarose gel. The primers flanking the *gfp11* insertion were used for the reaction. Invitrogen 1 Kb Plus DNA Ladder was used for A-E. For detailed primer information and expected results, see Table 1.2. All reactions were run at 150 V for at least 20 minutes.

Tables

Table 1.1. *C. elegans* strain information

Strain number	Genotype	Construction notes
CZ22434	<i>tba-1(ok1135) I</i>	The strain was outcrossed 6x by Dr. Naina Kurup. The strain was initially obtained from the Caenorhabditis Genetics Center (CGC) (initial strain number: RB1185).
CZ29879	<i>GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Constructed by Dr. Junxiang Zhou. <i>Prgef-1-GFP1-10(juIs409)</i> expresses <i>ttx-3::RFP</i> in the head region, which can be directly visualized using a fluorescence compound or dissecting microscope. <i>ben-1(ju1959)</i> is also visually genotyped using a fluorescence compound microscope for expression of GFP1-11::BEN-1(L246F) when in combination with <i>juIs409</i> . <i>ben-1(ju1959)</i> can also be genotyped using PCR. Primer information is included in Table 1.2.
CZ30341	<i>tba-2(ju2031 [D216S; E218D]) I</i>	Generated through CRISPR-Cas9 in N2 background (See Materials and Methods).
CZ30409	<i>tba-2(ju2031 [D216S; E218D]) I; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ29879 was crossed with N2 males. The crossed male progenies with the genotype <i>ben-1(ju1959)/+; juIs409/+</i> were crossed with CZ30341. <i>tba-2(ju2031)/+; ben-1(ju1959)/+; juIs409/+</i> F1 hermaphrodite progenies were isolated by genotyping <i>ben-1(ju1959)</i> and <i>juIs409</i> visually under a fluorescence compound microscope and left to self-reproduce ((<i>Prgef-1-GFP1-10(juIs409)</i> expresses <i>ttx-3::RFP</i> in the head region. <i>ben-1(ju1959)</i> shows expression of GFP1-11::BEN-1(L246F) in neurons when in combination with <i>juIs409</i>). The self-fertilized F2 progenies from F1s that express GFP1-11::BEN-1(L246F) were then single-housed and left to self-reproduce. After genotyping the F2

Table 1.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		animals for <i>ju2031</i> via PCR and genotyping the progenies of the F2s for <i>ben-1(ju1959)</i> and <i>juIs409</i> visually under a fluorescence compound microscope, CZ30409 <i>tba-2(ju2031); ben-1(ju1959); juIs409</i> was isolated.
CZ30554	<i>tba-2(ju2033 [D216S; E218D; S287N]) I; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Generated through CRISPR-Cas9 genome editing in CZ29879 (see Materials and Methods). Outcrossed using N2. CZ303554 was crossed with N2 males. The crossed F1 hermaphrodite progenies (<i>tba-2(ju2033)/+; ben-1(ju1959)/+; juIs409/+</i>) were isolated to self-reproduce. The F2 progenies that express GFP1-11::BEN-1(L246F) were isolated to further self-reproduce. After genotyping the F2s for <i>tba-2(ju2033)</i> via PCR and genotyping the progenies of the F2s for <i>ben-1(ju1959)</i> and <i>juIs409</i> visually under a fluorescence compound microscope, <i>tba-2(ju2033); ben-1(ju1959); juIs409</i> was re-isolated. The process was then repeated one more time.
CZ30634	<i>tba-2(ju2033 [D216S; E218D; S287N]) I tba-1(ok1135) I</i>	CZ30554 was crossed with N2 to generate males with the genotype <i>tba-2(ju2033)/+; ben-1(ju1959)/+; juIs409/+</i>. The males were then crossed with CZ22434. The crossed F1 hermaphrodite progenies have the genotype <i>tba-1(ok1135) tba-2(ju2033)?/+ +; ben-1(ju1959)?/+; juIs409?/+</i> (? = all possible genotypes). The crossed F1 progenies were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> under a fluorescence compound microscope. The crossed F1 progenies that did not contain <i>ben-1(ju1959)</i> but were heterozygous for <i>juIs409</i> were

Table 1.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		found. After the F1 worms self-reproduced, <i>tba-1(ok1135)</i> and <i>tba-2(ju2033)</i> were genotyped via PCR. F1s that have the genotype <i>tba-1(ok1135) tba-2(ju2033)/+ +; juIs409/+</i> were kept. The F2 progenies (from <i>tba-1(ok1135) tba-2(ju2033)/+ +; juIs409/+</i>) that did not express <i>juIs409 (ttx-3::RFP)</i> were single-housed. After the F2s self-reproduced, they were genotyped for <i>tba-2(ju2033)</i> and <i>tba-1(ok1135)</i> via PCR. F2s with the genotype <i>tba-1(ok1135) tba-2(ju2033)/+ tba-2(ju2033)</i> were isolated to self-reproduce again. The F3 progenies were single-housed and left to self-reproduce. The F3s were genotyped for <i>tba-1(ok1135)</i> via PCR. CZ30634 <i>tba-1(ok1135) tba-2(ju2033)</i> was then isolated.
CZ30700	<i>tba-2(ju2031 [D216S; E218D])</i> <i>I tba-1(ok1135) I</i>	CZ30341 was crossed with N2 to generate males with the genotype <i>tba-2(ju2031)/+</i>. The males were then crossed with CZ22434. The crossed F1 hermaphrodite progenies have the genotype <i>tba-1(ok1135) tba-2(ju2031)?/+ +</i> (? = all possible genotypes). The F1s were isolated to self-reproduce. They were then genotyped for <i>tba-2(ju2031)</i> via PCR. F1s with the genotype <i>tba-1(ok1135) tba-2(ju2031)/+ +</i> were found. The F2 progenies from <i>tba-1(ok1135) tba-2(ju2031)/+ +</i> were isolated to further self-reproduce. After the F2 worms self-reproduced, they were genotyped for <i>tba-2(ju2031)</i> and <i>tba-1(ok1135)</i> via PCR. Thereafter, <i>tba-1(ok1135) tba-2(ju2031)/tba-1(ok1135) +</i> was found. The F3 progenies from <i>tba-1(ok1135) tba-2(ju2031)/tba-1(ok1135) +</i> were isolated again to self-reproduce. They were then genotyped for <i>tba-</i>

Table 1.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		<i>2(ju2031)</i> via PCR. CZ30700 <i>tba-1(ok1135) tba-2(ju2031)</i> was then isolated.
CZ30884	<i>tba-2(ju2054 [S287N]) I; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Generated through CRISPR-Cas9 genome editing in CZ29879 (see Materials and Methods). The strain has not been examined for MT patch formation. <i>tba-2(ju2054)</i> has the same genotyping method as <i>tba-2(ju2033)</i> when they are not in combination (see Table 1.2 for more details). Outcrossed using N2. CZ30884 was crossed with N2 males. The crossed F1 hermaphrodite progenies (<i>tba-2(ju2054)/+; ben-1(ju1959)/+; juIs409/+</i>) were isolated to self-reproduce. The F2 progenies that express GFP1-11::BEN-1(L246F) were isolated to further self-reproduce. After genotyping the F2s for <i>tba-2(ju2054)</i> via PCR and genotyping the progenies of the F2s for <i>ben-1(ju1959)</i> and <i>juIs409</i> visually under a fluorescence compound microscope, <i>tba-2(ju2054); ben-1(ju1959); juIs409</i> was re-isolated.

Table 1.2. Allele information and genotyping primers

Allele	Gene	Mutation/s	Primer number/name	Sequence	Expected results
<i>ju1959</i>	<i>ben-1</i>	gfp11 with ELGSGSGSGS GSSAG linker inserted at the N-terminus of <i>ben-1</i> + SNPs in <i>ben-1</i> : A1853G, A1856T, C1860T	SD21166 (for <i>gfp11</i> insert)	CTCATATAAC TTCAAAAAGA ACTTGG	WT: 200 bp <i>ju1959</i>: 293 bp
			SD21167 (for <i>gfp11</i> insert)	CACCATTAGC CTCATTATAG TAGAC	
			YJ12495 (for L246F)	GATGAGACTT TCTGCATTGA CAACG	With MluCI digestion.
			YJ12496 (for L246F)	CACGTGGTGG AATATCACAG ACC	WT: 366 bp + 140 bp <i>ju1959</i>: 321 bp + 140 bp + 45 bp
<i>ok1135</i>	<i>tba-1</i>	1036 bp deletion	YJ11190 (External forward)	CTGTCTCGAG CATGGAATCC AG	WT: 1324 bp <i>ok1135</i>: 288 bp * must use in parallel with the internal primers listed below since the external primers can also amplify a non-specific band from <i>tba-1</i> .
			YJ11191 (External reverse)	CGACCTCTTC GTAGTCCTTT TCG	
			SD20485 (Internal forward)	GCTTCCCTCC GTTTCGATG	WT: 297 bp <i>ok1135</i>: no band
			SD20930 (Internal reverse)	GACATCTCCT CTGTACAAGA G	
<i>ju2031</i>	<i>tba-2</i>	SNPs in <i>tba-2</i> : C642T, G646A,	SD21170	CAAGCTCGAG TTCTCCATCT AC	With ApoI digestion.

Table 1.2. Allele information and genotyping primers (continued)

Allele	Gene	Mutation/s	Primer number/name	Sequence	Expected results
		A647G, G654T	SD21171	CTTGGGTATG GCACCAAGTT G	WT: 347 bp <i>ju2031</i>: 151 + 196 bp
<i>ju2033</i>	<i>tba-2</i>	SNPs in <i>tba-2</i> : C642T, G646A, A647G, G654T, C905A, C908T, G910A, C911T	SD21170	CAAGCTCGAG TTCTCCATCT AC	With MseI digestion. WT: 534 bp <i>ju2033</i>: 419 + 115 bp
			SD21172	CTTTGGAACG ACGTCTCCTC TG	
<i>ju2054</i>	<i>tba-2</i>	SNPs in <i>tba-2</i> : C905A, C908T, G910A, C911T	SD21170	CAAGCTCGAG TTCTCCATCT AC	With MseI digestion. WT: 534 bp <i>ju2054</i>: 419 + 115 bp (See gel image for <i>tba-2(ju2033)</i>)
			SD21172	CTTTGGAACG ACGTCTCCTC TG	To distinguish from <i>ju2033</i> , do a parallel PCR with primers SD21170 and SD21171. Then digest with ApoI. WT: 347 bp <i>ju2033</i>: 151 + 196 bp <i>ju2054</i>: 347 bp (See gel image for <i>tba-2(ju2031)</i>)

Table 1.3. CRISPR reagents

Allele	crRNA	HDR template (/ indicates Cas9 cut site; bold letters indicate nucleotide changes)
<i>tba-2(ju2031)</i>	GCUCAACAUCCAAGUUUCUG (PAM: <u>CGG</u>) <i>crRNA #1 in Figure 1.2</i>	TTGGAGCACTCCGACTG CGCCTTCATGGTCGATA ACGAGGCCATCTACGAC ATCTGCCCGCAG/AAATTT GAGT GTTGATCGACCAA GCTACACCAACCTCAAC AGAATCATCTCCCAGGT TTGTTGAGCTCAATTTG ATT
<i>tba-2(ju2033)</i> and <i>tba-2(ju2054)</i>	GCUAUUGGUGAUGUCGCUGA (PAM: <u>CGG</u>) <i>crRNA #2 in Figure 1.2</i>	TTCCCATTTGGCCGCCTA CACTCCACTCATCTCTG CTGAGAAGGCCTACCAC GAGGCTCTGTCAGTTA/ A TG ACATCACCAATAGCT GCTTCGAGCCGGCTAAC CAGATGGTCAAGTGTGA TCCACGTCACGGA

Chapter 2: Multiple bZIP transcription factors facilitate the formation of the MT patches

Background

The bZIP transcription factor CEBP-1 is the *C. elegans* homolog of CCAAT/enhancer-binding protein (C/EBP) and has been reported to act downstream of DLK-1 in the context of neuron development, axon regeneration, and stress responses to microtubule perturbation (Yan et al., 2009; Bounoutas et al., 2011). A previous study conducted a ChIP-sequencing experiment to investigate the DNA targets of CEBP-1 (Kim et al., 2016). They identified 212 gene targets, and many of the genes are targeted by CEBP-1 through a CEBP-1 (C/EBP) binding motif (Kim et al., 2016). The α -tubulin gene *tba-2* is one of the identified genes and contains a C/EBP binding motif in its promoter region. In the meantime, preliminary data from Dr. Junxiang Zhou suggests that DLK-1 promotes the formation of the MT patches by upregulating TBA-2 through the C/EBP binding motif in the *tba-2* promoter.

Removing CEBP-1 partially decreased MT patch formation in the reconstituted GFP::BEN-1(L246F) mutants (Figure 2.3). However, when I transgenically overexpressed (OE) CEBP-1 using an integrated array (*wgIs563*), it resulted in a significant increase in the number of MT patches (Figure 2.4). This evidence suggests the possibility that CEBP-1 is partially redundant with other downstream effectors of the DLK-1 MAPK pathway in facilitating the MT patch formation. As a *C. elegans* homolog of C/EBP, CEBP-1 contains a basic leucine zipper (bZIP) domain that enables homodimerization of C/EBP or heterodimerization with other bZIP domain-containing transcription factors to facilitate DNA binding and transcriptional regulations (Williams et al., 1991; Ramji & Foka, 2002). Aside from CEBP-1, ZIP-4 (shares 63% identity with CEBP-1), and ZIP-2 (shares 19.62% identity with CEBP-1) are two other bZIP transcription factors belonging to the C/EBP family (Figure 2.1)

(Tjahjono & Kirienko, 2017). CEBP-1, ZIP-4, and ZIP-2 have previously been reported to have overlapping functions in regulating the Ethanol and Stress Response Element (ESRE) network in response to mitochondrial dysfunction caused by *Pseudomonas aeruginosa* infection-induced pyoverdine exposure (Tjahjono & Kirienko, 2017). Therefore, I investigated if CEBP-1, ZIP-4, and ZIP-2 have overlapping or redundant roles downstream of DLK-1 in regulating the MT patch formation.

During my master's thesis research, I conducted complex genetic crosses to generate *zip-2; cebp-1* and *zip-4; cebp-1* double knockout mutants in the reconstituted GFP::BEN-1(L246F) background. I discovered that CEBP-1, ZIP-4, and ZIP-2 act redundantly in regulating the MT formation.

Materials and Methods

C. elegans strains and genetics

All worms were maintained on nematode growth media (NGM) plates seeded with the *E. coli* strain OP50 (Brenner, 1974). Strains were maintained at 20°C unless otherwise specified. The Bristol (N2) strain was used as the wild type. Strains used in this section are described in Table 2.1. The genotyping methods for the different alleles are described in Table 2.2. Alleles were either genotyped using standard PCR or using a fluorescence compound microscope (see Tables 2.1 and 2.2 for details). Figure 2.5 shows representative gel images of the PCR genotyping results.

DNA sequence and protein analysis

DNA sequence analysis was performed using ApE and SnapGene. Protein alignment was performed using T-Coffee (Tcoffee.crg.eu) and formatted using Boxshade (Notredame et al., 2000). Domain information of the bZIP transcription factors was obtained from UniProt with PROSITE-ProRule annotation (Sigrist et al., 2013; The UniProt Consortium, 2015).

MT patch quantification

A MT patch is a fiber-like structure enriched with reconstitutedGFP::BEN-1. A MT patch has an obviously higher reconstitutedGFP::BEN-1 intensity than its surroundings and is at least 1 μm in length. The number of patches on the ventral nerve cord (before the tail soma), dorsal nerve cord, and, for some, single-neurite commissures connecting the ventral and dorsal nerve cords were counted manually using a Zeiss Axio Imager M2 fluorescence compound microscope with a 63x objective oil immersion lens. Patch qualification experiments were performed in day

2 adult animals unless otherwise specified. To prepare day 2 adult animals for the analysis, animals at the L4 stage (easily distinguishable based on the white half-moon vulva) were picked and transferred to a new NGM plate. The L4 animals were left to grow at 20°C for 48 hours to reach the day 2 adult stage (a different temperature was used for the heat shock-induced DLK-1 experiments; see Chapter 3 for more details). The worms were then immobilized using 10 mM levamisole (in M9 buffer) on a 4% agar gel pad for the quantification.

Benomyl testing

To test if the bZIP transcription factor reporter strains (Table 2.1) respond to benomyl-induced DLK-1 activation, NGM plates with 10 μ M of benomyl were used. For each bZIP transcription factor reporter strain, 3 L4 hemihedrites were transferred to a 10 μ M benomyl NGM plate. The worms were left to grow in the presence of benomyl for 3-4 days until their progenies reached the L4 stage. In parallel, the no-benomyl control was prepared. For each bZIP transcription factor reporter strain, 3 L4 hemihedrites were transferred to a normal NGM plate. The worms were also left to grow for 3-4 days until their progenies reached the L4 stage. The L4 progenies of the benomyl and no-benomyl groups were then immobilized using 10 mM levamisole (in M9 buffer) on 4% agar gel pads. Using a Zeiss Axio Imager M2 fluorescence compound microscope with a 63x objective oil immersion lens, the expression levels of the corresponding bZIP transcription factor were visually examined and compared between the benomyl and no-benomyl groups. 2 independent experiments were performed for each bZIP transcription factor reporter strains.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9. The statistical tests were specified in the figure captions. ns: $P > 0.05$; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$

Results

To investigate if CEBP-1, ZIP-4, and ZIP-2 have overlapping or redundant roles downstream of DLK-1 in regulating the MT patch formation, I utilized worm strains that contain knockout allele of *cebp-1*, *zip-4*, or *zip-2* (Figure 2.2). Through genetic crosses, I combined the knockout allele of *cebp-1* with *zip-2* and *zip-4*, generating *zip-2; cebp-1* and *zip-4; cebp-1* double knockout alleles. I introduced *zip-2; cebp-1* and *zip-4; cebp-1* double knockout alleles into the reconstitutedGFP::BEN-1(L246F) background and analyzed the patch formation in day 2 adult animals. Removing *zip-2* or *zip-4* alone in the *ben-1(L246F)* mutants did not significantly decrease the MT patch numbers. However, removing *zip-2* or *zip-4* together with *cebp-1* significantly diminished the formation of the MT patches in the reconstitutedGFP::BEN-1(L246F) mutants (Figure 2.3). This evidence suggests that, in the *ben-1(L246F)* background, multiple bZIP transcription factors promote the formation of the MT patches.

Additionally, from my preliminary observation looking at the expression patterns of ZIP-2 and ZIP-4 through transgenic fluorescence reporters, ZIP-2 and ZIP-4 are expressed in the nucleus of many neurons at the nerve ring, ventral nerve cord, and tail ganglia similar to CEBP-1. The overlapping expression patterns help to further explain the redundant roles between CEBP-1, ZIP-2, and ZIP-4 in regulating neuronal MT properties in the *ben-1(L246F)* mutants.

After confirming the involvement of ZIP-2 and ZIP-4 in the MT patch formation, I examined if overexpression of ZIP-4 is also sufficient to increase the number of MT patches in the reconstitutedGFP::BEN-1(L246F) background. Very interestingly, overexpression of ZIP-4, unlike CEBP-1 OE, significantly decreased the number of MT patches in the ventral nerve cord while increasing the patch number in the dorsal nerve cord (Figure 2.4). This suggests that ZIP-4 likely gets activated through a different mechanism than CEBP-1 in this context, and

overexpression of ZIP-4 somehow depresses the MT patch formation in the ventral nerve cord through unknown mechanisms. In the meantime, to rule out any artifacts from overexpressing bZIP transcription factors, I tested the overexpression of CEBP-2 (another member of the C/EBP family whose role in the BEN-1(L246F) patch formation has not been tested yet). CEBP-2 OE did not affect the formation of the MT patches (Figure 2.4).

To further capture the potential downstream transcription factors of DLK-1, I activated the DLK-1 pathway through the BEN-1-targeting drug, benomyl (benzimidazole) (the activating effect of benomyl on DLK-1 was discovered by Dr. Junxiang Zhou). Using transgenic translational reporters, I examined the expression levels of 5 other bZIP transcription factors (HLH-30, CEBP-2, JUN-1, FOS-1, and DAF-19) along with ZIP-2 and ZIP-4 upon benomyl treatment (10 μ m benomyl). While CEBP-1 (the known downstream transcription factor of DLK-1) expression was highly upregulated in the neuronal soma upon DLK-1 activation by benomyl treatment, none of the 7 bZIP transcription factors (surprisingly, ZIP-2 and ZIP-4 included) showed any expression changes upon benomyl treatment. This evidence suggests that activation of bZIP transcription factors downstream of DLK-1 could occur either through an expression level increase (e.g., CEBP-1) or through potential post-translational modifications such as phosphorylation, which has been previously reported in several independent studies on different bZIP transcription factors (Shivers et al., 2010; Hattori et al., 2013).

Discussion

The context-specific activation and functions of bZIP transcription factors

The P38 MAPK pathway involving DLK-1, PMK-3, and CEBP-1 was found to play important roles in synaptogenesis and axon regeneration (Nakata et al., 2005; Yan et al., 2009). In neurons damaged by laser axotomy, genetic knockout of *cebp-1* abolishes axon regeneration to the same degree as genetic knockout of *dlk-1*, suggesting that CEBP-1 is likely the only bZIP transcription factor downstream of DLK-1 in the context of axon regeneration (Yan et al., 2009). In the touch receptor neurons of *C. elegans*, disruption of microtubules results in a general reduction of gene expression, and this reduction relies on the same pathway involving DLK-1, PMK-3, and CEBP-1 (Bounoutas et al., 2011). Similar to the context of axon regeneration, CEBP-1 appears to be the only downstream bZIP transcription factor of DLK-1 in regulating gene expression responding to microtubule disruption (Bounoutas et al., 2011). In my case of MT patch formation of the *ben-1(L246F)* mutants, however, I found 3 bZIP transcription factors of the C/EBP family CEBP-1, ZIP-2, and ZIP-4 that have overlapping and redundant roles likely downstream of the DLK-1 MAPK pathway.

While the neuronal functions of CEBP-1 have been somewhat characterized, ZIP-2 has been extensively studied in non-neuronal processes such as innate immune responses and mitochondrial maintenance during aging (Estes et al., 2010; Tjahjono & Kirienko, 2017; Hahm et al., 2020). ZIP-4, on the other hand, was scarcely studied and was, to my knowledge, only implicated in innate immune responses against *Pseudomonas aeruginosa* where it serves overlapping functions with CEBP-1, CEBP-2, and ZIP-2 (Tjahjono & Kirienko, 2017). In the works of Tjahjono and Kirienko (2017), removing CEBP-1, ZIP-2, or ZIP-4 alone caused a reduction in the expression of the same set of immune response genes and increased death after

infection. However, knocking down *zip-2* by RNAi in *zip-4* or *cebp-1* knockout background did not show synergistic effects in post-infection survival (only an additive effect between *zip-2* and *cebp-1*), suggesting the three bZIP transcription factors have overlapping but not completely redundant functions in terms of innate immune responses (Tjahjono & Kirienko, 2017). On the contrary, in my case of the BEN-1(L246F) patch formation, removing either *cebp-1*, *zip-2*, or *zip-4* alone had no or some effects on the MT patch formation, while double mutants of *zip-2*; *cebp-1* and *zip-4*; *cebp-1* showed a synergistic reduction in the MT patch formation, suggesting that the functions of the three bZIP transcription factors in the MT patch formation are more redundant than in the context of innate immune responses. This discrepancy could arise from the cell-type differences that affect how the bZIP transcription factors are activated in response to different stress signals (e.g., MT disruption vs. bacterial infection). Mechanistically, how the bZIP transcription factors were activated by the DLK-1 pathway remains unclear. It would also be interesting to more extensively test if CEBP-2 is redundantly involved in the MT patch formation as well.

Additionally, *zip-4*; *cebp-1* showed stronger effects on diminishing the MT patch formation than *zip-2*; *cebp-1*. The *zip-2(tm4067)* allele used in my study was previously deduced to be partial loss-of-function since *zip-2* RNAi produced a stronger suppression of the immune response gene *irg-1* than *zip-2(tm4067)* after *Pseudomonas aeruginosa* infection (Estes et al., 2010). Thus, it would be important to test a stronger knockout allele of *zip-2* (*zip-2(tm4248)*) to confirm my results and draw a more confident conclusion on whether *zip-2* or *zip-4* could be more involved in the MT patch formation (*zip-2(tm4248)* allele information is shown in Figure 2.2). That said, the involvement of *zip-2* in the DLK-1-dependent MT patch formation has been confirmed by my results.

Figures

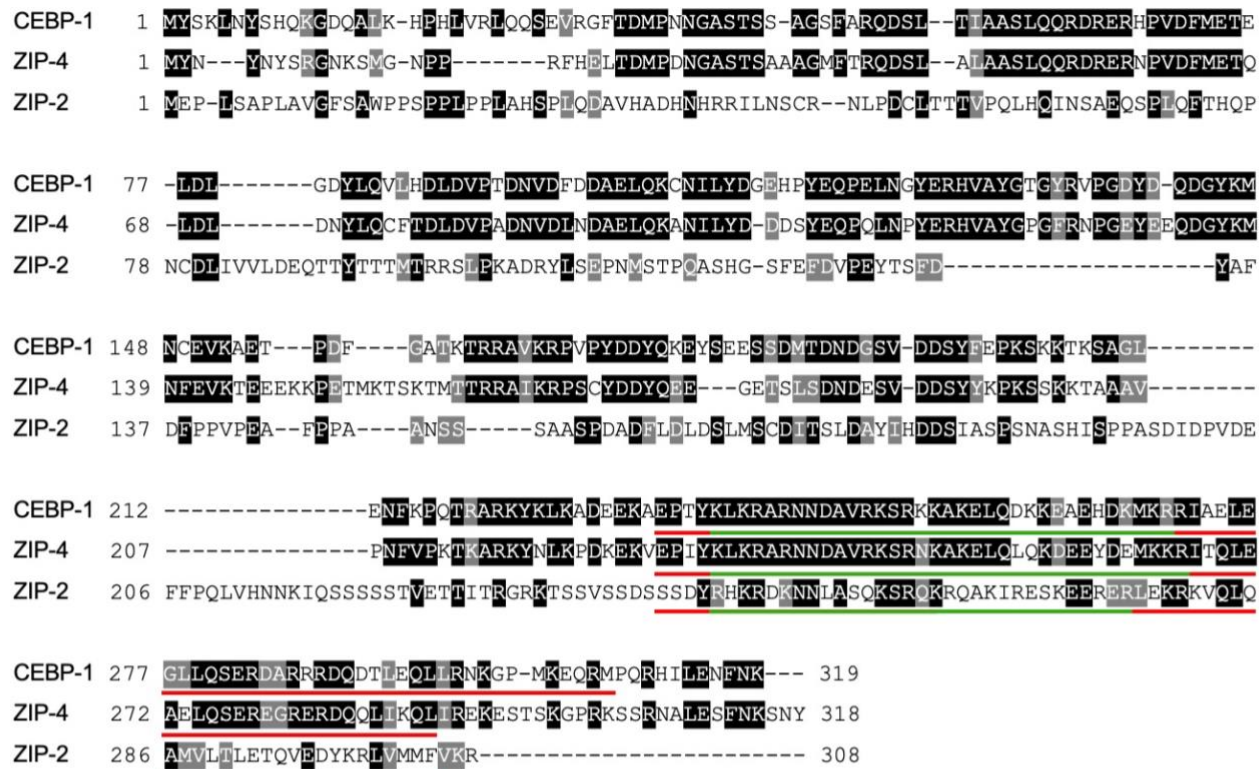


Figure 2.1. Protein sequence alignment of CEBP-1, ZIP-4, and ZIP-2. The bZIP domains are underlined in red. The basic motif of each bZIP domain is underlined in green. Domain and motif predictions from UniProt with PROSITE-ProRule annotation. When a residue is highlighted in black, it indicates an identical amino acid at that location to at least one other protein in the alignment. When a residue is highlighted in grey, it indicates a similar amino acid at that location to at least one other protein in the alignment.

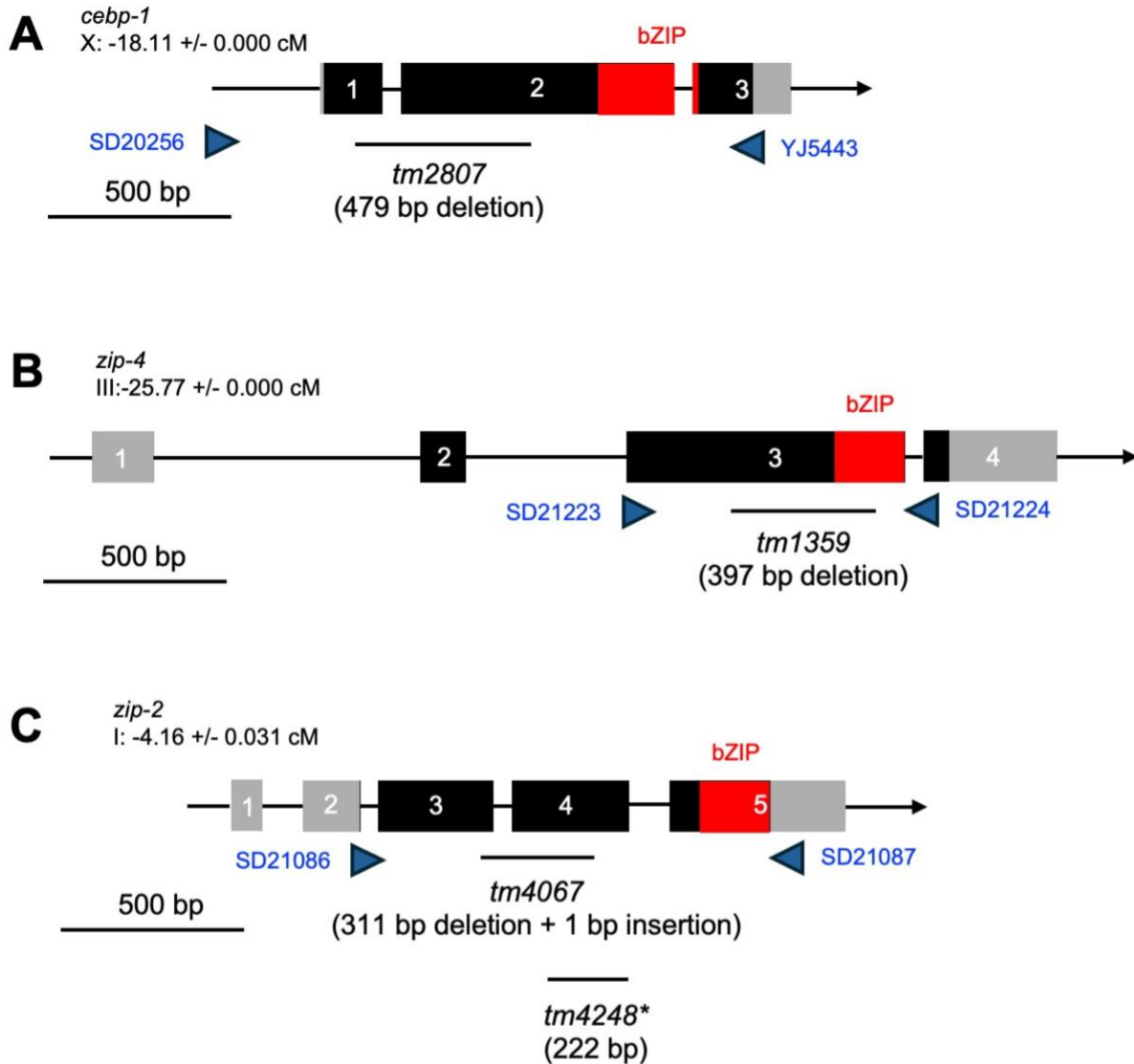


Figure 2.2. Gene illustrations for *cebp-1*, *zip-4*, and *zip-2* show the knockout alleles and genotyping primers. Black rectangles indicate the exons. Grey rectangles indicate the UTRs. Exon numbers are labeled. Black lines between the rectangles indicate the introns. Sequences that encode a bZIP domain are labeled in red (domain prediction from UniProt with PROSITE-ProRule annotation). Primers used to genotype the three alleles are indicated with blue arrowheads (details in Table 2.2). (A) Gene structure for *cebp-1*. The *tm2807* allele contains a 479 bp deletion mutation, causing a frameshift and a premature stop codon in exon 2. (B) Gene structure for *zip-4*. The *tm1359* allele contains a 397 bp deletion mutation, causing a frameshift and a premature stop codon in exon 3. (C) Gene structure for *zip-2*. The *tm4067* allele contains a 311 bp deletion and a 1 bp insertion, causing a frameshift and a premature stop codon in exon 4.

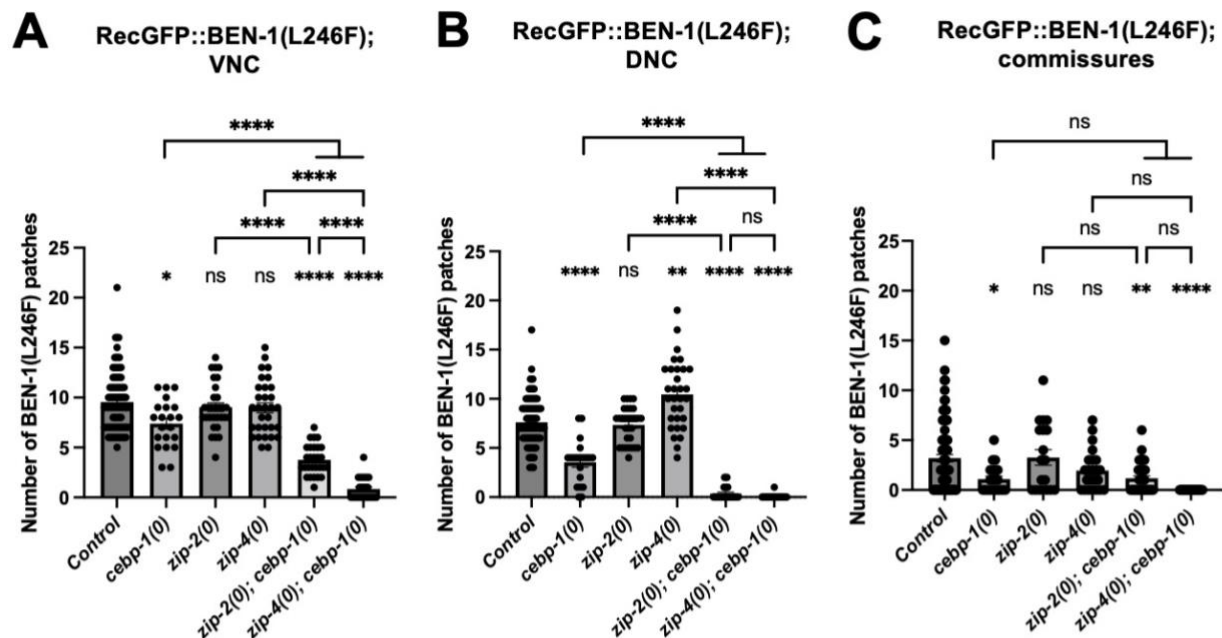


Figure 2.3. Multiple bZIP transcription factors of the C/EBP family redundantly facilitate the formation of the BEN-1(L246F) patches. (A-C) BEN-1(L246F) patch quantification of day 2 adults in different bZIP transcription factor knockout backgrounds. (A) Ventral nerve cord. (B) Dorsal nerve cord. (C) Commissures. Each dot indicates the patch quantification from 1 animal. n = (A) 96; 21; 30; 31; 28; 25. (B) 98; 20; 30; 31; 27; 24. (C) 90; 21; 19; 23; 36; 24. Error bar indicates mean $\pm$ SEM. Statistical analysis was performed using (A-B) Welch's ANOVA with Dunnett T3 multiple comparisons test (C) one-way ANOVA with Tukey's multiple comparisons test.

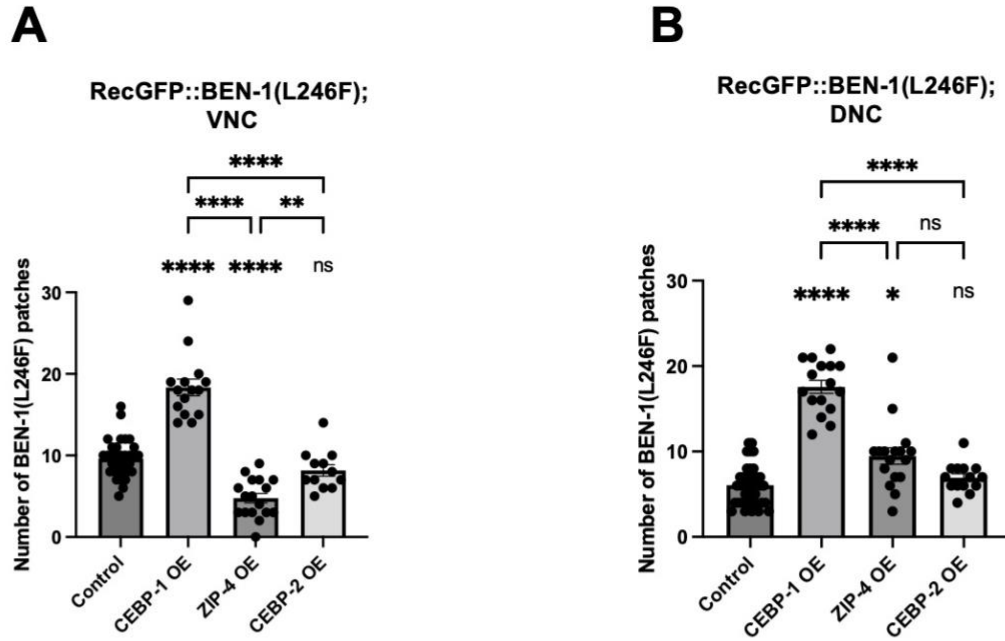


Figure 2.4. CEBP-1 overexpression (OE) significantly increases the formation of the BEN-1(L246F) patches across neurons, but ZIP-4 OE and CEBP-2 OE do not. (A-B) BEN-1(L246F) patch quantification of day 2 adults with endogenous expression (Control) and overexpression (OE) of CEBP-1, ZIP-4, and CEBP-2. (A) Ventral nerve cord. (B) Dorsal nerve cord. Each dot indicates the patch quantification from 1 animal. n = (A) 34; 17; 12; 15. (B) 40; 17; 14; 16. Error bar indicates mean $\pm$ SEM. Statistical analysis was performed using Welch's ANOVA with Dunnett T3 multiple comparisons test.

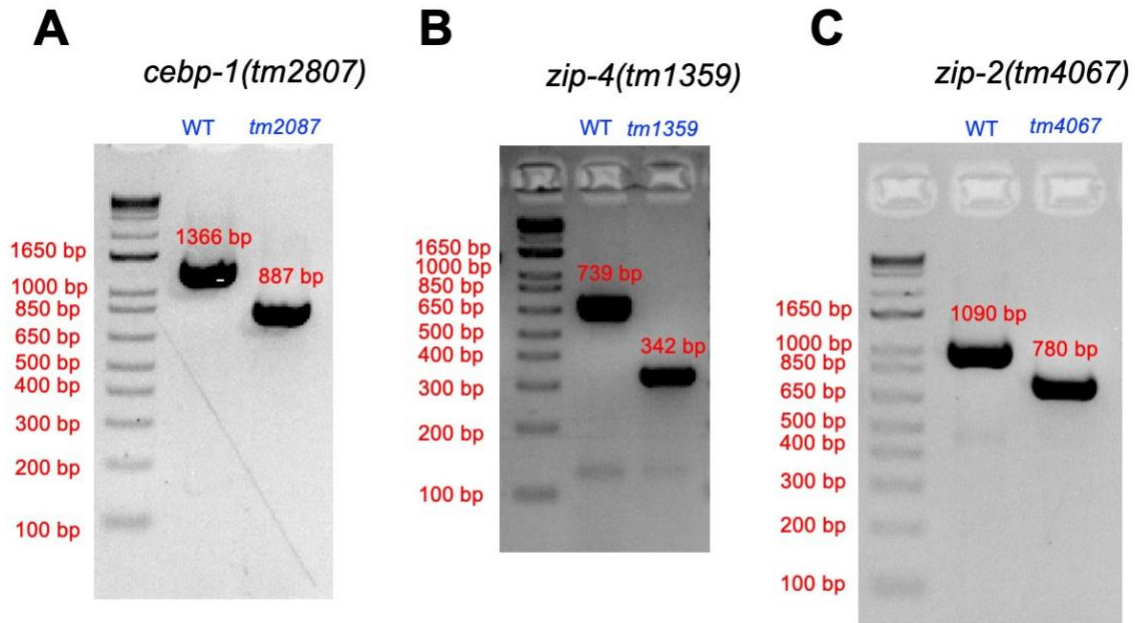


Figure 2.5. Representative gel images for the PCR genotyping of each allele. (A) Representative PCR results for WT and *ceb-1(tm2807)* run on ~3% agarose gel. (B) Representative PCR results for WT and *zip-4(tm1359)* run on ~3% agarose gel. (C) Representative PCR results for WT and *zip-2(tm4067)* run on ~3% agarose gel. Invitrogen 1 Kb Plus DNA Ladder was used for A-C. For detailed primer information and expected results, see Table 2.2. All reactions were run at 150 V for at least 20 minutes.

Tables

Table 2.1. *C. elegans* strain information

Strain number	Genotype	Construction notes
CZ11359	<i>zip-4(tm1359) I; mec-7-GFP(muIs32) II; cebp-1(tm2807) X</i>	Constructed by Dr. Dong Yan. Used to construct CZ30791. <i>muIs32</i> expresses <i>mec-7-GFP</i> in the touch receptor neurons, which can be genotyped visually under a fluorescence compound or dissecting microscope.
CZ28673	<i>[ceb-1::TY1::EGFP::3xFLAG + unc-119(+)](wgIs563)</i>	<i>wgIs563</i> was genotyped using a fluorescence compound microscope by looking at CEBP-1::GFP expression in the various tissues, such as head neuron soma and intestinal cells.
CZ29879	<i>GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Constructed by Dr. Junxiang Zhou.
CZ29894	<i>dlk-1(tm4024) I; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Constructed by Dr. Junxiang Zhou.
CZ29899	<i>GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV; cebp-1(tm2807) X</i>	Constructed by Dr. Junxiang Zhou.
CZ30701	<i>[ceb-1::TY1::EGFP::3xFLAG + unc-119(+)](wgIs563)/+; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ29894 was crossed with N2 to generate males. The male progenies have the genotype <i>dlk-1(tm4024)/+; ben-1(ju1959)/+; juIs409/+</i> . The male progenies were then crossed with CZ28673. The crossed F1 progenies were genotyped for <i>ben-1(ju1959)</i> , <i>juIs409</i> , and <i>wgIs563</i> under a fluorescence compound microscope. (<i>Prgef-1-GFP1-10(juIs409)</i> expresses <i>ttx-3::RFP</i> in the head region. <i>ben-1(ju1959)</i> shows expression of GFP11::BEN-1(L246F) in neurons when in combination with <i>juIs409</i> . <i>wgIs563</i> shows

Table 2.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		CEBP-1::GFP expression in various tissues, such as head neuron soma and intestinal cells). The F1s with the genotype <i>wgIs563/+; dlk-1(tm4024)?; ben-1(ju1959)/+; juIs409/+</i> were isolated and left to self-fertilize. The F2s that showed GFP1-11::BEN-1(L246F) and CEBP-1::GFP expression were isolated to self-reproduce. The F2s were genotyped for <i>dlk-1(tm4024)</i> , and their progenies were genotyped for <i>wgIs563</i> , <i>ben-1(ju1959)</i> , and <i>juIs409</i> under a fluorescence compound microscope. <i>wgIs563/+; ben-1(ju1959); juIs409</i> animals were found and left to self-reproduce. Their progenies (F3s) expressing <i>wgIs563</i> were isolated again and left to self-reproduce. None of the progenies was homozygous <i>wgIs563</i> . After repeating the last step twice, I concluded that <i>wgIs563; ben-1(ju1959); juIs409</i> is lethal. The lethality could be the result of background mutations from the transgenes.
CZ30712	<i>zip-2(tm4067) III</i> <i>GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ29879 was crossed with N2 to generate males. The male crossed progenies have the genotype <i>ben-1(ju1959)/+; juIs409/+</i> and were then crossed with FX04067. After genotyping the F1s from the cross for <i>ben-1(ju1959)</i> and <i>juIs409</i> , the F1s with the genotype <i>zip-2(tm4067) ben-1(ju1959)/++; juIs409/+</i> were found and left to self-reproduce. The F2 progenies that express GFP1-11::BEN-1(L246F) were isolated to self-reproduce. The F2s were then genotyped for <i>zip-2(tm4067)</i> , and their progenies were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> . CZ30712 <i>zip-2(tm4067) ben-1(ju1959); juIs409</i> was subsequently isolated.

Table 2.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
CZ30789	<i>[cebp-2::H1-wCherry + unc-119(+)](stIs11749); GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ29879 was crossed with N2 males to generate males. The crossed male progenies with the genotype <i>ben-1(ju1959)/+; juIs409/+</i> were then crossed with RW11749. <i>stIs11749/+ ben-1(ju1959)/+; juIs409/+</i> F1 hermaphrodites were isolated from the cross after genotyping <i>stIs11749</i> , <i>ben-1(ju1959)</i> , and <i>juIs409</i> under a fluorescence compound microscope. <i>stIs11749/+; ben-1(ju1959)/+; juIs409/+</i> F1 animals were left to self-reproduce, and their hermaphrodite F2 progenies that express CEBP-2::wCherry and GFP1-11::BEN-1(L246F) were isolated to self-reproduce again. After the F2 progenies self-reproduced, their progenies were genotyped for <i>stIs11749</i> , <i>ben-1(ju1959)</i> , and <i>juIs409</i> under a fluorescence compound microscope. CZ30789 <i>stIs11749; ben-1(ju1959); juIs409</i> was then isolated.
CZ30790	<i>[zip-4::TY1::EGFP::3xFLAG + unc-119(+)](wgIs590); GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ29879 was crossed with N2 to generate males. The crossed male progenies with the genotype <i>ben-1(ju1959)/+; juIs409/+</i> were then crossed with OP590. <i>wgIs590/+; ben-1(ju1959)/+; juIs409/+</i> F1 hermaphrodites were isolated from the cross after genotyping <i>wgIs590</i> , <i>ben-1(ju1959)</i> , and <i>juIs409</i> under a fluorescence compound microscope. <i>wgIs590/+; ben-1(ju1959)/+; juIs409/+</i> animals were found and left to self-reproduce, and their F2 progenies that express ZIP-4::EGFP and GFP1-11::BEN-1(L246F) were isolated to self-reproduce again. After the F2s self-reproduced, their progenies were genotyped for <i>wgIs590</i> , <i>ben-1(ju1959)</i> , and <i>juIs409</i> under a fluorescence compound microscope. CZ30790 <i>wgIs590; ben-1(ju1959); juIs409</i>

Table 2.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		was then isolated.
CZ30791	<i>zip-4(tm1359) I</i> ; <i>GFP11::BEN-1(ju1959 [L246F]) III</i> ; <i>Prgef-1-GFP1-10(juIs409) IV</i> ; <i>cebp-1(tm2807) X</i>	CZ29879 was crossed with N2 to generate males. The male crossed progenies have the genotype <i>ben-1(ju1959)/+</i> ; <i>juIs409/+</i> and were then crossed with CZ11359. The F1 hermaphrodite progenies from the cross with the genotype <i>zip-4(tm1359)/+</i> ; <i>muIs32/+</i> ; <i>ben-1(ju1959)/+</i> ; <i>juIs409/+</i> ; <i>cebp-1(tm2807)/+</i> were found after they were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> under a fluorescence compound microscope. <i>zip-4(tm1359)/+</i> ; <i>muIs32/+</i> ; <i>ben-1(ju1959)/+</i> ; <i>juIs409/+</i> ; <i>cebp-1(tm2807)/+</i> F1s were left to self-reproduce. Their F2 hermaphrodite progenies were genotyped for <i>muIs32</i> , <i>ben-1(ju1959)</i> , and <i>juIs409</i> . The F2 progenies that did not have <i>muIs32 (mec-7-GFP)</i> but have strong GFP1-11::BEN-1(L246F) expression were isolated to further self-reproduce. The F2s were then genotyped for <i>zip-4(tm1359)</i> and <i>cebp-1(tm2807)</i> via PCR, and their progenies were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> under a fluorescence compound microscope. CZ30791 <i>zip-4(tm1359)</i> ; <i>ben-1(ju1959)</i> ; <i>juIs409</i> ; <i>cebp-1(tm2807)</i> was subsequently isolated.
CZ30792	<i>zip-2(tm4067) III</i> ; <i>GFP11::BEN-1(ju1959 [L246F]) III</i> ; <i>Prgef-1-GFP1-10(juIs409) IV</i> ; <i>cebp-1(tm2807) X</i>	CZ29899 was crossed with N2 males to generate males. The crossed male progenies with the genotype <i>ben-1(ju1959)/+</i> ; <i>juIs409/+</i> ; <i>cebp-1(tm2807)/0</i> were then crossed with CZ30712. F1 hermaphrodite progenies from the cross with the genotype <i>zip-2(tm4067) ben-1(ju1959)/+ +?</i> ; <i>juIs409/+?</i> ; <i>cebp-1(tm2807)/+</i> were isolated. Once the F1 hermaphrodite progenies self-reproduced, their F2 hermaphrodite progenies were

Table 2.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> . The F2 worms that show strong GFP1-11::BEN-1(L246F) were isolated to further self-reproduce. The F2s were then genotyped for <i>zip-2(tm4067)</i> and <i>cebp-1(tm2807)</i> via PCR, and their progenies were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> under a fluorescence compound microscope. F2s with the genotype <i>zip-2(tm4067) ben-1(ju1959)/+ ben-1(ju1959) ; juIs409; cebp-1(tm2807)</i> were found and left to self-reproduce again. The F3s were isolated to self-reproduce. After the F3s self-reproduced, the F3s were genotyped for <i>zip-2(tm4067)</i> . CZ30792 <i>zip-2(tm4067) ben-1(ju1959); juIs409; cebp-1(tm2807)</i> was subsequently isolated.
CZ30870	<i>zip-4(tm1359) I; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ30791 was crossed with N2 males. The F1 hermaphrodite progenies from the cross with the genotype <i>zip-4(tm1359)/+; ben-1(ju1959)/+; juIs409/+; cebp-1(tm2807)/+</i> were isolated. After the F1 hermaphrodite progenies self-reproduced, the F2 progenies with strong GFP1-11::BEN-1(L246F) expression were isolated to further self-reproduce (the isolated F2 progenies have the genotype <i>zip-4(tm1359)?; ben-1(ju1959)/+?; juIs409/+?; cebp-1(tm2807)?</i>). Once the <i>zip-4(tm1359)?; ben-1(ju1959)/+?; juIs409/+?; cebp-1(tm2807)?</i> F2 worms self-reproduced, they were genotyped for <i>zip-4(tm1359)</i> and <i>cebp-1(tm2807)</i> via PCR and their progenies were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> under a fluorescence compound microscope. CZ30870 <i>zip-4(tm1359); ben-1(ju1959); juIs409</i> was subsequently isolated.
FX04067	<i>zip-2(tm4067) III</i>	From Dr. Shohei Mitani's lab.

Table 2.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
RW11749	<i>[cebp-2::H1-wCherry + unc-119(+)](stIs11749); unc-119(tm4063) III</i>	<i>stIs11749</i> expresses <i>cebp-2::H1-wCherry</i> which is directly visible under a fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).
OP234	<i>[jun-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)](wgIs234); unc-119(ed3) III</i>	<i>wgIs234</i> expresses <i>jun-1::TY1::EGFP</i> which is directly visible under a fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).
OP304	<i>[fos-1::TY1::EGFP::3xFLAG(92C12) + unc-119(+)](wgIs304); unc-119(ed3) III</i>	<i>wgIs304</i> expresses <i>fos-1::TY1::EGFP</i> which is directly visible under a fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).
OP432	<i>[zip-2::TY1::EGFP::3xFLAG + unc-119(+)](wgIs432) II; unc-119(tm4063) III</i>	<i>wgIs432</i> expresses <i>zip-2::TY1::EGFP</i> which is directly visible under a fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).
OP433	<i>[hlh-30::TY1::EGFP::3xFLAG + unc-119(+)](wgIs433); unc-119(tm4063) III</i>	<i>wgIs433</i> expresses <i>hlh-30::TY1::EGFP</i> which is directly visible under a fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).
OP590	<i>[zip-4::TY1::EGFP::3xFLAG + unc-119(+)](wgIs590);</i>	<i>wgIs590</i> expresses <i>zip-4::TY1::EGFP</i> which is directly visible under a

Table 2.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
	<i>unc-119(tm4063) III</i>	fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).
OP794	<i>[daf-19::TY1::EGFP::3xFLAG + unc-119(+)](wgIs794); unc-119(tm4063) III</i>	<i>wgIs794</i> expresses <i>daf-19::TY1::EGFP</i> which is directly visible under a fluorescence compound microscope and, thus, can be genotyped visually. Received through the Caenorhabditis Genetics Center (CGC).

Table 2.2. Allele information and genotyping primers

Allele	Gene	Mutation/s	Primer number/name	Sequence	Expected results
<i>ju1959</i>	<i>ben-1</i>	gfp11 with ELGSGSGSGS GSSAG linker inserted at the N-terminus of <i>ben-1</i> + SNPs in <i>ben-1</i> : A1853G, A1856T, C1860T	SD21166 (for <i>gfp11</i> insert)	CTCATATAAC TCAAAAAGA ACTTGG	WT: 200 bp <i>ju1959</i>: 293 bp
			SD21167 (for <i>gfp11</i> insert)	CACCATTAGC CTCATTATAG TAGAC	
			YJ12495 (for L246F)	GATGAGACTT TCTGCATTGA CAACG	With MluCI digestion. WT: 366 bp + 140 bp <i>ju1959</i>: 321 bp + 140 bp + 45 bp
			YJ12496 (for L246F)	CACGTGGTGG AATATCACAG ACC	
<i>tm2807</i>	<i>cebp-1</i>	479 bp deletion	YJ5442 Or <u>SD20256</u>	CTGATAACCT TCTGGGTGTG TCC Or <u>CTGATAACCT</u> <u>TCTGGGTGTG</u> <u>T</u>	WT: 1366 bp <i>tm2807</i>: 887 bp
			YJ5443	GCCGTGGAAT AAATATGAGG	
<i>tm1359</i>	<i>zip-4</i>	397 bp deletion	SD21223	GTGATCGGGA ACGAAATCCT G	WT: 739 bp. <i>tm1359</i>: 342 bp
			SD21224	CTACTGGCAC CTCACTTTTCG	
<i>tm4067</i>	<i>zip-2</i>	311bp deletion and 1 bp insertion	SD21086	GTTCAGCTAT CGGCTCCACT	WT: 1090 bp. <i>tm4067</i>: 780 bp
			SD21087	CGCTTTACAA ACATCATGAC C	

Chapter 3: DLK-1 is required at the late larval stage to promote the MT patch formation

Background

In the *ben-1(L246F)* mutants, DLK-1 is required for the formation of the MT patches, and DLK-1 is present since the early larval stage. However, the MT patches only start to form in the *ben-1(L246F)* mutants from the late larval stage (L4) to the adult stage. This raises questions about the age-dependent requirement of DLK-1 for the MT patch formation. Is DLK-1 only required at the L4 stage where the patches start to form? Or, is DLK-1 needed throughout the larval stages in order for the patches to form at the late larval stage to the adult stage? During my master's thesis research, I aimed to understand the age-dependent requirement of DLK-1 for the MT patch formation. I utilized a heat-shock-promoter-driven *dlk-1* gene construct (*Phsp-16.2-GFP::DLK-1*) to modulate the expression timing and levels of DLK-1 in *dlk-1(0)*; reconstitutedGFP::BEN-1(L246F) background. I found that acute expression of DLK-1 at the late larval stage, but not at the early larval stage, is able to induce MT patch formation in adult animals.

Materials and Methods

C. elegans strains and genetics

All worms were maintained on nematode growth media (NGM) plates seeded with the *E. coli* strain OP50 (Brenner, 1974). Strains were maintained at 20°C unless otherwise specified. The Bristol (N2) strain was used as the wild type. Strains used in this section are described in Table 3.1. The genotyping methods for the different alleles are described in Table 3.2. Alleles were either genotyped using standard PCR or using a fluorescence compound microscope (see Tables 3.1 and 3.2 for details). Figure 3.3 shows representative gel images of the PCR genotyping results.

MT patch quantification

A MT patch is a fiber-like structure enriched with reconstitutedGFP::BEN-1. A MT patch has an obviously higher reconstitutedGFP::BEN-1 intensity than its surroundings and is at least 1 μm in length. The number of patches on the ventral nerve cord (before the tail soma) and dorsal nerve cord were counted manually using a Zeiss Axio Imager M2 fluorescence compound microscope with a 63x objective oil immersion lens. The worms were then immobilized using 10 mM levamisole (in M9 buffer) on a 4% agar gel pad for the quantification.

Acute expression of DLK-1 induced by heat shock

juSi277 (Phsp-16.2-GFP::DLK-1) was generated by Dr. Kentaro Noma through *mos1*-mediated single copy insertion (MosSCI). *Phsp-16.2-GFP::DLK-1* was inserted into chromosome III (insertion genetic location: III: -0.85). After testing different heat-shock conditions, I found that a 35°C temperature upshift for 1 hour is sufficient to induce a high

expression level of *Phsp-16.2-GFP::DLK-1* within 1 hour after the heat shock without inducing obvious physiological defects in the animals (Figure 3.1). After removing the animals from the 35°C temperature upshift, the expression of *Phsp-16.2-GFP::DLK-1* peaks around 2 hours after the heat shock and starts to gradually decrease thereafter.

Additionally, I tested to if the downstream signaling of DLK-1 is activated after *Phsp-16.2-GFP::DLK-1* expression. I constructed CZ30714 to examine the response of CEBP-1::EGFP to *Phsp-16.2-GFP::DLK-1*. I picked >10 L4 animals from CZ30714, CZ30886, and CZ28894 (CZ30886 and CZ28894 are controls) each and transferred them to new NGM plates (in total, 3 plates; 1 for each strain). The animals were previously kept at 20°C. New NGM plates used were not pre-warmed (at room temperature at the time of use; ~22.5°C). I then transferred the three plates to a 35°C incubator and heat-shocked CZ30714, CZ30886, and CZ28894 for 1 hour. The plates were then transferred to a 25°C incubator. 1 hour and 4 hours after the 35°C heat shock, the levels of CEBP-1::EGFP were observed using a Zeiss Axio Imager M2 fluorescence compound microscope with a 63x oil immersion lens. The worms were immobilized using 10 mM levamisole (in M9 buffer) on a 10% agarose gel pad for the visual analysis of CEBP-1::EGFP. From my preliminary observation, CEBP-1::EGFP levels were not obviously increased at 1 hour after the heat shock in CZ30714. However, at 4 hours after the heat shock, CEBP-1::GFP levels significantly increased in the cell bodies of a wide range of cells including many neurons in CZ30714. The observations together suggest that 1 hour of 35°C heat shock is sufficient to induce *Phsp-16.2-GFP::DLK-1* expression within 1 hour and activate the DLK-1 pathway to activate CEBP-1 within 4 hours.

Thus, for the heat-shock experiment described in this chapter, *Phsp-16.2-GFP::DLK-1* expression was induced by 1 hour of 35°C heat shock in the *dlk-1(0); juSi277*

reconstitutedGFP::BEN-1(L246F) strain (CZ29803) at the L1 or L4 stage. CZ29879 and CZ29894 were always included as the control strains and were treated in the same heat-shock conditions as the CZ29803 (experimental group). None of the NGM plates used were pre-warmed (at room temperature at the time of use; ~22.5°C).

For heat shock at the L1 stage, 10 young adult animals (previously kept at 20°C) for CZ29803, CZ29879, and CZ29894 each were transferred to new NGM plates and left at 20°C to lay eggs for 2 hours (in total, 3 plates; 1 for each stain). After 2 hours, the 10 young adult animals from each plate were removed. The eggs were left to hatch at 20°C until the L1 stage (approximately 18 hours for CZ29803, CZ29879, and CZ29894). The plates containing the L1 animals were then transferred to a 35°C incubator and subjected to 1 hour of 35°C heat shock. After the 1-hour heat shock, the plates were transferred to a 25°C incubator until the animals reached the L4 stage (approximately 36 hours for CZ29879 and CZ29894; approximately 46 hours for CZ29803; CZ29803 grew significantly slower after the heat shock). The L4 animals from the plates were then picked and transferred to new NGM plates and still maintained at 25°C for another 48 hours. After 48 hours at 25°C, the animals reached the day 2 adult stage and were observed for MT patch quantification. The MT patch quantification for CZ29803 was conducted separately from CZ29879 and CZ29894 due to the growth rate difference after heat shock.

For heat shock at the L4 stage, >5 L4 animals (previously kept at 20°C) from CZ29803, CZ29879, and CZ29894 each were directly picked and transferred to new NGM plates (in total, 3 plates; 1 for each genotype). The plates containing the L4 animals were transferred to a 35°C incubator and subjected to 1 hour of 35°C heat shock. After the 1-hour heat shock, the plates were directly transferred to a 25°C incubator for 48 hours. After 48 hours at 25°C, the animals reached the day 2 adult stage and were observed for MT patch quantification.

Confocal imaging

Confocal images (showing *Phsp-16.2-GFP::DLK-1* expression) were taken through a Zeiss LSM800 confocal microscope with a 63x (oil immersion) objective lens. The images were taken 1 hour after the animals (CZ23716) were removed from 1 hour of 35°C heat shock (the animals were heat shocked at the L4 stage). The no heat-shock control consisted of L4 animals (CZ23716) without the heat-shock treatment. The worms were immobilized using 10 mM levamisole (in M9 buffer) on 10% agarose gel pads for at least 5 minutes before imaging. 488 nm laser was used, and snapshots were taken at approximately the same focal plane for all worms.

Statistical analysis

Statistical analysis was performed in GraphPad Prism 9. The statistical tests were specified in the figure captions. ns: $P > 0.05$; *: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; ****: $P \leq 0.0001$

Results

Using the optimized heat shock condition (1 hour at 35°C), I induced acute expression of DLK-1 in the *dlk-1(0)*; reconstitutedGFP::BEN-1(L246F) background at either the early larval stage (L1; normally no MT patch) or the late larval stage (L4; MT patches start to form) and analyzed the MT patch formation in day 2 adult animals (Figure 3.2 A and D). While acute expression of DLK-1 at the L1 stage was not sufficient to rescue the formation of MT patches in day 2 adult animals, acute expression of DLK-1 at the L4 stage resulted in partial rescue of the MT patch formation (Figure 3.2). The results suggest that DLK-1 is required at at least the late larval stage for the MT patch formation in adult animals.

Discussion

MT turnover rate may determine the age-specific formation of MT patches

The dynamic nature of microtubules plays a key role in neuronal development and functions. During different life stages of the animals, neuronal microtubule dynamics differs to enable the different stage-specific functions. For instance, after neurogenesis, microtubules in a specific neurite stabilized to determine the axon and neuron polarity (Witte et al., 2008). Subsequently, dynamic microtubules are required for growth cone progression needed for axon growth (Tanaka et al., 1995). During the motor neuron remodeling of young *C. elegans* larvae, microtubule dynamics is increased to enable trafficking of synaptic vesicles from the ventral to the dorsal nerve cord (Kurup & Jin, 2015). All of these neuronal activities occur in younger animals and need fast shuffling of the MT networks. A recent study in *Drosophila melanogaster* has shown that the tubulins within neuronal MTs are turned over significantly slower in aged animals compared to their younger counterparts (Okenve-Ramos et al., 2024).

In my research project, I found that acute expression of DLK-1 at the L4 but not the L1 stage is sufficient to rescue the MT patch formation in day 2 adults. Why would the acute activation of this pathway in younger animals fail to rescue the MT patch formation in adults? My hypothesis is that due to the constant growth of neurons in younger animals, microtubules undergo dynamic changes and replenishment that prohibit the accumulation of BEN-1(L246F)/TBA-2 heterodimers on MTs. As the worms reach adulthood, however, the sizes of the worms and neurons become mostly fixed. As the replenishment of microtubules also likely decreases, it favors the accumulation of the BEN-1(L246F)/TBA-2 heterodimers and, thus, the formation of the MT patches. Therefore, even though expression of DLK-1 at the L1 stage could induce the expression of TBA-2 to a level needed for the MT patch formation, the fast shuffling

of microtubules in young, growing neurons likely prohibits the accumulation of the BEN-1(L246F)/TBA-2 heterodimers to form the MT patches. Once the animals reach adulthood (~100 hours after the heat shock at the L1 stage), heat shock-induced DLK-1 is already diminished, and the animals can no longer form the MT patches. While acute expression of DLK-1 at the L4 stage also facilitates the formation of the BEN-1(L246F)/TBA-2 heterodimers by upregulating TBA-2, the MT turnover rate likely decreases with age, so the BEN-1(L246F)/TBA-2 heterodimers can accumulate in the microtubules and form the MT patches within 48 hours.

To further confirm my hypothesis, I should examine the levels of TBA-2 after heat shock-induced DLK-1 to verify the presence and duration of the upregulation. It would be also immensely interesting to test if decreasing the turnover rate of MTs through Taxol could promote the MT patch formation in the younger *ben-1(L246F)* mutants. This future study could further verify if the fast turnover rate of MTs indeed prohibits the accumulation of the BEN-1(L246F)/TBA-2 heterodimers and the MT patch formation in younger animals.

The DLK-1 pathway regulating MT patch formation is neuroprotective

According to previous findings by Dr. Junxiang Zhou, synapse formation, synaptic vesicle transport, and neuron morphology of the *ben-1(L246F)* mutants were not significantly different from the *ben-1(wt)* control. *dlk-1(0)*, *ben-1(0)*, and *tba-2(0)* alone also did not show any defects in the aforementioned neuronal parameters. However, removing *dlk-1* in the *ben-1(L246F)* mutants not only abolishes the formation of the MT patches but also disrupts synapse formation, synaptic vesicle transport, and neuron morphology in the *ben-1(L246F)* mutants. Removing *tba-2* in the *ben-1(L246F)* mutants also resulted in neuron morphology defects. These results suggest that *dlk-1* and *tba-2*, both involved in the pathway regulating the MT patch

formation in the *ben-1(L246F)* mutants, protect neurons from the genetic perturbation of MTs caused by the L246F mutation in *ben-1*.

Altogether, my findings further contributed to our understanding of the endogenous neuroprotective mechanisms in response to microtubule perturbation. Since tubulins and DLK-1 are both conserved between *C. elegans* and humans, my works further shed light on how MAPK signaling can protect neuron health and potentially be therapeutically targeted in neurodegenerative diseases that arise from microtubule perturbation.

Figures

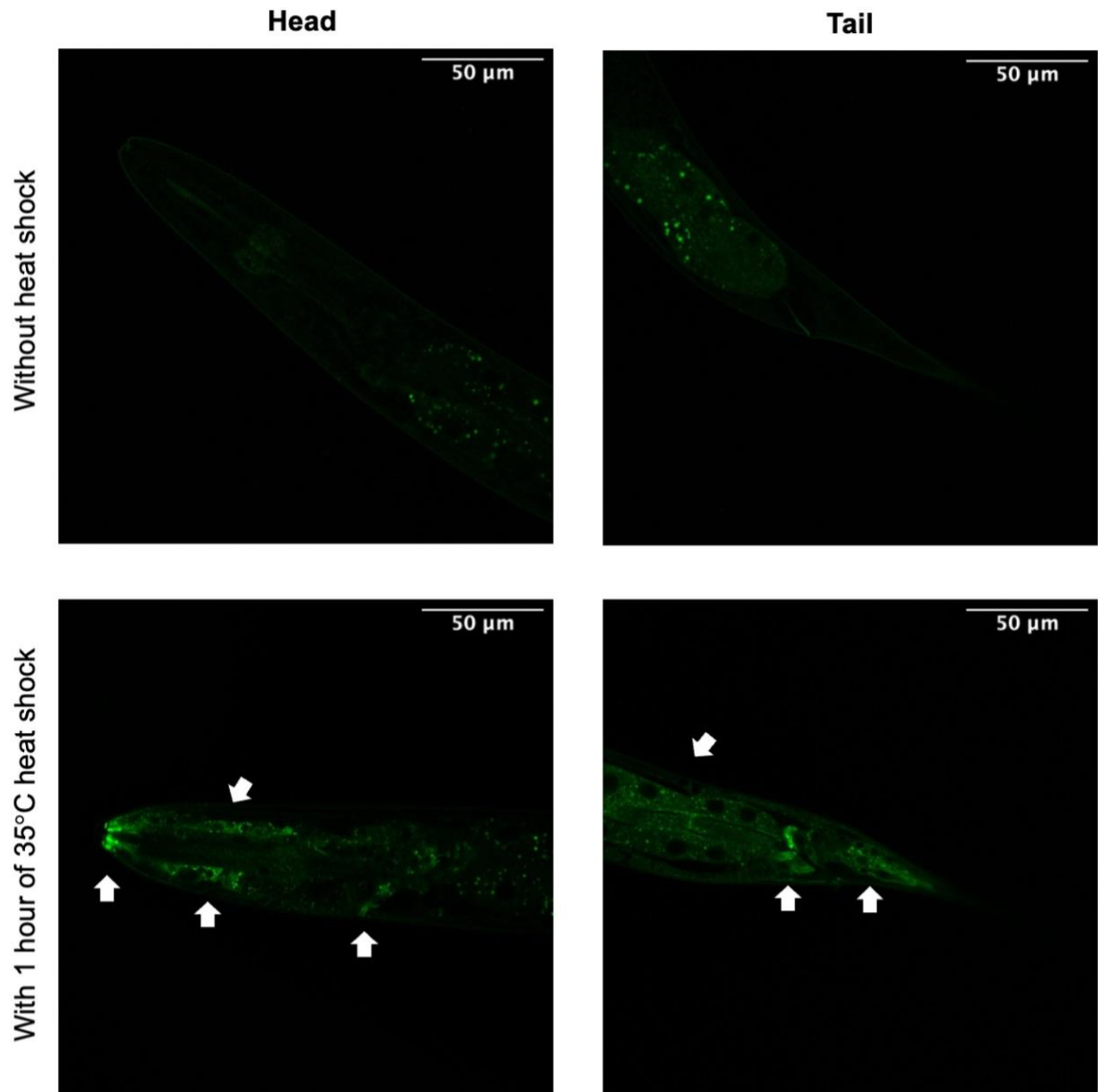


Figure 3.1. 1 hour of 35°C heat shock is sufficient to induce a high level of *Phsp-16.2-GFP::DLK-1* expression in 1 hour. Confocal images of *juSi277 (Phsp-16.2-GFP::DLK-1)* at the L4 stage with or without a 1 hour of 35°C heat shock. The images were taken 1 hour after the heat-shock group was removed from the 35°C heat shock. The head and tail regions of the worm are shown. White arrows indicate cells with high *Phsp-16.2-GFP::DLK-1* expression.

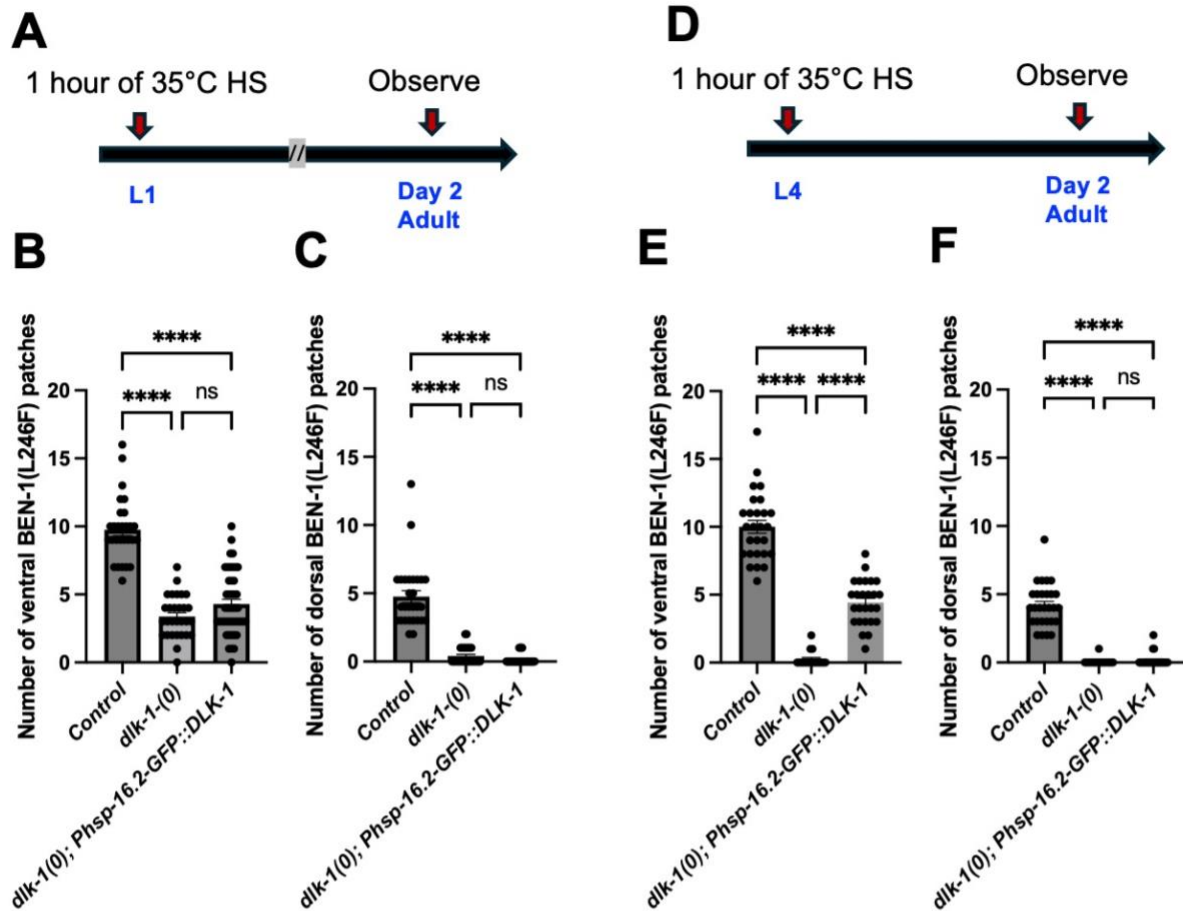


Figure 3.2. Acute expression of DLK-1 at the L4 stage is sufficient to rescue BEN-1(L246F) patch formation. (A) Timeline illustration showing the experimental setup for B and C. (B) Ventral nerve cord BEN-1(L246F) patch quantification of day 2 adults after 1 hour of 35°C heat shock at the L1 stage. (C) Dorsal nerve cord BEN-1(L246F) patch quantification of day 2 adults after 1 hour of 35°C heat shock at the L1 stage. (D) Timeline illustration showing the experimental setup for E and F. (E) Ventral nerve cord BEN-1(L246F) patch quantification of day 2 adults after 1 hour of 35°C heat shock at the L4 stage. (F) Dorsal nerve cord BEN-1(L246F) patch quantification of day 2 adults after 1 hour of 35°C heat shock at the L4 stage. Each dot indicates the patch quantification from 1 animal. $n =$ (B) 28; 29; 45. (C) 28; 29; 43. (E) 27; 17; 26. (F) 28; 17; 25. Error bar indicates mean $\pm$ SEM. Statistical analysis was performed using Welch's ANOVA with Dunnett T3 multiple comparisons test.

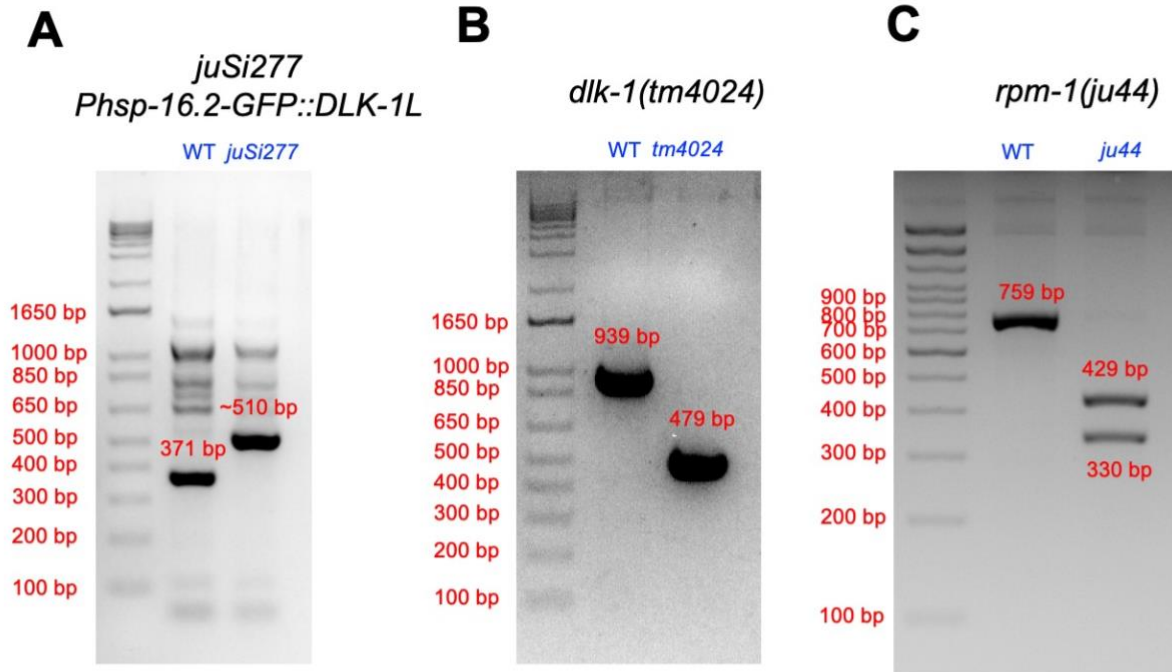


Figure 3.3. Representative gel images for the PCR genotyping of each allele. (A) Representative PCR results for WT and *juSi277* run on ~3% agarose gel. (B) Representative PCR results for WT and *dlk-1(tm4024)* run on ~3% agarose gel. (C) Representative PCR results for WT and *rpm-1(ju44)* run on ~3% agarose gel. Invitrogen 1 Kb Plus DNA Ladder was used for A-B. Invitrogen 100 bp DNA Ladder was used for C. For detailed primer information and expected results, see Table 3.2. All reactions were run at 150 V for at least 20 minutes.

Tables

Table 3.1. *C. elegans* strain information

Strain number	Genotype	Construction notes
CZ23716	<i>Phsp-16.2-GFP::DLK-1L(juSi277) III unc-119(ed3) III</i>	Generated by Dr. Kentaro Noma through <i>mos1</i> -mediated single copy insertion (MosSCI). <i>Phsp-16.2-GFP::DLK-1</i> was inserted into chromosome III (insertion genetic location: III:-0.85). Used to generate CZ29803.
CZ28894	<i>rpm-1(ju44ts) V; cebp-1::TY1::EGFP::3xFLAG(st12290) X</i>	Constructed by Dr. Yue Sun. Used to construct CZ30714 and CZ30886. CEBP-1::EGFP is directly visible under a fluorescence compound microscope and can be genotyped visually. <i>ju44</i> is genotyped via PCR (Table 3.2).
CZ29803	<i>dlk-1(tm4024) I; GFP11::BEN-1(ju1959 [L246F]) III Phsp-16.2-GFP::DLK-1L(juSi277) III; Prgef-1-GFP1-10(juIs409) IV</i>	CZ23716 was crossed with N2 males to generate males that were then crossed with CZ29894. The F1 hermaphrodite progenies from the cross were isolated to self-reproduce. The F1 hermaphrodites were then genotyped for <i>juSi277</i> via PCR. F1 worms with the genotype <i>dlk-1(tm4024)/+; ben-1(ju1959) juSi277/+ +; juIs409/+</i> were found and left to self-reproduce. The F2 worms with strong GFP1-11::BEN-1(L246F) expression were isolated (examined using a fluorescence compound microscope). The F2 worms were left to self-reproduce again. Once they produced progenies, the F2 worms were genotyped for <i>dlk-1(tm4024)</i> and <i>juSi277</i> via PCR. Once F2s with homozygous <i>dlk-1(tm4024)</i> and <i>juSi277</i> were found, their progenies were genotyped for <i>ben-1(ju1959)</i> and <i>juIs409</i> . CZ29803 <i>dlk-1(tm4024); ben-1(ju1959) juSi277; juIs409</i> was subsequently isolated.

Table 3.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
CZ29879	<i>GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Constructed by Dr. Junxiang Zhou.
CZ29894	<i>dlk-1(tm4024) I; GFP11::BEN-1(ju1959 [L246F]) III; Prgef-1-GFP1-10(juIs409) IV</i>	Constructed by Dr. Junxiang Zhou
CZ30714	<i>Phsp-16.2-GFP::DLK-1L(juSi277) III; cebp-1::TY1::EGFP::3xFLAG(st12290) X</i>	CZ28894 was crossed with N2 to generate males. The males with the genotype <i>rpm-1(ju44)/+; cebp-1(st12290)/0</i> were crossed with CZ23716. The F1 crossed progenies with CEBP-1::EGFP expression were isolated to self-reproduce (CEBP-1::EGFP can be genotyped using a fluorescence compound microscope). The isolated F1s have the genotype <i>juSi277/+; rpm-1(ju44)?/+; cebp-1(st12290)/+</i> . The F2 progenies with strong CEBP-1::EGFP expression were isolated to self-reproduce. The F2 progenies were genotyped for <i>juSi277</i> and <i>rpm-1(ju44)</i> via PCR. The progenies of the F2s were genotyped for <i>cebp-1(st12290)</i> . CZ30714 <i>juSi277; cebp-1(st12290)</i> was subsequently isolated.
CZ30886	<i>cebp-1::TY1::EGFP::3xFLAG(st12290) X</i>	CZ28894 was crossed with N2 to generate males. The males with the genotype <i>rpm-1(ju44)/+; cebp-1(st12290)/0</i> were crossed with CZ23716. The F1 progenies from the cross with CEBP-1::EGFP expression were isolated to self-reproduce. The isolated F1s have the genotype <i>juSi277/+; rpm-1(ju44)?/+; cebp-1(st12290)/+</i> . The F2 progenies with strong CEBP-1::EGFP expression were isolated to self-reproduce. The F2 progenies were genotyped for <i>juSi277</i> and <i>rpm-1(ju44)</i> . The progenies of the

Table 3.1. *C. elegans* strain information (continued)

Strain number	Genotype	Construction notes
		F2s were genotyped for <i>cebp-1(st12290)</i> . CZ30886 <i>cebp-1(st12290)</i> was subsequently isolated.

Table 3.2. Allele information and genotyping primers

Allele	Gene	Mutation/s	Primer number/name	Sequence	Expected results
<i>ju1959</i>	<i>ben-1</i>	gfp11 with ELGSGSGSGS GSSAG linker inserted at the N-terminus of <i>ben-1</i> + SNPs in <i>ben-1</i> : A1853G, A1856T, C1860T	SD21166 (for <i>gfp11</i> insert)	CTCATATAAC TTCAAAAAGA ACTTGG	WT: 200 bp <i>ju1959</i>: 293 bp
			SD21167 (for <i>gfp11</i> insert)	CACCATTAGC CTCATTATAG TAGAC	
			YJ12495 (for L246F)	GATGAGACTT TCTGCATTGA CAACG	With MluCI digestion. WT: 366 bp + 140 bp <i>ju1959</i>: 321 bp + 140 bp + 45 bp
			YJ12496 (for L246F)	CACGTGGTGG AATATCACAG ACC	
<i>tm4024</i>	<i>dlk-1</i>	460 bp deletion	SD20370	CCTAAGCTTG GTTGTAATGA ACTTC	WT: 939 bp <i>tm4024</i>: 479 bp
			SD20371	CCTCATTCGG GTATCCACGT AGC	
<i>ju44</i>	<i>rpm-1</i>	A SNP in <i>rpm-1</i> : G13930A	YJ2949	GGAAAGAAGA TGGAATGGTC	WT: 759 bp <i>ju44</i>: 429 + 330 bp
			YJ9183	CAAATTCCAC AACCCATTGC	
<i>juSi277</i>	<i>Phsp-16.2-GFP::DLK-1L</i>	Single-copy insertion of <i>Phsp-16.2-GFP::DLK-1L</i>	YJ10134 (SD20840)	AGGCCCAGCA ATTTCATCGC C	WT: 371 bp <i>juSi277</i>: ~510 bp
			oCF1513 (SD20841)	CAATTTTCAGG GGCGAAAGTA	
			oCF1514 (SD20842)	GCGTTCCGTA AATTGGAAGA	

References

- Arribere, J. A., Bell, R. T., Fu, B. X., Artiles, K. L., Hartman, P. S., & Fire, A. Z. (2014). Efficient marker-free recovery of custom genetic modifications with CRISPR/Cas9 in *Caenorhabditis elegans*. *Genetics*, 198(3), 837–846.
<https://doi.org/10.1534/genetics.114.169730>
- Baran, R., Castelblanco, L., Tang, G., Shapiro, I., Goncharov, A., & Jin, Y. (2010). Motor neuron synapse and axon defects in a *C. elegans* alpha-tubulin mutant. *PLoS ONE*, 5(3), e9655. <https://doi.org/10.1371/journal.pone.0009655>
- Brenner S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics*, 77(1), 71–94.
<https://doi.org/10.1093/genetics/77.1.71>
- Bounoutas, A., Kratz, J., Emtage, L., Ma, C., Nguyen, K. C., & Chalfie, M. (2011). Microtubule depolymerization in *Caenorhabditis elegans* touch receptor neurons reduces gene expression through a p38 MAPK pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 108(10), 3982–3987.
<https://doi.org/10.1073/pnas.1101360108>
- Chalfie, M., & Thomson, J. N. (1982). Structural and functional diversity in the neuronal microtubules of *Caenorhabditis elegans*. *The Journal of Cell Biology*, 93(1), 15–23.
<https://doi.org/10.1083/jcb.93.1.15>
- Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W., & Prasher, D. C. (1994). Green fluorescent protein as a marker for gene expression. *Science* (New York, N.Y.), 263(5148), 802–805.
<https://doi.org/10.1126/science.8303295>
- Desai, A., & Mitchison, T. J. (1997). Microtubule polymerization dynamics. *Annual Review of Cell and Developmental Biology*, 13, 83–117.
<https://doi.org/10.1146/annurev.cellbio.13.1.83>
- Davidse, L. C. (1986). Benzimidazole fungicides: mechanism of action and biological impact. *Annual Review of Phytopathology*, 24, 43–65.
<https://doi.org/10.1146/annurev.py.24.090186.000355>
- Driscoll, M., Dean, E., Reilly, E., Bergholz, E., & Chalfie, M. (1989). Genetic and molecular analysis of a *Caenorhabditis elegans* beta-tubulin that conveys benzimidazole sensitivity. *The Journal of Cell Biology*, 109(6 Pt 1), 2993–3003.
<https://doi.org/10.1083/jcb.109.6.2993>
- Dubey, J., Ratnakaran, N., & Koushika, S. P. (2015). Neurodegeneration and microtubule dynamics: death by a thousand cuts. *Frontiers in Cellular Neuroscience*, 9, 343.
<https://doi.org/10.3389/fncel.2015.00343>

- Erickson, H. P., & O'Brien, E. T. (1992). Microtubule dynamic instability and GTP hydrolysis. *Annual Review of Biophysics and Biomolecular Structure*, 21, 145–166. <https://doi.org/10.1146/annurev.bb.21.060192.001045>
- Estes, K. A., Dunbar, T. L., Powell, J. R., Ausubel, F. M., & Troemel, E. R. (2010). bZIP transcription factor zip-2 mediates an early response to *Pseudomonas aeruginosa* infection in *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences of the United States of America*, 107(5), 2153–2158. <https://doi.org/10.1073/pnas.0914643107>
- Fukushige, T., Siddiqui, Z. K., Chou, M., Culotti, J. G., Gogonea, C. B., Siddiqui, S. S., & Hamelin, M. (1999). MEC-12, an alpha-tubulin required for touch sensitivity in *C. elegans*. *Journal of Cell Science*, 112 (Pt 3), 395–403. <https://doi.org/10.1242/jcs.112.3.395>
- Ghosh-Roy, A., Goncharov, A., Jin, Y., & Chisholm, A. D. (2012). Kinesin-13 and tubulin posttranslational modifications regulate microtubule growth in axon regeneration. *Developmental Cell*, 23(4), 716–728. <https://doi.org/10.1016/j.devcel.2012.08.010>
- Gupta, K., Bishop, J., Peck, A., Brown, J., Wilson, L., & Panda, D. (2004). Antimitotic antifungal compound benomyl inhibits brain microtubule polymerization and dynamics and cancer cell proliferation at mitosis, by binding to a novel site in tubulin. *Biochemistry*, 43(21), 6645–6655. <https://doi.org/10.1021/bi036112v>
- Hahm, J. H., Jeong, C., Lee, W., Koo, H. J., Kim, S., Hwang, D., & Nam, H. G. (2020). A cellular surveillance and defense system that delays aging phenotypes in *C. elegans*. *Aging*, 12(9), 8202–8220. <https://doi.org/10.18632/aging.103134>
- Hattori, A., Mizuno, T., Akamatsu, M., Hisamoto, N., & Matsumoto, K. (2013). The *Caenorhabditis elegans* JNK signaling pathway activates expression of stress response genes by derepressing the Fos/HDAC repressor complex. *PLoS Genetics*, 9(2), e1003315. <https://doi.org/10.1371/journal.pgen.1003315>
- Hurd D. D. (2018). Tubulins in *C. elegans*. *WormBook*, 2018, 1–32. <https://doi.org/10.1895/wormbook.1.182.1>
- Hurd, D. D., Miller, R. M., Núñez, L., & Portman, D. S. (2010). Specific alpha- and beta-tubulin isoforms optimize the functions of sensory cilia in *Caenorhabditis elegans*. *Genetics*, 185(3), 883–896. <https://doi.org/10.1534/genetics.110.116996>
- Janke, C., & Magiera, M. M. (2020). The tubulin code and its role in controlling microtubule properties and functions. *Nature Reviews. Molecular Cell Biology*, 21(6), 307–326. <https://doi.org/10.1038/s41580-020-0214-3>

- Jumper, J., Evans, R., Pritzel, A., Green, T., Figurnov, M., Ronneberger, O., Tunyasuvunakool, K., Bates, R., Židek, A., Potapenko, A., Bridgland, A., Meyer, C., Kohl, S. A. A., Ballard, A. J., Cowie, A., Romera-Paredes, B., Nikolov, S., Jain, R., Adler, J., Back, T., Petersen, S., Reiman, D., Clancy, E., Zielinski, M., Steinegger, M., Pacholska, M., Berghammer, T., Bodenstein, S., Silver, D., Vinyals, O., Senior, A. W., Kavukcuoglu, K., Kohli, P., Hassabis, D. (2021). Highly accurate protein structure prediction with AlphaFold. *Nature*, 596(7873), 583–589. <https://doi.org/10.1038/s41586-021-03819-2>
- Kim, K. W., Thakur, N., Piggott, C. A., Omi, S., Polanowska, J., Jin, Y., & Pujol, N. (2016). Coordinated inhibition of C/EBP by Tribbles in multiple tissues is essential for *Caenorhabditis elegans* development. *BMC Biology*, 14(1), 104. <https://doi.org/10.1186/s12915-016-0320-z>
- Kurup, N., & Jin, Y. (2015). Neural circuit rewiring: insights from DD synapse remodeling. *Worm*, 5(1), e1129486. <https://doi.org/10.1080/21624054.2015.1129486>
- Lowery, L. A., & Van Vactor, D. (2009). The trip of the tip: understanding the growth cone machinery. *Nature reviews. Molecular Cell Biology*, 10(5), 332–343. <https://doi.org/10.1038/nrm2679>
- Lu, C., & Mains, P. E. (2005). Mutations of a redundant alpha-tubulin gene affect *Caenorhabditis elegans* early embryonic cleavage via MEI-1/katanin-dependent and -independent pathways. *Genetics*, 170(1), 115–126. <https://doi.org/10.1534/genetics.104.030106>
- Moore, J., Luduena, R., & Tuszynski, J. A. (2023). Editorial: The isotypes of α , β and γ tubulin: From evolutionary origins to roles in metazoan development and ligand binding differences. *Frontiers in Cell and Developmental Biology*, 11, 1176739. <https://doi.org/10.3389/fcell.2023.1176739>
- Mirdita, M., Schütze, K., Moriwaki, Y., Heo, L., Ovchinnikov, S., & Steinegger, M. (2022). ColabFold: making protein folding accessible to all. *Nature Methods*, 19(6), 679–682. <https://doi.org/10.1038/s41592-022-01488-1>
- Nishida, K., Tsuchiya, K., Obinata, H., Onodera, S., Honda, Y., Lai, Y. C., Haruta, N., & Sugimoto, A. (2021). Expression Patterns and Levels of All Tubulin Isotypes Analyzed in GFP Knock-In *C. elegans* Strains. *Cell Structure and Function*, 46(1), 51–64. <https://doi.org/10.1247/csf.21022>
- Mitchison, T., & Kirschner, M. (1984). Dynamic instability of microtubule growth. *Nature*, 312(5991), 237–242. <https://doi.org/10.1038/312237a0>
- Nakata, K., Abrams, B., Grill, B., Goncharov, A., Huang, X., Chisholm, A. D., & Jin, Y. (2005). Regulation of a DLK-1 and p38 MAP kinase pathway by the ubiquitin ligase RPM-1 is required for presynaptic development. *Cell*, 120(3), 407–420. <https://doi.org/10.1016/j.cell.2004.12.017>

- Nogales, E., Whittaker, M., Milligan, R. A., & Downing, K. H. (1999). High-resolution model of the microtubule. *Cell*, 96(1), 79–88. [https://doi.org/10.1016/s0092-8674\(00\)80961-7](https://doi.org/10.1016/s0092-8674(00)80961-7)
- Nogales E. (2000). Structural insights into microtubule function. *Annual Review of Biochemistry*, 69, 277–302. <https://doi.org/10.1146/annurev.biochem.69.1.277>
- Notredame, C., Higgins, D. G., & Heringa, J. (2000). T-Coffee: A novel method for fast and accurate multiple sequence alignment. *Journal of Molecular Biology*, 302(1), 205–217. <https://doi.org/10.1006/jmbi.2000.4042>
- Wang, H. W., & Nogales, E. (2005). Nucleotide-dependent bending flexibility of tubulin regulates microtubule assembly. *Nature*, 435(7044), 911–915. <https://doi.org/10.1038/nature03606>
- Williams, S. C., Cantwell, C. A., & Johnson, P. F. (1991). A family of C/EBP-related proteins capable of forming covalently linked leucine zipper dimers in vitro. *Genes & Development*, 5(9), 1553–1567. <https://doi.org/10.1101/gad.5.9.1553>
- Witte, H., Neukirchen, D., & Bradke, F. (2008). Microtubule stabilization specifies initial neuronal polarization. *The Journal of Cell Biology*, 180(3), 619–632. <https://doi.org/10.1083/jcb.200707042>
- O'Hagan, R., Avrutis, A., & Ramicevic, E. (2022). Functions of the tubulin code in the *C. elegans* nervous system. *Molecular and Cellular Neurosciences*, 123, 103790. <https://doi.org/10.1016/j.mcn.2022.103790>
- Okenve-Ramos, P., Gosling, R., Chojnowska-Monga, M., Gupta, K., Shields, S., Alhadyian, H., Collie, C., Gregory, E., & Sanchez-Soriano, N. (2024). Neuronal ageing is promoted by the decay of the microtubule cytoskeleton. *PLoS Biology*, 22(3), e3002504. <https://doi.org/10.1371/journal.pbio.3002504>
- Phillips, J. B., Lyczak, R., Ellis, G. C., & Bowerman, B. (2004). Roles for two partially redundant alpha-tubulins during mitosis in early *Caenorhabditis elegans* embryos. *Cell Motility and the Cytoskeleton*, 58(2), 112–126. <https://doi.org/10.1002/cm.20003>
- Ramji, D. P., & Foka, P. (2002). CCAAT/enhancer-binding proteins: structure, function and regulation. *The Biochemical Journal*, 365(Pt 3), 561–575. <https://doi.org/10.1042/BJ20020508>
- Shivers, R. P., Pagano, D. J., Kooistra, T., Richardson, C. E., Reddy, K. C., Whitney, J. K., Kamanzi, O., Matsumoto, K., Hisamoto, N., & Kim, D. H. (2010). Phosphorylation of the conserved transcription factor ATF-7 by PMK-1 p38 MAPK regulates innate immunity in *Caenorhabditis elegans*. *PLoS Genetics*, 6(4), e1000892. <https://doi.org/10.1371/journal.pgen.1000892>

- Sigrist, C. J., de Castro, E., Cerutti, L., CuChe, B. A., Hulo, N., Bridge, A., Bougueleret, L., & Xenarios, I. (2013). New and continuing developments at PROSITE. *Nucleic Acids Research*, 41(Database issue), D344–D347. <https://doi.org/10.1093/nar/gks1067>
- Silva, M., Morsci, N., Nguyen, K. C. Q., Rizvi, A., Rongo, C., Hall, D. H., & Barr, M. M. (2017). Cell-Specific α -Tubulin IsoType Regulates Ciliary Microtubule Ultrastructure, Intraflagellar Transport, and Extracellular Vesicle Biology. *Current Biology*, 27(7), 968–980. <https://doi.org/10.1016/j.cub.2017.02.039>
- Tanaka, E., Ho, T., & Kirschner, M. W. (1995). The role of microtubule dynamics in growth cone motility and axonal growth. *The Journal of Cell Biology*, 128(1-2), 139–155. <https://doi.org/10.1083/jcb.128.1.139>
- Ti, S. C., Alushin, G. M., & Kapoor, T. M. (2018). Human β -Tubulin IsoTypes Can Regulate Microtubule Protofilament Number and Stability. *Developmental Cell*, 47(2), 175–190.e5. <https://doi.org/10.1016/j.devcel.2018.08.014>
- Tjahjono, E., & Kirienko, N. V. (2017). A conserved mitochondrial surveillance pathway is required for defense against *Pseudomonas aeruginosa*. *PLoS Genetics*, 13(6), e1006876. <https://doi.org/10.1371/journal.pgen.1006876>
- The UniProt Consortium (2015). UniProt: a hub for protein information. *Nucleic Acids Research*, 43(Database issue), D204–D212. <https://doi.org/10.1093/nar/gku989>
- Yan, D., Wu, Z., Chisholm, A. D., & Jin, Y. (2009). The DLK-1 kinase promotes mRNA stability and local translation in *C. elegans* synapses and axon regeneration. *Cell*, 138(5), 1005–1018. <https://doi.org/10.1016/j.cell.2009.06.023>
- Zhen, M., Huang, X., Bamber, B., & Jin, Y. (2000). Regulation of presynaptic terminal organization by *C. elegans* RPM-1, a putative guanine nucleotide exchanger with a RING-H2 finger domain. *Neuron*, 26(2), 331–343. [https://doi.org/10.1016/s0896-6273\(00\)81167-8](https://doi.org/10.1016/s0896-6273(00)81167-8)