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TRANSCRIPTIONAL COORDINATION OF NEURONAL IDENTITY AND SYNAPSE ORGANIZATION IN *C. ELEGANS* MOTOR NEURONS

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ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AChR	Acetylcholine receptor
AD	Alzheimer's disease
AID	Auxin inducible degron
ATAC-seq	Assay for transposase-accessible chromatin followed with sequencing
ChAT	Choline acetyltransferase
ChIP-seq	Chromatin immunoprecipitation with sequencing
COE	Collier/Olf/Ebf
DNC	Dorsal nerve cord
EtOH	Ethanol
FI	Fluorescent intensity
$GABA_AR$	Type-A GABA receptors
GAD	Glutamic acid decarboxylase
GABA	Gamma-aminobutyric acid

GAT	GABA re-uptake transporter
GFP	Green fluorescent protein
Glu	Glutamate
L-AChR	Levamisole-sensitive acetylcholine receptor
L1	Larval stage 1
L4	Larval stage 4
LAMP	Lysosomal associated membrane protein
MN	Motor neurons
mNG	mNeonGreen
N-AChR	Nicotine-sensitive acetylcholine receptor
NT	Neurotransmitter
PD	Parkinson's disease
Prom	Promoter
RFP	Red fluorescent protein
RNA-seq	RNA sequencing
RNAi	RNA interreference

smRNA FISH	Single-molecule RNA fluorescence in situ hybridization
VAChT	Vesicular acetylcholine transporter
VGAT	Vesicular GABA transporter
VNC	Ventral nerve cord
YA	Young adult

ACKNOWLEDGMENTS

It has been almost seven years since I embarked on this journey. I left Puerto Rico as a naïve scientist, a naïve individual even. However, nothing feels better than reflecting on the fact that this journey has been filled with growth, and for that, I am grateful. Amidst the growth, there were moments of frustration and joy, but most importantly, there were many people who were always there, and that is what I treasure the most. Whether I have been able to convey the extent of my gratitude to them, I conclude this journey with the following words, hoping they now grasp how grateful I am.

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ABSTRACT

The study of synapse development has uncovered multiple factors that are implicated in neurotransmitter biosynthesis and neurotransmitter receptor clustering. However, the molecular mechanisms that control these processes, in a presynaptic and a postsynaptic cell, remain elusive. Leveraging the *C. elegans* neuromuscular system, this thesis unveils the role of UNC-30 (PITX), the homeodomain terminal selector of GABAergic motor neuron identity, in postsynaptic organization. We show that loss of *unc-30* (PITX) or the short isoform of the synapse-organizing molecule *madd-4B* (*Punctin/ADAMTSL*) results in severe GABA receptor clustering defects in postsynaptic muscle cells. Mechanistically, we identify UNC-30 as a direct regulator of *madd-4B*. In addition, we uncover a repertoire of novel targets regulated by UNC-30, revealing its dual role as both an activator and repressor of gene expression in GABAergic motor neurons. Specifically, UNC-30 represses genes normally expressed in other neuron types. We also uncover that UNC-30 is continuously required, from embryonic development through adulthood, to maintain *madd-4B* and GABA biosynthesis gene (e.g., unc-25/GAD, unc-47/VGAT) expression. These findings support the concept of coordinated presynaptic and postsynaptic differentiation, ensuring effective neurotransmission. Altogether, this work uncovers a transcriptional co-regulatory strategy essential for synapse functionality, demonstrating how neuronal identity and synapse organization are coordinated. The findings are expected to resonate with the developmental biology community and the broader field of gene regulation, offering insights into fundamental questions regarding transcriptional control of cell identity and function. Beyond its developmental biology contributions, this

study holds biomedical relevance, as UNC-30/PITX mutations have been associated with diseases such as the Axenfeld-Rieger syndrome.

CHAPTER 1

INTRODUCTION

1.1 Prelude

Neuronal development is a sophisticated and dynamic process that spans from the early stages of embryogenesis to the formation of mature neural circuits. Initiated during gastrulation, neural induction gives rise to the neural plate. As development progresses, the neural plate eventually folds and undergoes arrangement to form the precursor of the central nervous system, known as the neural tube. Throughout this process, neural stem cells generate neurons (i.e., neurogenesis). This event is followed by their migration to their final destinations within the brain. Once in place, neurons extend axons, guided by molecular cues, to establish connections with target cells, ultimately forming synapses. This intricate connectivity (i.e., wiring) enables communication between neurons, ultimately shaping the complex neural circuits crucial for sensory perception, motor function, and cognitive processes, laying the foundation for the functionality of the nervous system.

1.2 Establishing functional synapses: a multistep process

Synapses are specialized junctions that serve as the fundamental interface for communication in the nervous system. They function as signaling hubs, facilitating the

transmission of information between connected neurons, and contributing to the formation of the neural circuits that drive behavior (i.e., locomotion and movement).

Establishing synapses is not a singular event but rather the result of an intricate multistep process. These steps include neurogenesis, neuron migration, axon guidance, dendrite arborization, and neuron-to-neuron recognition (i.e., selecting synaptic partners), ultimately leading to the precise juxtaposition of presynaptic and postsynaptic elements (i.e., synaptic specificity). It is the effectiveness and strength of the transmitted signal, which occurs between the presynaptic and postsynaptic elements, that defines functional synapses. In essence, this effectiveness is a measure of how well the signal is diffused from the transmitting (i.e., presynaptic) neuron to the receiving (i.e., postsynaptic) neuron, while the strength refers to the magnitude or intensity of the signal that influences the activity of the postsynaptic neuron.

Overall, the establishment of functional synapses is an essential landmark during neuron development, which highlights the functionality of the nervous system. Understanding the mechanisms that drive this process remains an important question in the field of neurobiology. In this thesis, I focus on characterizing the molecular mechanisms that coordinate the establishment and maintenance of presynaptic and postsynaptic elements, thus controlling synapse functionality. Insights from these studies offer valuable perspectives into the pathological mechanisms underlying neurological disorders that arise from irregular synaptic function and development.

1.3 Synapse assembly and refinement drive synaptic specificity

Synaptic specificity is defined as the process by which presynaptic and postsynaptic cells selectively choose each other as synaptic partners from a range of neighboring cells. Several anatomical studies indicate that neurons showcase this unique ability. For instance, through electron microscopy, studies reveal retinal ganglion cells select only four cells out of 43 cells as their synaptic partner.[1,2] Further, a study examining the *C.* elegans nervous system shows that one out of six neurons synapse with each other.[2,3] This phenomenon raises the question of how such precise synaptic specificity is generated during development, a topic I will discuss below. Moreover, this thesis aims to shed light on the molecular mechanisms underlying this process, specifically, the establishment of functional synapses.

1.3.1. Establishing synaptic partners: an overview of axon guidance

The initial stages of synapse assembly involve a series of events during development. These include neurons selecting their synaptic partners, a process driven by spatiotemporal cues arising from diverse cellular and molecular mechanisms.

First, there are positive regulators that play a critical role in guiding neurons to select their synaptic partners (i.e., axon guidance). They are present on the membranes of presynaptic and postsynaptic neurons to promote attraction between them (**Figure 1.1a**). Specifically, this process occurs through the ability of neurons to express specific sets of adhesion molecules or secreted anterograde and retrograde signals. A model that

demonstrates positive regulators through adhesion molecules are the cadherins and immunoglobulin-superfamily (IgSF) of proteins.[2,4,5] This superfamily of proteins can generate a diverse range of isoforms. Given that each IgSF isoform engages exclusively in homophilic interactions, this diversity is believed to form the basis for a molecular code governing neuron-neuron interaction.[2,6,7] For instance, retinal ganglion cells expressing the same IgSF isoform select each other as synaptic partners. Studies have shown that alterations in the IgSF molecular code within these cells result in the selection of incorrect synaptic partners. This model strongly indicates that synaptic partners possess specific molecular mechanisms that actively promote neuron-to-neuron synapse assembly.

Next, inhibitory regulators also participate in axon guidance. They can be presented by inappropriate synaptic partners or appear in the form of morphogenic gradients released by other cells. As their name implies, inhibitory regulators prevent synapse assembly by repelling axons (i.e., presynapse) seeking their postsynaptic targets (**Figure 1.1b**). An example of this mechanism arises from studies of synapse assembly in the fruit fly. In *Drosophila*, different presynaptic neurons select particular postsynaptic cells (M12 and M13) as their synaptic partner.[8] M13, unlike M12, expresses the inhibitory regulator Wnt4. Upon WNT4 depletion in M13, presynaptic neurons normally repelled (i.e., normally only select M12 as their postsynaptic partner) now also select M13 as their postsynaptic partner. Another instance of inhibitory regulators in synapse assembly comes from studies of morphogenic gradients in the nematode. In the nematode *C. elegans*, LIN-44/WNT is at a low level ventrally and at a high level dorsally, repelling axons of a particular neuron DA9.[9] The loss of LIN-44 results in abnormal synapses of

DA9. This model strongly indicates that specific synaptic partners possess molecular mechanisms that actively inhibit synapse assembly with inappropriate synaptic partners.

Beyond the initial stages of synapse assembly (i.e., establishing synaptic partners), there are essential processes that play a crucial role in shaping synaptic refinement. These processes include synapse elimination and remodeling (to be discussed below), and additional cellular processes emerging from cell-cell interactions (e.g., secreted anterograde and retrograde signals). Furthermore, thesis will focus on uncovering the molecular mechanisms governing secreted anterograde signals that influence synapse assembly in *C. elegans* synapses.

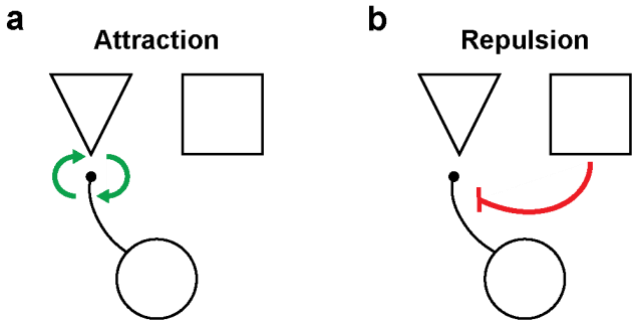

Figure 1.1: Axon guidance mechanisms dictating synaptic specificity.
(a) Synaptic partners attract through positive regulators (green).
(b) Selective repulsion through negative regulators (red) originated from inappropriate synaptic partners.

1.3.2 Synaptic elimination and remodeling during development

During synapse assembly, synapses refine or rewire their connections to achieve such precise synaptic specificity. In the early stages of nervous system development, there is an abundance of synapses between neurons. However, not all these synapses (i.e., connections) are necessary for efficient neuron-neuron communication or proper nervous system development. As the nervous system matures, synapse elimination comes into play, selectively removing weaker synapses (**Figure 1.2a**).[10–12] For instance, before mice open their eyes, each lateral geniculate nucleus neuron synapses with multiple (+20) retinal ganglion neurons.[13] However, two weeks later, only one to three synapses remain. Overall, this process highlights how crucial synapse elimination is to promote synaptic specificity thereby ensuring efficient neuron-neuron communication.

Aside from synapse elimination, synaptic refinement can also be acquired through the remodeling of synapses (**Figure 1.2b**). This process involves alterations in synapses, often influenced by various factors such as neural activity, environmental stimuli, or developmental changes. For example, studies using electron microscopy show that the DD neurons in *C. elegans* remodel their synapses during larval development.[3] In newly born larvae, DD neurons connect to ventral muscles and receive input (i.e., connections) from DA and DB neurons. However, by the end of the first larval stage (L1), DD neurons remodel their connections – they connect to dorsal muscles and receive input from newly born VA and VB neurons, while now DA and DB neurons relocate output to newly born VD neurons.

For DD neurons to undergo synaptic remodeling, they need to eliminate their connection to ventral muscles and redirect synapses toward the dorsal muscle. These events are facilitated by CYY-1/CCNY, a cyclin box-containing protein, and CDK-5/CDK5, a cyclin-dependent kinase, respectively.[14] CDK5 also regulates synapse remodeling in *Drosophila*.[15] This highlights the idea that conserved mechanisms may govern synaptic specificity throughout development by controlling synaptic assembly across species.

Synaptic remodeling occurs at particular time points throughout development. For example, studies reveal that IRX-1/IRX, an Iroquois homeodomain protein, facilitates synapse remodeling, and its depletion leads to delayed remodeling.[16] The timing of DD synaptic remodeling is subject to the control of a transcriptional program. This same study revealed that UNC-30/PITX, a homeodomain transcription factor, regulates the expression of IRX-1, thereby promoting DD synaptic remodeling. Here, I aim to highlight the extent of UNC-30's function in controlling synapse assembly. In Chapter 2, I will unravel the emerging roles of UNC-30 in establishing functional synapses by regulating secreted anterograde signals influencing synapse assembly in *C. elegans*.

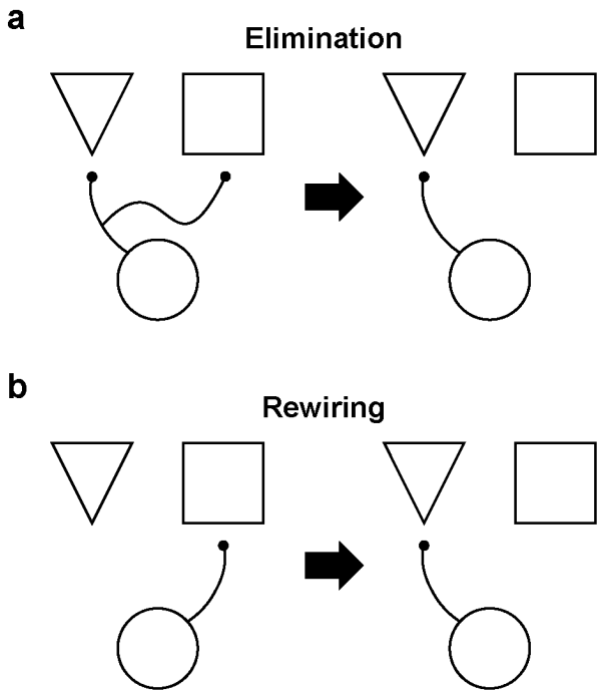

Figure 1.2: Mechanisms altering synapses for precise synaptic specificity. (a-b) Elimination or rewiring of synapses with inappropriate targets results in synaptic specificity.

1.4 The role of neurotransmitters and neurotransmitter receptors in the nervous system

For the nervous system to function properly, it heavily relies on efficient communication occurring at synapses between neurons. This fundamental process depends on the ability of neurons to synthesize, package, and release, specific neurotransmitters. Neurotransmitters are chemical messengers secreted by neurons that facilitate the transmission of impulses from one neuron to another, thereby, enabling effector organs to execute precise functions. Depending on the neurotransmitter secreted by neurons, these chemical messengers are categorized into various systems, such as cholinergic,

glutamatergic, and GABAergic. To receive and recognize these chemical messengers, the postsynaptic side must present and properly cluster a specific neurotransmitter receptor corresponding to the messenger secreted by the presynaptic side. The thesis aims to elucidate the molecular mechanisms regulating neurotransmitter synthesis, packaging, and release, and the clustering of neurotransmitter receptors, all of which are critical in synapses for efficient neuron communication.

1.4.1 Excitatory and inhibitory synapses at neuromuscular junctions

Neuron-neuron synapses are crucial for the function of the nervous system, yet equally vital are the neuromuscular synapses (i.e., neuromuscular junctions), which distinctly involve connections between motor neurons and their synaptic partners: muscle cells. These neuromuscular junctions comprise three primary components: (1) the presynaptic motor neuron, (2) the synaptic gap, and (3) the postsynaptic muscle cell. At the presynaptic side, motor neurons synthesize the neurotransmitters and subsequently load them into synaptic vesicles using vesicular transporters unique to each neurotransmitter. These synaptic vesicles then dock at the membrane and fuse, releasing neurotransmitters into the synaptic gap. The neurotransmitters diffuse and interact with their corresponding neurotransmitter receptors, facilitating neurotransmission from motor neurons to muscle cells. Altogether, this process triggers a cascade of events that lead to muscle contraction or relaxation, thereby generating a behavioral response like movement or locomotion.

To contract or relax muscles, neuromuscular junctions employ different neurotransmitters across various organisms. For instance, vertebrate neuromuscular junctions predominantly rely on acetylcholine, while in *Drosophila*, glutamate is the main neurotransmitter — both acetylcholine and glutamate act as excitatory neurotransmitters, causing muscle contraction.[17–20] Conversely, in *C. elegans*, there are two distinctive neuromuscular junction types (**Figure 1.3**).[21,22] At excitatory neuromuscular junctions, muscle contraction is triggered by cholinergic motor neurons expressing acetylcholine biosynthesis genes, encoding proteins involved in acetylcholine synthesis, packaging, and release (e.g., CHA-1/ChAT: choline acetyltransferase, UNC-17/VAChT: vesicular acetylcholine transporter) (**Figure 1.3**). However, at inhibitory neuromuscular junctions, muscle relaxation is induced by GABAergic motor neurons expressing γ-aminobutyric acid (GABA) biosynthesis genes, encoding proteins involved in GABA synthesis, packaging, and release (e.g., UNC-25/GAD: glutamic acid decarboxylase, UNC-47/VGAT: vesicular GABA transporter) (**Figure 1.3**). In contrast to *Drosophila*, where a single muscle cell can be innervated (i.e., connected) by two or more distinct motor neurons, in *C. elegans*, a muscle cell receives input from both cholinergic and GABAergic motor neurons. It is the balance between excitatory and inhibitory neurotransmission at these distinct neuromuscular junctions that modulates muscle activity in *C. elegans*, enabling precise motor control and sinusoidal locomotion[21].

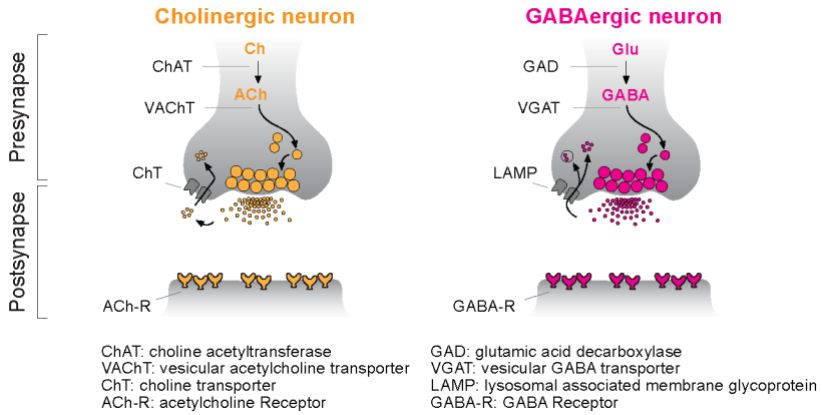

Figure 1.3: Schematic of excitatory and inhibitory synapses.
A detailed description is provided in this section (Excitatory and inhibitory synapses at neuromuscular junctions).

In parallel with the variety of neurotransmitters used at neuromuscular junctions, the efficiency of neuronal communication (i.e., neurotransmission) not only relies on neurotransmitter release but also on the precise organization of neurotransmitter receptors at the postsynaptic side (**Figure 3**). In muscle cells, these receptors are packed at high densities in neuromuscular synapses, forming clusters. However, a fundamental question arises: how do these clusters assemble precisely opposite the presynaptic motor neurons? Studies show that in many instances, neurotransmitters are initially evenly distributed throughout the muscle.[23] As innervation from motor neurons occurs (i.e. synapse formation), these neurotransmitters cluster opposite the presynaptic side.[24–27] This phenomenon, known as prepatterning, is akin to the chicken-and-egg problem. For instance, in mouse aneural muscles, acetylcholine receptors are initially evenly distributed at embryonic day 12.5. By embryonic day 13.5, the presynaptic motor neuron overlaps some primitive acetylcholine receptor clusters. Then, by embryonic day 18.5, the

innervated primitive acetylcholine receptor clusters become densely packed, while those outside of the innervated muscle (i.e., extrasynaptic area) disperse and disappear.[28–30] These findings suggest an active role of the postsynaptic muscle in neuromuscular junction formation, whereas the enlargement of some primitive neurotransmitter receptor clusters is also induced by the presynaptic motor neuron (discussed below).

Neurotransmitter receptors (e.g., acetylcholine, glutamate, GABA) can be further characterized by their structure and function. For instance, in *C. elegans* excitatory (i.e., cholinergic) neuromuscular junctions, there are two types of ionotropic acetylcholine neurotransmitter receptors: heteromeric levamisole-sensitive acetylcholine receptors (L-AChRs) and homomeric nicotine-sensitive acetylcholine receptors (N-AChRs).[31,32] Upon activation, L-AChRs trigger a cascade of events that lead to muscle contraction, whereas the physiological role of N-AChRs is not fully understood.[33,34] These receptors can be further characterized by their structural subunits: L-AChRs comprised of three α subunits (LEV-8, UNC-38, and UNC-63) and two non-α subunits (LEV-1 and UNC-29).[35–37] In inhibitory (i.e., GABAergic) neuromuscular junctions, type-A GABA receptors ($GABA_AR$s/ UNC-49) induce muscle relaxation.[31] They are composed of three different subunits (A, B, and C) produced via alternative splicing.[32] However, analyses of $GABA_AR$s neuromuscular synapses suggest their composition predominantly consists of UNC-49 B/C heteromers.

In *C. elegans,* presynaptic innervation promotes UNC-49 $GABA_AR$ clustering. This process relies on specific molecular cues presented by motor neurons, such as the synaptic organizers like neurexin and punctin.[31] These synaptic organizers (discussed in the

following section) play a pivotal role in establishing functional neuromuscular synapses. In this thesis, I uncover molecular mechanisms involved in regulating synapse-organizing molecules in GABAergic neuromuscular junctions. Furthermore, I expand on the ability of presynaptic GABAergic motor neurons to promote proper UNC-49 $GABA_AR$ clustering.

1.5 Molecular mechanisms of synapse assembly

After initial synapse assembly events (e.g., selecting synaptic partners), both the presynaptic and postsynaptic sides undergo dynamic structural changes to ensure efficient cell-cell communication. This process, known as synaptic differentiation, is primarily driven by synapse-organizing molecules (i.e., synaptic organizers). For instance, synaptic organizers situated at nearby opposing presynaptic and postsynaptic sides play a crucial role in promoting the proper clustering of neurotransmitter receptors. This thesis will specifically focus on uncovering the molecular mechanisms that regulate these synaptic organizers, which ultimately control the establishment of functional synapses. In the following sections, I will discuss our current understanding of the pivotal role played by synapse-organizing molecules in the development of both excitatory and inhibitory synapses.

1.5.1 Defining synaptic organizers

Synaptic organizers are complex molecules that coordinate the intricate processes governing nervous system development at the level of individual synapses. They play a

crucial role in differentiating synapses by facilitating the recruitment of presynaptic and postsynaptic elements, thus determining synapse type, location, and plasticity in a cell type-specific manner. The dynamic range of cell type-specific synapse organization arises from the range of synaptic organizer (e.g., neurexins, neuroligins, and punctin) and their specific synapse-organizing properties.[23,31] Studies indicate that when the function of synaptic organizing molecules is impaired, it leads to compromised and deficient synaptic functionality.[38–40] These deficiencies have been linked to major nervous system disorders and behavioral features of neurodevelopmental and neuropsychiatric diseases.[41–44] For example, the synaptic organizer MADD-4/PUNCTIN is essential for the proper clustering of neurotransmitter receptors in *C. elegans*.[31,38,40] Thus, when MADD-4/PUNCTIN is depleted, electrophysiological analysis shows a decrease in signaling (i.e., communication) between the presynaptic and postsynaptic sides in cholinergic synapses.[40] In the second chapter of my thesis, I will unravel the molecular mechanisms that regulate *madd-4*/Punctin in *C. elegans* GABAergic synapses. Below, I will delve deeper into the molecular mechanisms underlying the distinct categories of synaptic organizers.

1.5.2 Categories of synaptic organizers

Cell-cell interaction is pivotal in shaping synaptic specificity and facilitating synapse differentiation.[23] The close juxtaposition of presynaptic and postsynaptic sides enables synaptic organizers to coordinate the recruitment of specific presynaptic and postsynaptic

elements, ensuring functional synapses. However, these mechanisms are mediated through distinct molecular processes.

First, synaptic organizers can interact bidirectionally to orchestrate presynaptic and postsynaptic differentiation (**Figure 1.4a**).[2,31,45–47] Synaptic adhesion molecules, present at both sides of the synapse, play a role in organizing and structuring synapses – an example being the neurexin-neuroligin complexes. Neurexins and neuroligins are single-pass transmembrane proteins implicated in both excitatory and inhibitory synapse differentiation.[2,23,31] Neurexin, expressed at the presynaptic side, is involved in regulating presynaptic differentiation, including the neurotransmitter release machinery and synapse numbers. For example, upon depletion of neurexin in glutamatergic synapses in *Drosophila*, there is a severe reduction in the number of synapses and the detachment of the juxtaposed presynaptic and postsynaptic sides.[48] Neurexins, as well as neuroligins, have been shown to interact with intracellular and extracellular partners (e.g., calcium/calmodulin-dependent serine protein kinase, calsyntenins, neurexophilins), serving as serve as context-dependent synaptic organizers.[49–55] Notably, neurexin also interacts with neuroligins.[56,57] Neuroligins are expressed at the postsynaptic and play a crucial role in postsynaptic differentiation, including proper neurotransmitter receptor clustering.[17,45,58,59] For instance, upon neuroligin depletion in the hippocampus of mice, there is a significant reduction in GABA neurotransmitter receptor accumulation.[60] Studies investigating the loss-of-function of bidirectional synaptic organizers in mice and invertebrate organisms (e.g., *C. elegans*) emphasize their indispensable role in structuring functional synapses.

Next, synaptic organizers, also known as anterograde organizers, are secreted from the presynaptic side to promote postsynaptic differentiation (**Figure 1.4b**).[2] In vertebrate organisms, one extensively studied retrograde synaptic organizer is agrin.[61,62] Found at excitatory synapses, agrin plays an indispensable role in the proper clustering of acetylcholine receptors at the postsynaptic side in mice.[63,64] In *C. elegans*, postsynaptic differentiation of excitatory and inhibitory synapses relies on the anterograde synaptic organizer named PUNCTIN/MADD-4.[31,38,40,59] This synaptic organizer promotes proper acetylcholine and GABA neurotransmitter receptor clustering in cholinergic and GABAergic synapses through cell-type-specific isoform expression and function (discussed below). Importantly, PUNCTIN/MADD-4 has been shown to interact with neurexin and neuroligin, which contribute to synapse differentiation, ensuring synaptic specificity and functionality.

On the other hand, retrograde synaptic organizers, secreted from the postsynaptic side, promote presynaptic differentiation, including the number and structure of synapses (**Figure 1.4c**).[2] For instance, in *Drosophila* mutants with defective 'Wishful thinking', the ortholog of the human BMP type II receptor, there is a significant reduction in the number of synapses and abnormal synapse structure (e.g., the detachment of the juxtaposed presynaptic and postsynaptic sides).[65,66] In addition, electrophysiological analysis reveals a decrease in neurotransmission (i.e., communication) between the presynaptic and postsynaptic sides in these mutants.

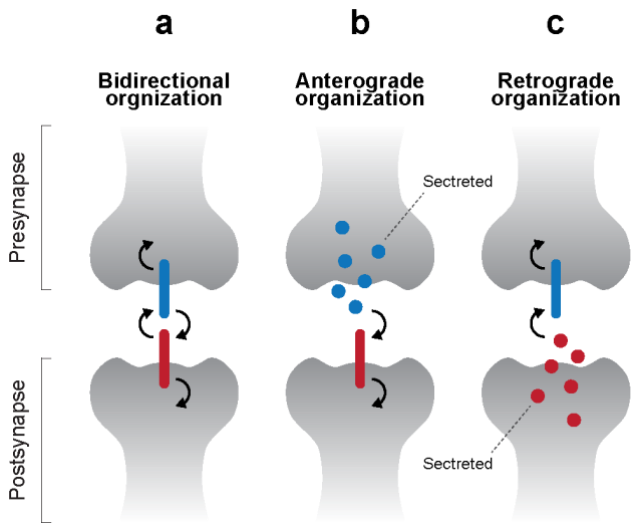

Figure 1.4: Mechanistic models of synapse organization signals.
(a-c) A detailed description is provided in this section (Categories of synaptic organizers).

Overall, the cell-cell interaction and coordination between the presynaptic side and the postsynaptic side via synaptic organizers – such as bidirectional, anterograde, retrograde, and glia-derived synaptic organizers (not discussed) – is crucial to ensure synaptic specificity (**Figure 1.4**). These synaptic organizing molecules exhibit cell-type-specific expression and function (discussed below), contributing to the diversity governing synapse organization and the establishment of functional synapses. This thesis will focus on elucidating the molecular mechanisms that control the expression of the anterograde synaptic organizer PUNCTIN/MADD-4 inhibitory (i.e., GABAergic) synapses in *C. elegans*.

1.5.3 Isoforms of synaptic organizers drive synaptic specificity

To ensure synaptic specificity and function, neurons must express a specific set of genes, effectively forming a 'molecular code' responsible for encoding synapse-organizing molecules. These molecules, each with distinct roles, play a pivotal role in establishing functional synapses. Yet, one might wonder: how is such a diverse molecular code produced, and how does it influence synaptic differentiation? A significant factor contributing to this diversity is the process of alternative splicing in genes encoding synapse-organizing molecules.

For instance, mammals express three genes encoding the synaptic organizer neurexin/NRXN, designated as NRXN1, NRXN2, and NRXN3.[42] These genes undergo alternative splicing, producing thousands of isoforms.[67] However, each gene generates two main neurexin isoforms via two independent promoters – a long isoform and a short isoform, referred to as NRXNα and NRXNβ, respectively.[68,69] While NRXN1α, NRXN2β, and NRXN3β are found to be widely expressed in the brain, NRXN1β, NRXN2α, and NRXN3α tend to exhibit high expression levels only in specific regions (e.g., thalamus, cerebellum, cerebral cortex).[47] This evidence illustrates the ability of these isoforms to be expressed in a neuron-type-specific manner. Although most neurons express multiple neurexins, each showcases a unique molecular code.

Another example is the synapse-organizing molecule MADD-4/PUNCTIN in *C. elegans*. Through alternative promoters, *madd-4* generates two isoforms – a long isoform and a short isoform, referred to as *madd-4L* and *madd-4B*, respectively.[31,38,40,59] *madd-4L* is exclusively expressed in cholinergic neurons and secreted to the synaptic gap, where

it promotes acetylcholine neurotransmitter receptor clustering at the cholinergic postsynaptic side. On the other hand, *madd-4B* is expressed in both cholinergic and GABAergic neurons. In cholinergic neurons, MADD-4B upon secretion to the synaptic gap, inhibits GABA neurotransmitter receptor clustering at the GABAergic postsynaptic side through heterodimerization with MADD-4L. However, in GABAergic neurons, secreted MADD-4B promotes GABA neurotransmitter receptor clustering at the GABAergic postsynaptic side. Genetic depletion of MADD-4B leads to improper GABA neurotransmitter receptor clustering at the postsynaptic side of GABAergic synapses. In these mutants, GABA neurotransmitter receptors relocate and cluster at the postsynaptic side of cholinergic synapses. Conversely, ectopic expression of MADD-4L in GABAergic neurons of madd-4 mutant animals leads to the clustering of both acetylcholine and GABAergic receptors at the postsynaptic side of GABAergic synapses.

Collectively, these scenarios illustrate a neuron-type-specific molecular code responsible for encoding synapse-organizing molecules and how the distinct roles of these synaptic organizers are intricately linked to the establishment of functional synapses. In the second chapter of this thesis (Chapter 2), I will uncover the molecular mechanisms that control the expression of the synaptic organizer PUNCTIN/MADD-4 – activating MADD-4B expression and repressing MADD-4L expression – in GABAergic neurons of C. elegans.

1.5.4 Implications in disease

The diversity of synapse-organizing molecules and their unique functions is a significant factor contributing to the functionality of synapses. Consequently, each of these molecules has the potential to contribute differently to diseases due to their distinct roles.

Human genetic screenings across various disorders have identified NRXN genetic variants and their isoforms as significant contributors to the pathogenesis of these disorders.[42,70,71] For instance, mutations in NRXN1 have been identified more frequently than in other NRXNs concerning neurodevelopmental disorders.[72] However, NRXN1α isoforms have been predominantly linked to schizophrenia compared to the NRXN1β isoform. Studies in mice have also shown that the contribution of NRXN2α to brain function might be "weaker" than that of other NRXN genes, as the phenotypes in Nrxn2α KO mice are milder than those of NRXN1β or other NRXNs.[73] Lastly, NRXN3α has been implicated in the pathogenesis of Alzheimer's disease.[44] Elevated levels of this specific isoform have been detected in the cerebrospinal fluid of individuals in the preclinical stage of Alzheimer's disease. This, in turn, could lead to an increase in GABAARs and affect neurotransmission (i.e., communication) at inhibitory synapses, synaptic plasticity, learning, and memory, ultimately promoting Alzheimer's disease progression.

In the second chapter of my thesis, I highlight (1) the distinct roles of the synaptic organizer PUNCTIN/MADD-4 isoforms in C. elegans motor neurons (e.g., synapse functionality) and (2) the molecular mechanisms that control the expression of these isoforms. Although the role of PUNCTIN remains unknown in humans, these scenarios

collectively illustrate how critical the diversity of synapse-organizing molecules and their unique functions contribute to the onset or progression of particular diseases.

1.6 A historical perspective of defining neurons

The journey to understand the fundamental unit of the nervous system – the neuron – has evolved significantly, interconnecting eras and scientific breakthroughs. Technological advancements within the last decade, particularly in microscopy and molecular profiling, have sparked a growing interest in defining cell types in the brain. The classification of neuron types has been a central aim in the field of neuroscience, providing a framework to understand the brain's structure and supporting both neuroscience research and the development of treatments for neurological disorders. The following section will delve into existing schemes for classifying neuron types over the years. This historical journey not only outlines the evolution of our understanding of neurons but also highlights the complexity and diversity of these cells in the function of the nervous system.

1.6.1. Features that classify neuron types: defining neuronal identity

The nervous system, composed of billions of neurons, forms the intricate framework behind behavior (i.e., thoughts, sensations, and actions). Neurons represent a diverse cell population traditionally divided into types and subtypes based on qualitative criteria:

such as cell morphology, connectivity, function, and molecular features (discussed below).[74–77]

Historically, schemes for classifying neuron cell types initially relied on morphology. For instance, Purkinje cells are a unique type of neuron found in the cerebellum of mammals like mice. They are immediately recognizable for their extensive and elaborate dendritic arborization (i.e., branching), allowing them to receive input from multiple synaptic partners.[78] Another example is the D-type neurons in *C. elegans*, recognized by their distinct morphology: a unipolar axon and a dendritic arbor that extends in a stereotyped pattern.[21] Moreover, neurons can be characterized by their connectivity. For instance, olfactory projection neurons in *Drosophila* connect sensory input from the antennal lobe to higher brain centers, facilitating the processing of olfactory information.[79] In mice, retinal ganglion cells establish connections between photoreceptors and the brain, with different ganglion cell types conveying specific visual information.[80]

While traditional schemes for classifying neurons centered on morphology and and connectivity, the understanding of neural diversity has expanded significantly by characterizing the functional properties of neurons. For instance, interneurons facilitate communication between sensory and motor neurons, enabling an organism's response to stimuli. In *C. elegans*, the LUA interneuron, located at the posterior of the nematode, triggers a behavioral response when touched in that region.[81–83] Another example is motor neurons, which facilitate muscle contraction or inhibition. As previously discussed, in organisms like *Drosophila,* glutamatergic motor neurons trigger muscle contractions.[18]

In *C. elegans,* while muscle contraction is triggered by cholinergic motor neurons, muscle relaxation is triggered by GABAergic motor neurons.[21]

However, how do the anatomy-based classification schemes match these functional properties? The cell-type-specific genetic expression (i.e., molecular code: quantitative features) of distinct neurons plays a critical role in their differentiation and consequent classification.[74] For instance, cholinergic motor neurons must express acetylcholine biosynthesis genes, encoding proteins like ChAT (choline acetyltransferase) and VAChT (vesicular acetylcholine transporter), involved in acetylcholine synthesis, packaging, and release.[84] Conversely, GABAergic motor neurons need to express γ-aminobutyric acid (GABA) biosynthesis genes, encoding proteins such as UNC-25/GAD (glutamic acid decarboxylase) and UNC-47/VGAT (vesicular GABA transporter), crucial for GABA synthesis, packaging, and release.[22]

Overall, defining neuronal identity (i.e., neuron types) has been a fundamental and historical challenge in neuroscience. Today, researchers complement anatomical classifications with detailed molecular data, revolutionizing our understanding of neuronal diversity and shedding light on the precise role neurons play in the nervous system. In this thesis, and the following section, I elucidate the role of transcriptional programs controlling the molecular features (i.e., genetic expression) that define neurons and their function (i.e., synapse functionality). In Chapter 2, I specifically focus on characterizing the role of the transcriptional program controlling the expression of GABA biosynthesis genes.

1.7 Transcriptional programs controlling neuron-type identities

The foundation of the nervous system is rooted in a diverse array of neuron types and their functions. It is critical that, throughout development, each neuron undergoes differentiation (i.e., acquires its identity) and establishes functional synapses. This process relies on the ability of neurons (i.e., presynaptic side) to express terminal identity genes - genes required for neurotransmitter biosynthesis (e.g., neuropeptides, ion channels, enzymes).[85,86] Understanding how the expression of these genes is regulated and how specific neuron types acquire their identity has been a long-standing focus in the field of neuron development.

Studies uncover transcription factors (i.e., terminal selectors) that control the expression of terminal identity genes in neurons.[87] These transcription factors, present from development through adulthood, directly bind to the cis-regulatory regions of neuron type-specific terminal identity genes and activate their transcription.[88–90] They can act in combinations, which in turn establishes neuron terminal identity and gives rise to a diverse array of neuron types. For instance, the transcription factor CHE-1 (chemotaxis-defective) is required for ASE chemosensory neuron identity in *C. elegans*.[91,92] It directly controls ASE-specific neuron terminal identity genes by binding their cis-regulatory regions through a consensus binding site. In *Drosophila,* the CHE-1 ortholog in *Drosophila* (Glass) also acts as a terminal selector in the sensory neurons of the fruit fly's retina.[93,94]

Another example is the transcription factor UNC-3 in *C. elegans* motor neurons. UNC-3, the orthologue of the COE (Collier/Olf/Ebf) transcription factor family, acts

directly to establish cholinergic motor neuron identity and indirectly to prevent the adoption of alternative motor neurons (e.g., GABAergic).[39,95–100] In *C. intestinalis*, the UNC-3 ortholog (COE) is necessary to establish cholinergic MN identity, while the ortholog in mice (EBF2), it is required for the proper differentiation of neurons in the medial columns of the developing spinal cord.[101]

Altogether, these scenarios emphasize the conserved role of terminal selectors in controlling the expression of neuron terminal identity genes across species, suggesting a transcriptional program that governs neuron terminal identity and differentiation. In the following section, I delve deeper into the roles of the transcription factor PITX in neuron differentiation, including the control of GABA terminal identity gene expression. Chapter 2 of this thesis highlights novel UNC-30/PITX transcriptional targets in *C. elegans*, solidifying this transcription factor as a terminal selector in GABAergic motor neurons.

1.7.1 The role of the transcription factor PITX in the nervous system

PITX belongs to the bicoid-related subclass of the homeodomain transcription factor family, conserved across species. Vertebrates possess three *Pitx* paralogs, *Pitx1, Pitx2,* and *Pitx3* while non-vertebrates have a single *Pitx* gene. Although PITX was initially discovered to be involved in regulating left–right asymmetry (i.e., tissue patterning), new roles continue to emerge because of advances in science and technology.[102]

For instance, based on their expression pattern, *Pitx2* and *Pitx3* have been implicated to have roles in the development of the nervous system.[103] *Pitx3* is expressed

within the developing midbrain, specifically in mesencephalic dopaminergic neurons.[104] Mice lacking *Pitx3* failed to develop mesencephalic dopaminergic neurons, highlighting its role in neuron differentiation.[103–105] On the other hand, *Pitx2* is expressed within the prosencephalon and mesencephalon, associated with GABAergic neurogenesis.[106–108] In mice, *Pitx2* seems to directly activate *Gad1*, which codes for the glutamate decarboxylase (GAD).[109] This evidence suggests a role for this transcription factor as a regulator of GABAergic differentiation during mammalian neuron development.

The PITX ortholog UNC-30 is also required for GABAergic motor neuron differentiation in the nematode *C. elegans*. UNC-30 acts as a terminal selector, as it directly controls GABA-specific neuron terminal identity genes (e.g., *unc-25*/GAD, *unc-47*/VGAT, and *unc-46*/LAMP) by binding their cis-regulatory regions through a consensus binding site.[21,98,110–115] Animals lacking UNC-30 not only display GABAergic differentiation defects (i.e., loss of GABA motor neuron terminal identity) but also axon guidance and behavioral abnormalities. Notably, *Pitx2* rescues these GABAergic differentiation defects (e.g., GABA motor neuron terminal identity, axon guidance, behavioral phenotypes).[109] Taken together, these findings point to a notable conservation in the role of these evolutionarily related genes, emphasizing the transcriptional program that directs GABAergic terminal identity and differentiation. Understanding the intricacies of this transcriptional program – and the role of PITX – becomes particularly crucial when considering its potential implications for neurological diseases and disorders (discussed below).

1.7.1.1 Implications of PITX in neurological diseases

PITX is evolutionarily conserved and has been identified in nearly all members of the animal kingdom. Animals lacking PITX exhibit phenotypic defects, such as abnormal neuron differentiation (mice and *C. elegans*).[98,103–108,110–115] In humans, these defects have been strongly correlated with and linked to the onset or progression of neurological diseases.

For example, Parkinson's disease is characterized by the loss of mesencephalic dopamine neurons, pivotal for an individual's motor-behavioral function[43]. Findings from analyzing these cells in Parkinson's patients and animal models (mice and rats) consistently support the role of *Pitx3* in mesencephalic dopamine neuron differentiation.[103,116] Thus, *Pitx3* outlines a pathway crucial for survival of neurons associated with Parkinson's disease and vital for locomotion.

On the other hand, *Pitx2* has also been associated with ocular – or optic nerve – development, as mice lacking *Pitx2* exhibit defected lens (aniridia) or loss of the extraocular muscle.[117,118] In zebrafish, *Pitx3* has a similar role – it is required for lens development and retinal cell differentiation and survival.[119] These findings, in conjunction with human genetic studies, reveal the involvement of PITX in medical conditions such as glaucoma in Axenfeld–Rieger patients (*Pitx2*) and congenital cataracts in the Chinese population (*Pitx3*).[118,120,121]

Overall, understanding the role of PITX (UNC-30) and the molecular mechanisms explored in this thesis deepens our understanding of the nervous system's development

and function, contributing to efforts aimed at advancing medical science for better disease diagnosis, treatment, and prevention.

1.7.2 Coordination of neuron identity and synapse assembly

To function, the nervous system relies on the efficient communication between neurons. To accomplish this, a neuron must synthesize, pack, and release specific neurotransmitters, ultimately enabling proper synaptic output. This process, happening in the presynaptic side, is coordinated by terminal selectors – transcription factors that regulate the expression of genes that code for the biosynthesis of a specific neurotransmitter, thus determining a neuron's identity. To receive input from the presynaptic neuron, the postsynaptic side must differentiate and present the corresponding neurotransmitter receptors. While extensive research has elucidated the role of terminal selectors in regulating neuron identity, our understanding of the factors controlling postsynaptic differentiation remains limited.

Recent studies have broadened our understanding of terminal selectors in neurons, revealing their role not only in regulating a neuron's identity (i.e., presynaptic differentiation) but also in postsynaptic differentiation (e.g., specifically in terms of neurotransmitter receptor clustering). In *C. elegans*, for example, the terminal selector UNC-3 not only controls the expression of cholinergic biosynthesis genes, determining cholinergic motor neuron identity but also regulates the expression of genes coding for synaptic organizers (e.g., *madd-4*/Punctin).[122] These organizers, synthesized in the

presynaptic neuron, are subsequently secreted and localized at the postsynaptic side to facilitate the clustering of neurotransmitter receptors, thereby exerting control over postsynaptic differentiation.[40]

The extent to which the recently uncovered characteristic of the terminal selector UNC-3 — controlling and coordinating both presynaptic and postsynaptic differentiation — applies to other selectors remains uncertain. In this thesis (Chapter 2) I present evidence supporting an expanded definition of a terminal selector by using UNC-30, the terminal selector of GABAergic motor neurons in C. elegans, as an example.

1.8 Maintaining synaptic functionality throughout life

During development, each neuron must acquire its identity for the establishment of functional synapses. These neurons are terminally differentiated and post-mitotic, thereby no longer go cell division and must maintain synapse functionality throughout the life of an organism. Therefore, it is critical to understand the molecular mechanisms involved in not only establishing functional synapses but also maintaining their function. This process hinges on the ability of neurons (i.e., presynaptic side) to continuously express terminal identity genes.[85] These terminal identity genes encode genes essential for the synthesis, packaging, and release of a specific neurotransmitter (e.g., neuropeptides, ion channels, enzymes), also known as neurotransmitter biosynthesis genes. While studies in the past have focused on understanding how functional synapses are established during development, much less is known about the molecular

mechanisms involved in maintaining terminal identity gene expression and proper neurotransmitter receptor clustering (i.e., synapse functionality) throughout the life of an organism.

Evidence has emerged indicating that a terminal selector in *C. elegans*, CHE-1/Glass, is not only crucial for establishing ASE chemosensory neuron identity but also for its continuous maintenance. Studies reveal that the transient depletion of CHE-1 protein in adult animals, even after ASE identity has been established, results in the loss of ASE identity and associated chemotactic behavior.[123] Similarly, UNC-3/COE is indispensable for both establishing and maintaining cholinergic motor neuron identity.[98] Despite its absence in the postsynaptic side (i.e., muscle), UNC-3 indirectly regulates neurotransmitter receptor clustering at the muscle by regulating the expression of genes coding for synaptic organizers (e.g., *madd-4*/Punctin), which are secreted from the presynaptic side and locate at the muscle to recruit postsynaptic elements (e.g., neurotransmitter receptors).[122] The continuous requirement for a terminal selector to maintain the expression of a synaptic organizer (e.g., *madd-4*/Punctin) is currently unknown. Additionally, it remains uncertain whether the synaptic organizer MADD-4/Punctin is continuously essential for maintaining the proper clustering of neurotransmitter receptors at the postsynaptic side. This thesis will elucidate the role of the terminal selector UNC-30/PITX in maintaining the expression of genes essential for GABA biosynthesis (i.e., GABAergic motor neuron identity) and genes encoding synaptic organizers that facilitate the appropriate clustering of receptors at the postsynaptic side.

1.9 *C. elegans* as a model to study the establishment and maintenance of functional synapses and motor neuron identity

C. elegans was initially adopted as a model system by Sydney Brenner in 1963 to study the nervous system. Since then, this 1mm-long nematode has presented several advantages as a model organism. It has a fast life cycle, and despite it being semi-transparent, it offers a robust set of genetic tools. It holds the distinction of being the first multicellular organism to have its entire genome sequenced and the first organism to have its connectome fully mapped. In the following discussion, I dive into the advantages of using *C. elegans* to study the establishment and maintenance of functional synapses and motor neuron identity.

1.9.1. Advantages of using C. elegans as a model organism

In this study, I adopt *C. elegans* as a model organism for several reasons. First, its size and life cycle provide convenient culturing conditions. *C. elegans* progresses from an egg to a fertile adult in three days at 20^{o}C, transitioning through four larval stages (L1 – L4) in between. It has two sexes: (1) self-fertilizing hermaphrodites, enabling the production of homozygous progeny, and (2) males, facilitating cross-fertilization.[21]

Second, the semi-transparency of the worm allows easy visualization of proteins tagged with fluorescent reporters in live animals. The *C. elegans* research community has established an extensive collection of transgenic reporter strains that are widely shared around the world. In addition, a diverse array of cell-specific promoters has been

characterized, allowing spatial and temporal control of transgene expression via microinjection techniques.

Third, an array of genetic tools has been well established in *C. elegans*. Powerful large-scale forward and reverse genetic screens drive genetic discoveries. CRISPR and RNAi are commonly used for genetic manipulation. State-of-the-art techniques, such as ChIP-seq, RNA-seq, ATAC-seq, and smRNA FISH, are available.

Fourth, the *C. elegans* genome has been sequenced. Moreover, single-cell RNA-seq data spanning from embryonic stages to adulthood of the nervous system have been extensively characterized.[74,124] Although compact, comprising approximately 20,000 genes, around 40% of its predicted protein products exhibit mammalian homologs.[125] In addition, progeny carrying mutations in genes that are essential for viability in mammals, are in many cases, viable – enabling research that focuses on conserved gene function. Lastly, the comprehensive mapping of its neuronal connections (motor circuit) has been well characterized[21]. Further, I delve deeper into *C. elegans* motor circuit as a model.

1.9.2 The C. elegans motor circuit

The hermaphrodite motor circuit consists of two distinct types of motor neurons: (1) cholinergic and excitatory, and (2) GABAergic and inhibitory.[21] These categories could be subdivided into six cholinergic (DA, DB, VA, VB, VC, AS) and two GABAergic (DD, VD) motor neuron subtypes that coordinate sinusoidal movement (DA, DB, VA, VB, AS, DD, VD) or egg-laying (VC) (**Figure 1.5**). Each class, delineated by in by its unique

morphology, consists of a specific number of motor neurons (DA = 9, DB = 7, VA = 12, VB = 11, VC = 6, DD = 6, VD = 13, and AS = 11). These intermingle along the ventral nerve cord, accounting for 58 motor neurons, and within its surrounding ganglia, totaling 17 motor neurons. Altogether, there are 75 motor neurons that comprise this intricate motor circuit.

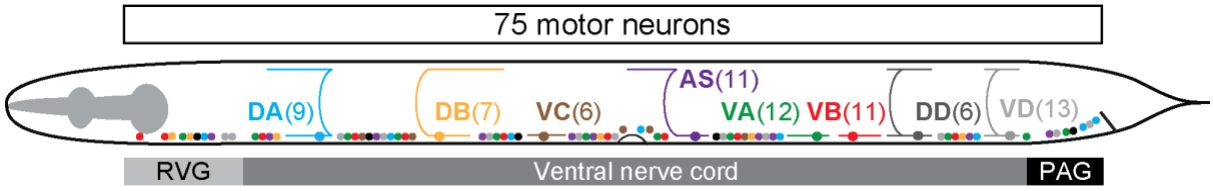

Figure 1.5: Schematic of the nematode *C. elegans*.
A detailed description is provided in this section (The *C. elegans* motor circuit).

The motor neurons in *C. elegans* are born during two developmental stages. DA, DB, and DD motor neurons originate during embryogenesis, whereas VA, VB, AS, and VD motor neurons are born at the first larval stage after embryogenesis (L1).[126,127] In adult animals, the cholinergic motor neurons form dyadic synapses, innervating both muscles and GABAergic motor neurons.[21,128] Specifically, DA, DB, and AS motor neurons provide excitatory input to both dorsal muscles and VD motor neurons, while VA, VB, and VC motor neurons provide excitatory input to both ventral muscles and DD motor neurons. Consequently, VD and DD motor neurons deliver inhibitory input to ventral and dorsal muscles, respectively. Altogether, these '*en passant*' synapses along the ventral and dorsal nerve cords, constitute a significant component of the C. elegans motor circuit.

1.9.2.1 C. elegans motor neurons as a simple yet powerful system

Throughout development, motor neurons must acquire the ability to synthesize, package, and release a specific neurotransmitter. This process involves obtaining distinct features that define these functional characteristics. Such features (e.g., neurotransmitter synthesis enzymes and packaging proteins, neuropeptides, and ion channels) must be properly established and maintained throughout life to uphold the distinct terminal identity unique to individual motor neurons. While early stages of motor neuron development have been extensively studied, the molecular mechanisms governing the terminal identity of motor neurons remain less explored. The motor neurons in the *C. elegans* ventral nerve cord, despite their simplicity, present a sophisticated and well-characterized research system. This characteristic renders them an ideal model for investigating the establishment and maintenance of motor neuron identity.

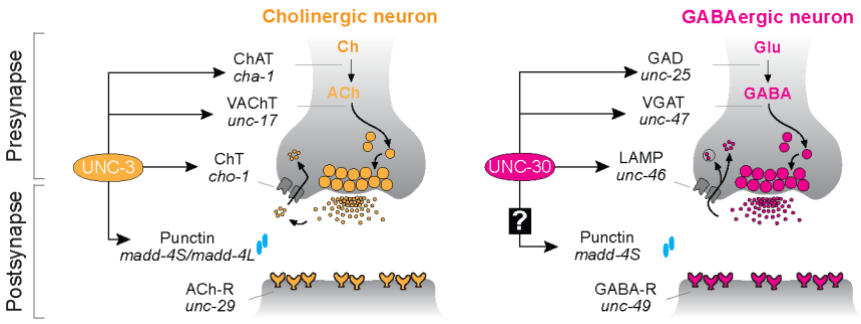

Figure 1.6: Schematic of terminal selectors at cholinergic and GABAergic motor neurons in *C. elegans*.
A detailed description is provided in this section (C. elegans motor neurons as a simple yet powerful system).

The two motor neuron types in *C. elegans* ventral nerve chord, cholinergic and GABAergic, showcase unique functional characteristics.[21,77,82] Cholinergic motor neurons express specific identity genes associated with the acetylcholine (ACh) pathway, such as *cho-1*/ChT, *unc-17*/VAChT, and *ace-2*/AChE, as well as genes encoding crucial ion channels like *del-1*/ENaC and *slo-2*/K-channel.[84] The cholinergic motor neuron terminal identity of cholinergic motor subtypes DA, DB, VA, VB, and AS is controlled by the terminal selector UNC-3, the sole ortholog of the Collier/Olf/Ebf (COE) family of transcription factors.[39,88–90] UNC-3 exerts its function by directly binding conserved COE motifs found in the cis-regulatory regions of genes related to cholinergic identity (e.g., cho-1/ChT, unc-17/VAChT, ace-2/AChE). UNC-3 plays a crucial role not only in establishing cholinergic terminal identity during embryogenesis but also in maintaining this identity throughout adulthood.[98] Consequently, the depletion of UNC-3 leads to compromised expression of nearly all its target genes, resulting in the loss of cholinergic terminal identity. In addition, without UNC-3, cholinergic motor neurons exhibit a mixed identity, expressing genes typical of other motor neuron types.[95] This underscores the crucial role of UNC-3 in preventing the expression of genes usually associated with different motor neuron identities, thus, safeguarding cholinergic motor neuron identity.

On the contrary, GABAergic motor neurons express specific identity genes linked to the GABAergic (GABA) pathway, such as *unc-25*/GAD, *unc-47*/VGAT, and *unc-46*/LAMP, along with genes encoding essential ion channels like s*fn-1*/GAT.[22] The terminal identity of all GABAergic motor neuron subtypes (DD, VD) is under the control

of the terminal selector UNC-30/PITX, a member of the homeodomain family of transcription factors. UNC-30 functions by directly binding conserved motifs (TAATCC) found in the cis-regulatory regions of genes related to GABAergic identity (e.g., unc-25/GAD, unc-47/VGAT, unc-46/LAMP), thus, playing a crucial role in establishing GABAergic terminal identity.[109,111,115] Depletion of UNC-30 compromises the expression of its target genes, leading to the loss of GABAergic terminal identity. However, whether UNC-30 is involved in maintaining this identity throughout adulthood remains unclear. Additionally, the molecular mechanism within GABAergic motor neurons that prevents the expression of genes typically seen in other motor neuron types is unknown.

Given that motor neurons must establish and maintain their terminal identity throughout life to sustain the functionality of synapses, further, I will discuss the emerging role of UNC-30. In the second chapter of my thesis (Chapter 2), I will highlight its crucial function in (1) not only establishing GABAergic terminal identity during embryogenesis but also maintaining this identity into adulthood, and (2) repressing cholinergic identity genes in GABAergic motor neurons, thereby preventing mixed terminal identity.

1.9.3 Relevance to understanding the synapse functionality

Besides motor neurons acquiring their terminal identity and consequently presenting a specific neurotransmitter, it is crucial for the muscle to acquire features that recognize that neurotransmitter. These features, such as presenting and clustering the corresponding neurotransmitter receptors, must be properly established and maintained in the muscle (i.e., postsynaptic domain) throughout life to sustain the functionality of

synapses. The postsynaptic domain is partly shaped and defined by a variety of synaptic organizers (e.g., Neuroligin, Neurexin, and Punctin), crucial for facilitating the proper clustering of neurotransmitter receptors.[31] Thus, the depletion of certain synaptic organizers compromises the appropriate clustering of neurotransmitter receptors, consequently leading to the loss of the functionality of synapses.

Although the roles of these synaptic organizers have been elucidated, the molecular mechanisms governing their function remain unknown. The neuromuscular junctions within the *C. elegans* ventral nerve cord and the molecular components delineating its structure and function have been extensively characterized. This makes *C. elegans* an ideal model for investigating the establishment and maintenance of the functionality of synapses.

1.9.3.1 Punctin drives synapse differentiation in C. elegans

The functionality of synapses strongly relies on the postsynaptic assembly of cholinergic and GABAergic neuromuscular junctions. In *C. elegans*, this process is shaped by an anterograde synaptic organizer known as MADD-4/Punctin, a member of the ADAMTS-like protein family.[31,40] Through the alternative usage of distinct promoters, *madd-4* generates two isoforms, a long and a short isoform. Each *madd-4* isoform is expressed in specific motor neuron types and showcases a unique function. The long isoform, *madd-4L*, is specifically expressed in cholinergic motor neurons, where it is secreted from the presynaptic side and facilitates the proper clustering of acetylcholine neurotransmitter receptors (e.g., *unc*-29/L-AChR) at the cholinergic postsynaptic side.[40]

On the other hand, the short isoform, *madd-4b*, is expressed in both cholinergic and GABAergic motor neurons.[40] In cholinergic motor neurons, it inhibits the clustering of GABA neurotransmitter receptors (e.g., *unc-49*/$GABA_AR$) at the cholinergic postsynaptic side through heterodimerization with MADD-4L. In GABAergic motor neurons, MADD-4B is secreted from the presynaptic side and facilitates the proper clustering of GABA neurotransmitter receptors (e.g., *unc-49*/$GABA_AR$) at the GABAergic postsynaptic side. The depletion of MADD-4 does not alter the presynaptic side, but electrophysiological analyses showed that it compromises the functionality of synapses.[40] Although the functions of MADD-4 have been well characterized, the molecular mechanisms governing their role are still being elucidated.

1.9.3.2 Transcriptional programs coordinate synapse functionality in C. elegans

Recent studies have uncovered a transcriptional program that coordinates both the presynaptic and postsynaptic sides, controlling motor neuron identity and the proper clustering of neurotransmitter receptors, respectively. In cholinergic neuromuscular junctions, the terminal selector UNC-3/COE is expressed in the presynaptic site and directly controls the expression of cholinergic motor neuron identity.[39] Despite not being expressed on the postsynaptic side, UNC-3 promotes the proper clustering of acetylcholine neurotransmitter receptors (e.g., *unc*-29/L-AChR by controlling the expression of both *madd-4L* and *madd-4B* in the presynaptic side.[122] UNC-3 exerts its function by directly binding conserved COE motifs found in the cis-regulatory regions specific to each *madd-4* isoform, controlling the proper clustering of neurotransmitter

receptors non-cell-autonomously, and consequently, controlling synapse functionality in cholinergic neuromuscular junctions.

Whether a transcriptional program coordinates GABAergic neuromuscular junctions, by controlling both motor neuron identity (i.e., the presynaptic side) and the proper clustering of neurotransmitter receptors (i.e., the postsynaptic side), remains elusive. Specifically, the molecular mechanisms governing the function of MADD-4 in GABAergic motor neurons (i.e., the presynaptic side) – (1) activating *madd-4B* expression and non-cell-autonomously promoting the proper clustering of GABA neurotransmitter receptors (e.g., *unc-49*/$GABA_AR$) in the GABAergic postsynaptic, while (2) avoiding *madd-4L* expression and non-cell-autonomously inhibiting the clustering of acetylcholine neurotransmitter receptors (e.g., *unc*-29/L-AChR) in the GABAergic postsynaptic – are unknown. In the second chapter of my thesis (Chapter 2), I highlight a novel transcriptional program in GABAergic neuromuscular synapses and the emerging role of UNC-30/PITX driving this program.

1.10 Aims of this study

The primary aims of this study are centered around three main aspects. First, we aim to expand the conventional definition of terminal selectors, specifically focusing on UNC-30/PITX, the terminal selector of GABAergic motor neuron identity in *C. elegans*. Our study seeks to demonstrate that UNC-30 not only regulates GABA terminal identity genes but also plays a critical role in synaptic differentiation by influencing synapse organizers.

We will investigate the transcriptional co-regulatory strategy employed by UNC-30/PITX to ensure GABA neurotransmission.

Second, we aim to unravel the temporal function of UNC-30/PITX, particularly its continuous requirement in maintaining GABA identity gene expression in *C. elegans* motor neurons. By addressing the gap in our understanding of how GABA neurotransmission is sustained from embryo to adulthood, we aim to contribute valuable insights into the dynamic regulation of neurotransmitter identity over time.

Lastly, our study aims to translate developmental insights into biomedical relevance. We seek to explore the potential biomedical implications of UNC-30 orthologs (PITX1-3) in the mammalian brain. Through these aims, we anticipate contributing to the broader fields of developmental neurobiology and gene regulation.

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CHAPTER 2

UNC-30/PITX COORDINATES NEUROTRANSMITTER IDENTITY WITH POSTSYNAPTIC GABA RECEPTOR CLUSTERING

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2.1 Abstract

Terminal selectors are transcription factors that control neuronal identity by regulating the expression of key effector molecules, such as neurotransmitter (NT) biosynthesis proteins, ion channels, and neuropeptides. Whether and how terminal selectors control neuronal connectivity is poorly understood. Here, we report that UNC-30 (PITX2/3), the terminal selector of GABA motor neuron identity in *C. elegans*, is required for NT receptor clustering, a hallmark of postsynaptic differentiation. Animals lacking *unc-30* or *madd-4B,* the short isoform of the MN-secreted synapse organizer *madd-4* (*Punctin/ADAMTSL*), display severe GABA receptor type A ($GABA_AR$) clustering defects in postsynaptic muscle cells. Mechanistically, UNC-30 acts directly to induce and maintain transcription of *madd-4B* and GABA biosynthesis genes (e.g., *unc-25/GAD*, *unc-47/VGAT*). Hence, UNC-30 controls $GABA_AR$ clustering on postsynaptic muscle cells and GABA biosynthesis in presynaptic cells, transcriptionally coordinating two critical

processes for GABA neurotransmission. Further, we uncover multiple target genes and a dual role for UNC-30 both as an activator and repressor of gene transcription. Our findings on UNC-30 function may contribute to our molecular understanding of human conditions, such as Axenfeld-Rieger syndrome, caused by PITX2 and PITX3 gene mutations.

2.2 **Introduction**

In the nervous system, neuronal communication critically depends on the proper transmission of signals through chemical and electrical synapses. In the context of chemical synapses, presynaptic neurons must be able to synthesize and package into synaptic vesicles specific chemical substances known as neurotransmitters (NTs), such as acetylcholine (ACh), gamma-aminobutyric acid (GABA), and glutamate (Glu). Upon secretion into the synaptic cleft, each NT molecule binds to its cognate receptors located at the postsynaptic cell membrane, thereby evoking postsynaptic electrical responses.

Genes encoding proteins for NT biosynthesis and packaging (e.g., enzymes, transporters) are co-expressed in specific neuron types. The co-expression of these proteins defines the NT identity (or NT phenotype) of individual neuron types (e.g., cholinergic, GABAergic, dopaminergic). Although instances of NT identity switching have been described in the nervous system,[1,2] it is generally the case that individual neuron types acquire a specific NT identity during development and maintain it throughout life. The continuous expression of NT identity genes is fundamental for the ability of a

presynaptic neuron to signal to its postsynaptic targets. For efficient neurotransmission, however, it is equally important that cognate NT receptors cluster at postsynaptic domains precisely juxtaposed to presynaptic boutons.[3,4] Whether and how these two critical processes, i.e., NT identity of the presynaptic neuron and NT receptor clustering at the postsynaptic cell, are coordinated remains poorly understood.

Genetic studies in *C. elegans*, flies, and mice have revealed a phylogenetically conserved principle for the control of NT identity: neuron type-specific transcription factors, termed "terminal selectors", coordinate the expression of NT identity genes, thereby coordinating the synthesis of enzymes and transporters necessary for NT biosynthesis and signaling.[5,6] In addition, terminal selectors broadly control batteries of genes encoding proteins essential for neuronal identity and function (e.g., ion channels, neuropeptides).[6,7] To date, terminal selectors have been described for 111 of the 118 *C. elegans* neuron types.[8,9] Beyond *C. elegans*, terminal selectors have also been identified in fruit flies (*Drosophila)*, cnidarians (*Nematostella vectensis)*, marine chordates (*Ciona intestinalis)*, and mice (*Mus musculus*),[6] suggesting a deeply conserved role for these critical regulators of NT identity. A defining feature of terminal selectors is their continuous expression - from development throughout adulthood - in specific neuron types.[5] While the essential roles of terminal selectors in establishing NT identity during development are well-attested across model organisms, their involvement in maintaining NT identity in later-life stages remains poorly examined,[10] partially due to the lack of genetic tools for inducible terminal selector depletion in late-life stages.

In the case of GABAergic neurons, NT identity is defined by the co-expression of highly conserved proteins, including (a) the enzyme glutamic acid decarboxylase (GAD) which synthesizes GABA from its precursor, (b) the vesicular GABA transporter (VGAT) which packages GABA into synaptic vesicles, and (c) the GABA re-uptake transporter (GAT) (**Figure 2.1**).[12] Importantly, reduced expression of these GABA identity determinants, as well as impaired GABA transmission, lead to a variety of neuropsychiatric diseases, including schizophrenia, autism, epilepsy, or anxiety.[13] [14]

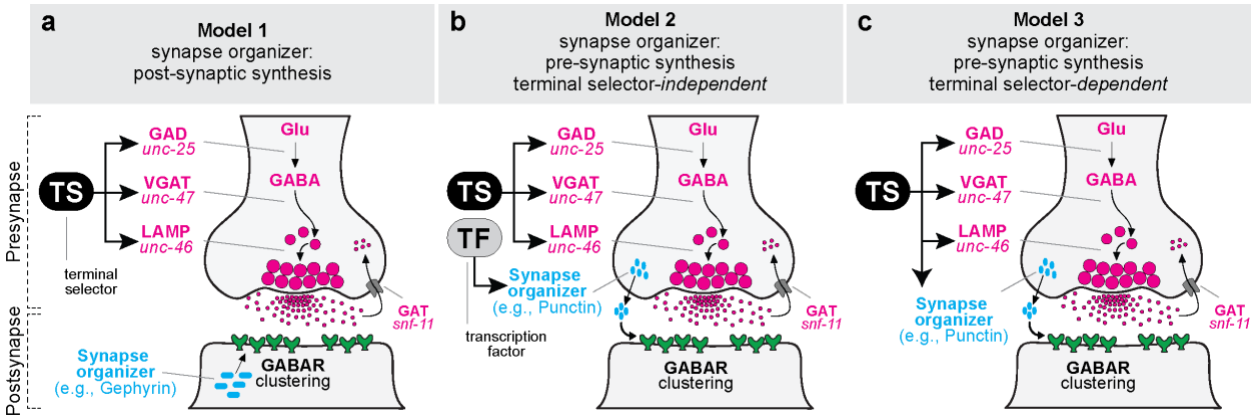

Figure 2.1: Three hypothetical models for transcriptional control of GABA synapse organizers.
(a-c) A detailed description of the three models is provided in this section (Introduction).

Despite GABA being the most abundant inhibitory NT both in invertebrate and vertebrate nervous systems, it is poorly understood how the expression of GABA identity genes is controlled over time, from development through adulthood, to ensure GABA neurotransmission. To date, a handful of studies in *C. elegans* and mice have identified

terminal selectors in various GABAergic neuron types. Examples include homeodomain proteins (e.g., UNC-30/PITX),[15-18] nuclear hormone receptors (e.g., NHR-67/NR2E1),[12] and GATA-type (GATA2/3) transcription factors, each necessary for expression of GABA identity genes during development.[19-21] However, whether any of these factors is required for maintaining GABA identity gene expression during post-embryonic life is unknown.[10]

During neuronal development, GABA receptor (GABAR) clustering is fundamental for postsynaptic differentiation, a process primarily driven by synapse-organizing molecules that can be either secreted or bound to the cell membrane.[13] In mice, the cell adhesion molecule Neuroligin-2, the scaffold protein Gephyrin, and the transmembrane protein b-dystroglycan act as synapse organizers to control GABAR clustering.[22-24] In the nematode C. elegans, the secreted molecule MADD-4 (Muscle Arm Development Defect-4), also known as human Punctin/ADAMTSL, acts as an anterograde synapse organizer at neuromuscular synapses.[25] Specifically, the short MADD-4 isoform (MADD-4B) activates DCC (Deleted in Colorectal Cancer)/UNC-40 signaling, recruiting an intracellular postsynaptic scaffold composed of FRM-3, a FERM (p4.1, Ezrin, Radixin, Moesin) domain protein, and LIN-2/CASK (Calcium Calmodulin dependent Serine/threonine Kinase).[26,27] Moreover, MADD-4B controls GABAR positioning at synapses by recruiting the sole *C. elegans* neuroligin homolog, NLG-1, which binds to LIN-2.[26-29] On the other hand, the long *madd-4* isoform, MADD-4L, promotes ACh receptor (AChR) clustering on muscle cells through the formation of an extracellular scaffold.[25,30-32]

In vertebrates, there are two *madd-4* orthologs: Punctin1/Adamtsl1 and Punctin2/Adamtsl3.[33,34] Although the brain function of Punctin1/Adamtsl1 remains unknown, recent data identified Adamtsl3 as an extracellular synapse organizer in the rodent hippocampus, where it supports glutamatergic and GABAergic synapse formation in vivo.[35] Furthermore, in the adult brain, Adamtsl3 signals via DCC at GABAergic synapses and facilitates synapse maintenance, synaptic plasticity, and memory. In humans, Adamtsl3 is widely expressed in the brain and has been identified as a candidate gene for schizophrenia.[36] Despite their well-established roles in GABAR clustering, the transcriptional mechanisms that control the expression of the aforementioned synapse organizers remain poorly understood.

GABA neurotransmission critically relies on (a) the ability of the presynaptic neuron to continuously express GABA identity genes (e.g., GAD, VGAT, GAT) and (b) the ability of the postsynaptic neurons to properly cluster GABARs.[3,4] Whether these two processes, that occur in two synaptically-connected cells, are coordinated remains poorly understood. In principle, at least three non-mutually exclusive models can be envisioned for the transcriptional control of a GABA synapse organizer (**Figure 2.1**). GABAR clustering at the post-synaptic (target) cell could be achieved via the activity of a synaptic organizer (membrane-bound or secreted) produced in the post-synaptic cell. For example, Gephyrin, a synapse organizer produced in the target cell, is essential for GABAR clustering (**Figure 2.1a,** model 1). Alternatively, GABAR clustering in the post-synaptic cell may rely on secreted synaptic organizers, like MADD-4/Punctin, produced in the presynaptic GABAergic neuron (**Figure 2.1b-c**, models 2 - 3). In that case, transcription of the synapse organizer gene may or may not require the activity of the

terminal selector of the presynaptic neuron (model 2 versus 3). Our previous work in *C. elegans* provided support for model 3 in the context of cholinergic neuromuscular synapses;[37] the terminal selector UNC-3 (Collier, Ebf) is not only required for AChR clustering in the postsynaptic neuron but also controls NT identity genes in the presynaptic cell. However, whether this principle of transcriptional coordination extends beyond cholinergic motor neurons (MNs) was unclear.

C. elegans has been a prime model for dissecting molecular mechanisms underlying NT identity and synapse formation.[4,8] Here, we show that the *C. elegans* terminal selector of GABAergic MN identity, UNC-30/PITX, is required for the clustering of type A GABARs, a major type of inhibitory NT receptors.[38,39] We find that UNC-30 acts directly to activate the transcription of the synapse organizer *madd-4B*. Hence, the terminal selector UNC-30 coordinates GABAR clustering on postsynaptic muscle cells (via control of *madd-4B/Punctin*) with the acquisition of GABAergic identity in presynaptic MNs (**Figure 2.1**, model 3). Further, we find that UNC-30 acts directly to maintain the expression of *madd-4B* and NT identity genes (e.g., *unc-25*/GAD, *unc-47*/VGAT) in late larval and adult stages. Intriguingly, UNC-30 also represses transcription of the long *madd-4* isoform (*madd-4L)*, which is normally required for AChR clustering in postsynaptic muscle cells.[25] Hence, our work in GABA MNs highlights that NT receptor clustering, a central event of postsynaptic differentiation, is transcriptionally coordinated with the acquisition and maintenance of NT identity, significantly extending previous observations made in *C. elegans* cholinergic MNs to other neuron types.[37] Last, we uncovered additional target genes that are either positively or negatively regulated by UNC-30/PITX, indicating direct activator and repressor

functions. Such mechanistic insights may help us understand the molecular mechanisms underlying human genetic disorders caused by *PITX* gene mutations, such as Axenfeld-Rieger syndrome.[40-42]

2.3 **Materials and methods**

2.3.1 C. elegans strains

Worms were grown at 15˚C, 20˚C, or 25˚C on nematode growth media (NGM) plates seeded with bacteria (*E.coli* OP50) as a food source. All *C. elegans* strains used in this study are listed in **Table 2.1**.

Table 2.1: Key resources

Reagent type (species) or resource	**Designation**	**Source or reference**	**Identifier**
Strain, strain background (C. elegans)	*unc-30(e191)*	Caenorhabditis Genetics Center	CB845
Strain, strain background (C. elegans)	*otIs354 [cho-1(fosmid)::SL2::YFP::H2B]; him-5(e1490)]*	Caenorhabditis Genetics Center	OH13470

Table 2.1 continued.

Strain, strain background (C. elegans)	*otIs564 [unc-47(fosmid)::SL2::H2B::mChopti + pha-1(+)]; him-5(1490)]*	Caenorhabditis Genetics Center	OH13105
Strain, strain background (C. elegans)	*otIs549 [unc-25prom::unc-25(partial)::mChopti::unc-54 3' UTR + pha-1(+)]; him-5(1490)]*	Caenorhabditis Genetics Center	OH13526
Strain, strain background (C. elegans)	*otEx5663 [unc-30prom::GFP::rab-3::unc-10 3'UTR + rol-6(su1006)]*	Caenorhabditis Genetics Center	OH12499
Strain, strain background (C. elegans)	*kr296 [unc-49::rfp]*	Bessereau lab (Universite Claude Bernard Lyon 1)	
Strain, strain background (C. elegans)	*krIs67 [unc-47prom::SNB-1::BFP]*	Bessereau lab (Universite Claude Bernard Lyon 1)	
Strain, strain background (C. elegans)	*madd-4(tr185)*	Bessereau lab (Universite Claude Bernard Lyon 1)	
Strain, strain background (C. elegans)	*krSi92 [unc-47prom::T7::MADD-4S::GFP*	Bessereau lab (Universite Claude Bernard Lyon 1)	

Table 2.1 continued.

Strain, strain background (C. elegans)	*syb623 [2xNLS::mScarlet::SL2::madd-4B]*	Bessereau lab (Universite Claude Bernard Lyon 1)	PHX623
Strain, strain background (C. elegans)	*syb624 [2xNLS::mScarlet::SL2::madd-4L]*	Bessereau lab (Universite Claude Bernard Lyon 1)	PHX624
Strain, strain background (C. elegans)	*syb623 [2xNLS::mScarlet::SL2::madd-4B]; otIs354 [cho-1(fosmid)::SL2::NLS::YFP::H2B]*	Bessereau lab (Universite Claude Bernard Lyon 1)	EN7454
Strain, strain background (C. elegans)	*syb624 [2xNLS::mScarlet::SL2::madd-4L]; otIs354[cho-1(fosmid)::SL2::NLS::YFP::H2B]*	Bessereau lab (Universite Claude Bernard Lyon 1)	EN7455
Strain, strain background (C. elegans)	*otEx5601 [madd-4b(4.4kb prom)::GFP, line 1]*	Hobert lab (Columbia University, NY, USA)	OH12398
Strain, strain background (C. elegans)	*syb3561 [madd-4b(Δ506bp)::NLS::Scarlet::SL2]*	Kratsios lab (University of Chicago, IL, USA)	PHX3561

Table 2.1 continued.

Strain, strain background (C. elegans)	*syb2344 [unc-30::mNG::3xFlag::AID]; ieSi57 [Peft-3::TIR1::mRuby::unc-54 3' UTR, cb-unc-119(+)]*	Kratsios lab (University of Chicago, IL, USA)	KRA575
Strain, strain background (C. elegans)	*syb2344 [unc-30::mNG::3xFlag::AID]*	Kratsios lab (University of Chicago, IL, USA)	PHX2344
Strain, strain background (C. elegans)	*ieSi57 [eft-3prom::TIR1::mRuby::unc-54 3' UTR, cb-unc-119(+)]*	Caenorhabditis Genetics Center	CA1200
Strain, strain background (C. elegans)	*sEx11477 [rCes F55D10.1::GFP + pCeh361]*	Caenorhabditis Genetics Center	BC11477
Strain, strain background (C. elegans)	*wdEx351 [tsp-7::GFP + unc-119(+)]*	Caenorhabditis Genetics Center	NC850
Strain, strain background (C. elegans)	*icIs270 [glr-5::GFP]*	Alfonso lab (University of Illinois Chicago, IL, USA)	AL270
Strain, strain background (C. elegans)	*kasEx214 [nhr-40(peak 1, +938 to +1846bp from ATG)::RFP::unc-54 3'UTR, line 1]*	Kratsios lab (University of Chicago, IL, USA)	KRA500

Table 2.1 continued.

Strain, strain background (C. elegans)	*kasEx216 [nhr-40(peak 2, +4360 to +5522bp from ATG)::RFP::unc-54 3'UTR, line 1]*	Kratsios lab (University of Chicago, IL, USA)	KRA502
Strain, strain background (C. elegans)	*kasEx220 [nhr-49 (-803 to +58bp from ATG)::RFP::unc-54 3'UTR, line 1]*	Kratsios lab (University of Chicago, IL, USA)	KRA506
Strain, strain background (C. elegans)	*kasEx232 [mab-9 (-5569 to -3768bp from ATG)::RFP::unc-54 3'UTR, line 1]*	Kratsios lab (University of Chicago, IL, USA)	KRA518
Strain, strain background (C. elegans)	*pha-1(e2123); otEx4949 [madd-4B(1.9kb prom)::GFP, line 9.1]*	Hobert lab (Columbia University, NY, USA)	OH11007
Strain, strain background (C. elegans)	*pha-1(e2123); otEx4948 [madd-4B(1.9kb prom)::GFP, line 4.1]*	Hobert lab (Columbia University, NY, USA)	OH11006
Strain, strain background (C. elegans)	*pha-1(e2123); kasEx315 [madd-4B(1.9kb prom_TAATCC mutated to GCGCGC)::GFP, line 1]*	Kratsios lab (University of Chicago, IL, USA)	KRA852

Table 2.1 continued.

Strain, strain background (C. elegans)	*pha-1(e2123); kasEx316 [madd-4B(1.9kb prom_TAATCC mutated to GCGCGC)::GFP, line 2]*	Kratsios lab (University of Chicago, IL, USA)	KRA853
Strain, strain background (C. elegans)	*dpy-5(e907) I; sEx14990 [madd-4L(2.9kb prom)::GFP]*	Caenorhabditis Genetics Center	BC14990
Strain, strain background (C. elegans)	*hdIs1 [unc-53p::GFP + rol-6(su1006)] X; ufIs26*	Caenorhabditis Genetics Center	NC2913
Strain, strain background (C. elegans)	*kasEx222 [nhr-19 (+1250 to +2302bp from ATG)::RFP::unc-54 3'UTR, line 1]*	Kratsios lab (University of Chicago, IL, USA)	KRA508
Strain, strain background (C. elegans)	*sEx10749 [rCes F08B12.3b::GFP + pCeh361]; dpy-5(e907)*	Caenorhabditis Genetics Center	BC10749
Strain, strain background (C. elegans)	*wdIs3 [del-1::GFP + dpy-20(+)]; dpy-20(e1282)*	Caenorhabditis Genetics Center	NC138
Strain, strain background (C. elegans)	*otIs224 [cat-1::GFP]*	Caenorhabditis Genetics Center	OH8249

Table 2.1 continued.

Strain, strain background (C. elegans)	*otIs266 [cat-1p::mCherry]*	Caenorhabditis Genetics Center	OH9279
Strain, strain background (C. elegans)	*otIs456 [lin-11::mCherry + myo-2::GFP]*	Caenorhabditis Genetics Center	OH11954
Strain, strain background (C. elegans)	*sEx14820 [rCes F58A6.6::GFP + pCeh361]; dpy-5(e907)*	Caenorhabditis Genetics Center	BC14820
Strain, strain background (C. elegans)	*inIs179 [ida-1p::GFP]; him-8(e1489)*	Caenorhabditis Genetics Center	BL5717
Strain, strain background (C. elegans)	*sEx11128 [gpd-2p::GFP + (pCeh361)dpy-5(+)]*	Caenorhabditis Genetics Center	CF2893
Strain, strain background (C. elegans)	*wdIs4 [unc-4::GFP + dpy-20(+)]; dpy-20(e1282)*	Caenorhabditis Genetics Center	NC197
Strain, strain background (C. elegans)	*otIs544 [cho-1(fosmid)::SL2::mCherry::H2B + pha-1(+)]; pha-1(e2123); him-5(e1490)*	Caenorhabditis Genetics Center	OH13646

2.3.2 Generation of transgenic reporter animals

Reporter gene fusions for *cis*-regulatory analysis were made using either PCR fusion or Gibson Assembly Cloning Kit (NEB #5510S)[88]. Targeted DNA fragments were fused (ligated) to *tagrfp* or *gfp* coding sequence, followed by *unc-54 3' UTR*. Mutations of UNC-30 binding sites were introduced via PCR mutagenesis. The product DNA fragments were either injected into young adult *pha-1(e2123)* hermaphrodites at 50ng/µl using *pha-1* (pBX plasmid) as a co-injection marker (50 ng/µl),and further selected for survival, or injected into young adult N2 hermaphrodites at 50ng/µl (plus 50ng/µl pBX plasmid) using *myo-2::gfp* as co-injection marker (3 ng/µl) and further selected for GFP signal. Primer sequences used for reporter construct generation are provided in **Table 2.2**.

Table 2.2: Primer sequences

Gene	Primers	5' to 3'	Additional information
nhr-40	FWD	ACTTGGTCATTTCCCCATTGG	UNC-30 Peak 1
nhr-40	REV	TCCGCACTCTTGATATTATC	
nhr-40	FWD	TCAAAGTTTAACCCTTTCCAGTG	UNC-30 Peak 2
nhr-40	REV	TTAACAAGTTGGACATTTGGG	

Table 2.2 continued.

nhr-19	FWD	TTGCAGTGAAATGTGATTCGG	
nhr-19	REV	ATACCAACTACCTTCGAAGC	
nhr-49	FWD	TGAATTTCCGGTTTTGACAC	
nhr-49	REV	AATGTCGCTTGATGTGTCCG	
mab-9	FWD	AACCAAAATATCTCGGAGCTCC	
mab-9	REV	TCAGTGTTTTTTGCTCCAGC	

Notes for Table 2.2:

- These primers only amplify the sequence of the genes fused to the *tagrfp*, not to amplify the *tagrfp* itself.

2.3.3 Targeted genome engineering

CRISPR/Cas9 genome editing was performed by SunyBiotech following standard procedures[89]. SunyBiotech generated the unc-30 endogenous reporter allele syb2344 [unc-30::mNG::3xFlag::AID] via CRISPR/Cas9 by inserting the *mNG::3xFLAG::AID* cassette immediately before the *unc-30* termination codon. The endogenous *madd-4L* reporter allele syb624 *[2xNLS::mScarlet::SL2::madd-4L]* was generated by inserting the *2xNLS::mScarlet::SL2* cassette immediately after the ATG of *madd-4L*. The endogenous *madd-4B* reporter allele *syb623 [2xNLS::mScarlet::SL2::madd-4B]* was generated by inserting the *2xNLS::mScarlet::SL2* cassette immediately after the ATG of

madd-4B. The endogenous *madd-4B* reporter allele *syb3561 [2xNLS::mScarlet::SL2::madd-4B* $^{\Delta 506\ bp}$] was generated by creating a 506 bp-long deletion (-1433bp to -927bp from the *madd-4B* ATG) in the background strain carrying the endogenous *madd-4B* reporter allele *syb623 [2xNLS::mScarlet::SL2::madd-4B].*

2.3.4 Temporally controlled protein degradation

AID-tagged proteins are conditionally degraded when exposed to auxin in the presence of TIR1[53,54]. Animals carrying auxin-inducible alleles of *unc-30 (syb2344[unc-30::mNG::3xFLAG::AID]) IV* were crossed with *ieSi57* animals that express TIR1 pan-somatically. Auxin (indole-3-acetic acid [IAA], Catalog number A10556, Alfa Aesar) was dissolved in ethanol (EtOH) to prepare 400 mM stock solutions which were stored at 4°C for up to one month. NGM agar plates were poured with auxin or ethanol added to a final concentration of 4 mM and allowed to dry overnight at room temperature. Plates were seeded with OP50 bacteria. To induce protein degradation, worms of the experimental strains were transferred onto auxin-coated plates and kept at 20°C. As a control, worms were transferred onto EtOH-coated plates instead. Auxin solutions, auxin-coated plates, and experimental plates were shielded from light.

2.3.5 Microscopy

For Figure 2, young adult *C. elegans* were mounted on 2% agarose (w/v in water) dry pads immersed in 2% polystyrene bead (0.1 mm diameter, Polyscience, 00876-15)

diluted in M9 buffer. Images were taken using a Nikon-IX86 microscope (Olympus) equipped with an Andor spinning disk system (Oxford Instruments), a 60x/NA1.42 oil immersion objective, and an Evolve EMCCD camera. For each animal (Figure 2), an image of the dorsal nerve cord at the first quarter of the worm was acquired as a stack of optical sections (0.2 mm apart). The Pearson's coefficient was calculated as described.[26] For the remaining figures, worms were anesthetized using 100mM of sodium azide (NaN_3) and mounted on a 4% agarose pad on glass slides. Images were taken using an automated fluorescence microscope (Zeiss, Axio Imager.Z2). Several z-stack images (each ~1 μm thick) were acquired with a Zeiss Axiocam 503 mono using the ZEN software (Version 2.3.69.1000, Blue edition). Representative images are shown following max-projection of 1-8 μm Z-stacks using the maximum intensity projection type. Image reconstruction was performed using Image J/FIJI software.[90]

2.3.6 MN identification

MNs were identified based on a combination of the following factors: i) co-localization with fluorescent markers with known expression pattern, ii) invariant cell body position along the ventral nerve cord, or relative to other MN subtypes, iii) MN birth order, and (iv) number of MNs that belong to each subtype.

2.3.7 Fluorescence intensity (FI) quantification

To quantify FI of individual MNs in the VNC, images of worms from different genetic backgrounds were taken with identical parameters through full-thickness z-stacks that cover the entire cell body. Image stacks were then processed and quantified for FI via FIJI. The focal plane in Z-stacks that has the brightest FI was selected for quantification. The background signal was minimized by using FIJI's background subtraction feature (rolling ball at 50 pixels). Cell outline was manually selected, and FIJI was used to quantify the FI and area to get the mean value for FI.

2.3.8 Statistical analysis and reproducibility

For quantification, box and whisker plots were adopted to represent the quartiles in the graphs. The box includes data points from the first to the third quartile value with the horizontal line in the box representing the median value. Upper and lower limits indicate the max and min, respectively. An unpaired t-test with Welch's correction was performed and p-values were annotated. Visualization of data and p-value calculation were performed via GraphPad Prism Version 9.2.0 (283). Each experiment was repeated twice.

2.3.9 Immunofluorescence staining

For **Figure 2.2**, immunofluorescence staining was performed as described.[26] Images were acquired using a Leica 5000B microscope equipped with a spinning disk CSU10 (Yokogawa) and a Coolsnap HQ2 camera.

2.3.10 Acknowledgements

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2.3.11 Author contributions

Conceptualization: E.C.,M.M.,B.P.L,J.L.B.,P.K.; Methodology: E.C.,M.M.,M.C.; Investigation: E.C.,M.M.,M.C.; Formal analysis: E.C., M.M.,M.C; Visualization: E.C., M.M.,B.P.L,P.K.; Funding acquisition: J.L.B.,P.K.; Writing original draft.

E.C.,B.P.L.,P.K.; Writing - review and editing: E.C.,M.M.,M.C.,B.P.L,J.L.B.,P.K.; Supervision: B.P.L,J.L.B.,P.K.

2.3.12 Competing interests

The authors declare no competing interests.

2.4 Results

2.4.1 The experimental system: GABAergic neuromuscular synapses in C. elegans

C. elegans locomotion relies on both cholinergic and GABAergic MNs, whose cell bodies intermingle along the ventral nerve cord (equivalent to vertebrate spinal cord) (**Figure 2.2a**). Based on anatomical criteria, cholinergic and GABAergic MNs are respectively divided into six (VA, VB, DA, DB, AS, VC) and two (DD, VD) classes, which form *en passant* synapses along the ventral and dorsal nerve cords (**Figure 2.2a**).[4,43] The coordinated activity of excitatory cholinergic and inhibitory GABAergic MNs generates sinusoidal locomotion in *C. elegans,* with each muscle cell receiving dual innervation from cholinergic and GABAergic MNs. Along the dorsal nerve cord of adult animals, three cholinergic MN classes (DA, DB, and AS) form dyadic synapses, providing excitatory input not only to dorsal muscles but also to VD GABAergic neurons, which in turn innervate and inhibit ventral muscles (**Figure 2.2a**).[44] Along the ventral nerve cord, another three cholinergic MN classes (VA, VB, and VC) also form dyadic synapses with

ventral muscles and DD GABAergic neurons, which innervate and inhibit dorsal muscles (**Figure 2.2a**). Because each muscle cell receives both excitatory (ACh) and inhibitory (GABA) inputs, the *C. elegans* neuromuscular system represents a powerful model to study how different NT receptors precisely cluster in front of their corresponding neurotransmitter release sites.[4]

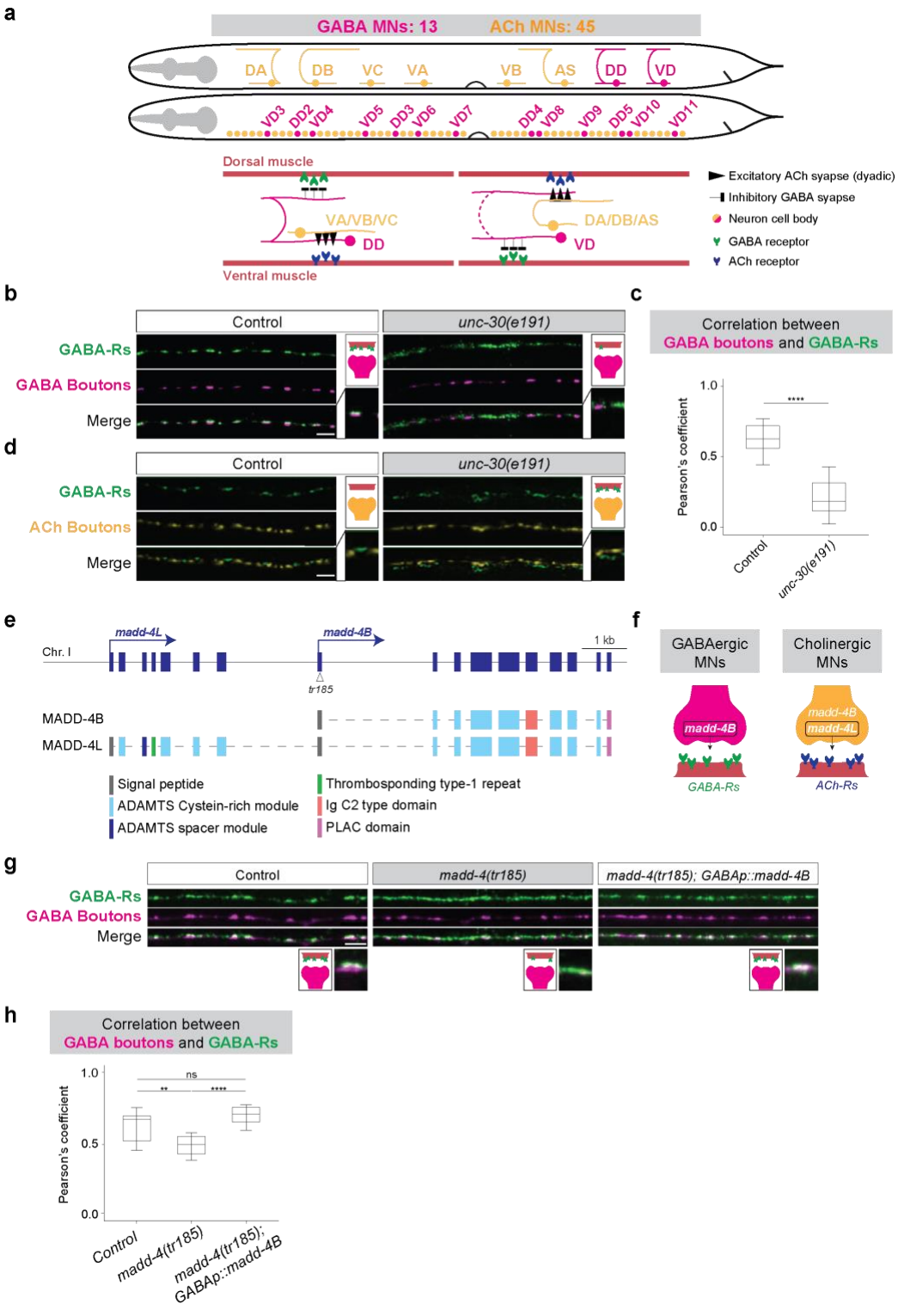

Figure 2.2: *unc-30* and *madd-4B* control GABAR clustering at *C. elegans* neuromuscular synapses.

Figure 2.2 continued.

(a) MN connectivity in the *C. elegans* ventral nerve cord. Cholinergic (DA, DB, VC, VA, VB, AS) and GABAergic (DD, VD) MN cell bodies intermingle. See text for details.
(b) Fluorescence micrographs of $GABA_AR$ (*kr296 [unc-49::rfp],* green pseudocolor) and GABAergic presynaptic boutons (*otEx5663 [unc-30prom::GFP::rab-3],* magenta pseudocolor) in control and *unc-30(e191)* animals.
(c) Pearson's correlation coefficient between $GABA_AR$ and GABAergic presynaptic boutons as shown in (b). Box and whisker plots show median, lower, and upper quartiles – whiskers represent SD. ANOVA followed by Tukey post-hoc test. ****p<0.0001. Control: n=11, *unc-30(e191)*: n=10.
(d) Immunofluorescence staining of $GABA_AR$ (anti-UNC-49 antibody) and cholinergic presynaptic boutons (anti-UNC-17 antibody) in control and *unc-30(e191)* animals.
(e) *madd-4* locus with both *(madd-4L*, *madd-4B*) isoforms. Protein domains are shown. The *madd-4(tr185)* allele carries a mutation in exon 1 of *madd-4B*.
(f) Schematic of *madd-4B* and *madd-4L* expression in *C. elegans* MNs.
(g) Fluorescence micrographs of $GABA_AR$ (*kr296[unc-49::rfp],* green pseudocolor) and GABAergic boutons (*krIs67[unc-47prom::SNB-1::BFP]*, magenta pseudocolor) in control and *madd-4(tr185)* animals, and a rescue strain: *madd-4B* in GABAergic MNs (*krSi92 [unc-47prom::T7::MADD-4S::GFP]*. Scale bar: 5 μm.
(h) Pearson's correlation coefficient between $GABA_AR$ and GABAergic presynaptic boutons as shown in (g). Kruskal-Wallis followed by Dunn's post-test. ns: non-significant, *p < 0.01, ****p < 0.0001. Wild-type: n=18; *madd-4(tr185):* n=22; rescue strain: n=21.

2.4.2 UNC-30/PITX controls $GABA_A$ receptor clustering at inhibitory neuromuscular synapses in a non-cell autonomous manner

Within the *C. elegans* ventral nerve cord, the transcription factor UNC-30/PITX2-3 is specifically expressed in GABAergic (DD, VD) MNs.[17] In these cells, UNC-30 controls the expression of GABA identity genes (**Figure 2.1**).[15-18,45] Recent studies also implicated UNC-30 in synaptic remodeling, as it prevents premature synapse rewiring of DD cells and aberrant synapse rewiring of VD cells.[46,47] However, whether UNC-30 is necessary for the postsynaptic differentiation of target muscle cells remains unknown.

We, therefore, asked whether genetic loss of *unc-30* affects GABAR clustering in *C. elegans* muscle cells innervated by GABAergic MNs. To this end, we used an endogenous *RFP* reporter for *unc*-49 (UNC-49::RFP), which encodes a type-A GABAR ($GABA_AR$) expressed both in ventral and dorsal body wall muscles (**Figure 2.2b**).[27] Upon crossing this strain to a presynaptic marker (*unc-30prom::GFP::RAB-3)* for GABAergic MNs (DD, VD), we visualized in young adult (day 1) animals the juxtaposition of GABAergic presynaptic boutons and $GABA_AR$ clusters in body wall muscles (**Figure 2.2b**). We focused our analysis on DD synapses onto dorsal muscle (**Figure 2.2a**) because the dorsal nerve cord (DNC) does not contain MN cell bodies, thereby facilitating the visualization of UNC-49::RFP and *unc-30prom::GFP::RAB-3* fluorescent signals (**Figure 2.2a-b**).

In homozygous animals carrying a strong loss-of-function *unc-30* allele (*e191*),[17,48] we found that $GABA_ARs$ are present on dorsal muscle (DNC), but no longer cluster opposite presynaptic GABA (DD) boutons at young adult (day 1) stages (**Figure 2.2b-c**). Because in control animals GABAergic (DD) and cholinergic (DA, DB, AS) neurons form *en passant* neuromuscular synapses with dorsal muscle (**Figure 2.2a**), we considered two possibilities: either GABARs on the dorsal muscle of *unc-30(e191)* animals are not juxtaposed to any presynaptic terminal, or they are inappropriately juxtaposed to presynaptic boutons of cholinergic (DA, DB, AS) MNs. We therefore performed double immunofluorescence staining against UNC-49 and UNC-17 (VAChT/SLC18A3), a marker of cholinergic presynaptic boutons. We indeed found that $GABA_ARs$ incorrectly localize opposite to cholinergic presynaptic boutons in the *unc-30(e191)* mutants (**Figure 2.2d**). Therefore, *unc-30/PITX* is necessary for the correct positioning of $GABA_ARs$ at

neuromuscular synapses along the dorsal nerve cord. Because *unc-30* is present in GABAergic MNs but not expressed in body wall muscles or muscle progenitor cells (**Figure 2.2.1**),[17] we conclude that *unc-30* controls $GABA_AR$ clustering in an indirect (non-cell autonomous) manner.

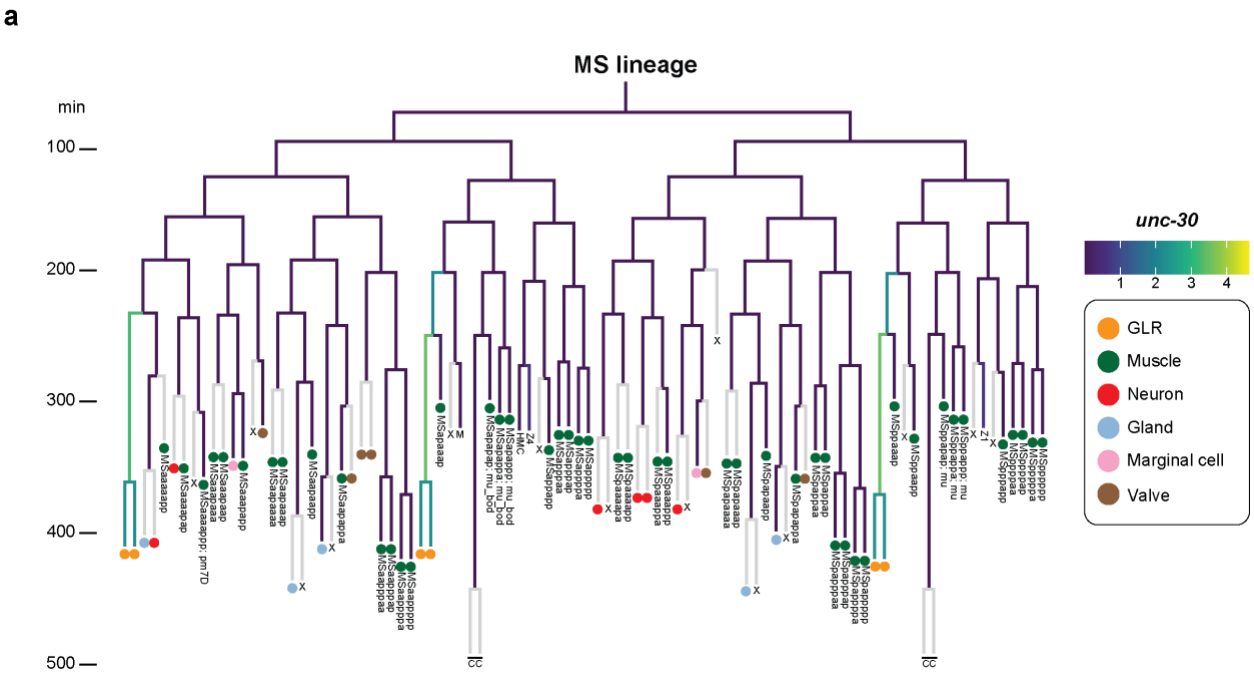

Figure 2.2.1: *unc-30* is not expressed in *C. elegans* body wall muscle cells.
(a) Lineage of the blast cell MS. Gradient depicts *unc-30* mRNA expression, while grey color depicts cells that were not characterized for *unc-30*.[92]

2.4.3 The short isoform of madd-4 (Punctin) controls GABA$_A$R clustering at neuromuscular synapses in a non-cell autonomous manner

In a previous study, we demonstrated that *madd-4/Punctin*, a secreted synapse organizer, is critical for GABAR and AChR clustering at *C. elegans* neuromuscular synapses.[25] The *madd-4* locus generates two isoforms through alternative promoter usage (**Figure 2.2e**).[37,49] The long isoform (*madd-4L*) is produced by cholinergic MNs and required for levamisole-sensitive AChR (L-AChR) clustering at neuromuscular synapses (**Figure 2.2e-f**).[25,37] The short isoform (*madd-4B*) is required for GABAR clustering (**Figure 2.2e-f**).[25] Because *madd-4B* is produced by both GABAergic and cholinergic MNs,[25,26,37] it remained unclear whether *madd-4B* from GABAergic and/or cholinergic MNs is required for GABA$_A$ receptor clustering at neuromuscular synapses.

To test this, we first analyzed animals specifically lacking *madd-4B* gene activity using the *madd-4(tr185)* allele (**Figure 2.2e**). Confirming their previously reported synaptic phenotype,[26,27] we found that UNC-49::RFP fluorescence signal on the dorsal muscle of *madd-4B(tr185)* animals is no longer restricted to sites opposite of GABA (DD) boutons (**Figure 2.2g-h**). Instead, UNC-49::RFP is detected along the dorsal nerve cord. Because both GABAergic and cholinergic neuromuscular synapses are located *en passant,* the continuous distribution of UNC-49::RFP along the dorsal nerve cord suggests that GABA$_A$R clusters face both GABAergic (DD) and cholinergic (DA, DB, AS) presynaptic boutons (**Figure 2.2a**). Importantly, the expression of *madd-4B* specifically in GABAergic MNs led to a complete rescue of this phenotype (**Figure 2.2g-h**). Because *madd-4B* is expressed in GABAergic neurons, but not in muscle cells,[25,37,49] these findings

consolidate a non-cell autonomous role for the secreted synaptic organizer *madd-4B* in $GABA_AR$ clustering at postsynaptic muscle cells.

2.4.4 UNC-30 controls madd-4B transcription in GABAergic MNs

Because both *unc-30* and *madd-4B* mutants display defects in $GABA_AR$ localization (**Figure 2.2c, h**), we hypothesized that the transcription factor UNC-30 regulates *madd-4B* in GABAergic MNs. To test this, we employed CRISPR/Cas9 genome editing and generated an endogenous fluorescent reporter of *madd-4B* transcription. Before the ATG of *madd-4B*, we inserted a *mScarlet* sequence preceded by two copies of a nuclear localization signal (2xNLS) and followed by the SL2 trans-splicing element (**Figure 2.3a**). The *2x::NLS::mScarlet* sequence and endogenous *madd-4B* are transcribed as one mRNA, but each is translated independently due to the SL2 element. In agreement with transgenic *madd-4B* reporters,[37] this endogenous *2xNLS::mScarlet::SL2::madd-4B* transcriptional reporter (*mScarlet::madd-4B* hereafter) is expressed both in cholinergic and GABAergic MNs, albeit higher levels are observed in GABAergic MNs (**Figure 2.3b**). To test the effect of *unc-30* gene loss in *madd-4B* expression specifically in GABAergic MNs, we crossed a nuclear marker for cholinergic MNs (*cho-1::SL2::YFP::H2B*) to the *mScarlet::madd-4B* reporter in the context of control and *unc-30(e191)* animals (**Figure 2.3b**). We observed a significant decrease in the number of GABAergic cells (defined by the absence of *cho-1::SL2::YFP::H2B* signal) expressing *mScarlet::madd-4B* in *unc-30(e191)* mutants at the fourth larval (L4) stage (**Figure 2.3b-c**). That is, all 13 GABAergic neurons (DD2-DD5, VD3-VD11) of the ventral nerve

cord express *mScarlet::madd-4B* in control animals, but only ~10 neurons in *unc-30(-)* mutants (**Figure 2.3a, c**). Importantly, the remaining *mScarlet::madd-4B* expression in these 10 GABAergic neurons is also decreased, as revealed by quantification of *mScarlet::madd-4B* fluorescence intensity with single-cell resolution (e.g., VD3, DD2, VD4, VD5, DD3, VD6) (**Figure 2.3d**). The remaining *madd-4B* expression suggests that additional, yet-to-be-identified factors cooperate with UNC-30 to activate *madd-4B* expression in these cells. We note that throughout our analysis we excluded six GABAergic MNs (DD1, DD6, VD1, VD2, VD13) because their location (outside the ventral nerve cord) makes their identification less straightforward.

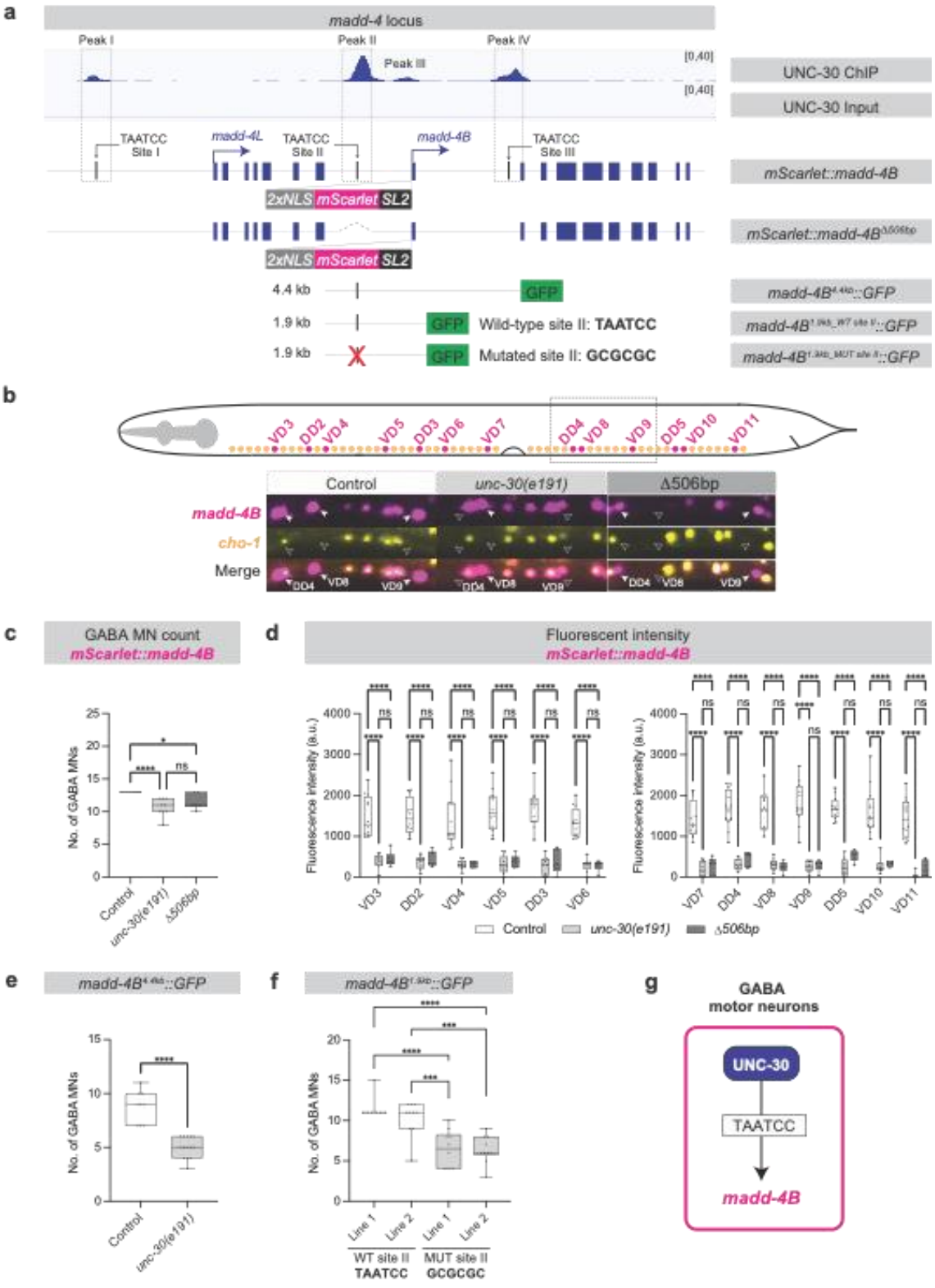

Figure 2.3: UNC-30 directly activates *madd-4B* in GABAergic motor neurons.

Figure 2.3 continued.

(a) UNC-30 ChIP-Seq and Input (negative control) on *madd-4* locus. Four UNC-30 binding peaks (peaks I, II, III, IV) and three UNC-30 binding sites (TAATCC sites I, II, II) are shown. Depicted below: endogenous *madd-4B* reporter *(syb623[2xNLS::mScarlet::SL2::madd-4B])*, mutant reporter allele (*syb3561[2xNLS::mScarlet::SL2:: madd-4B$^{\Delta 506bp}$]*), *madd-4B* transgenic reporters: *(otEx5601 [madd-4B$^{4.4kb}$::GFP], otEx4948-9[madd-4B$^{1.9kb}$::GFP]), kasEx315-6[madd-4B$^{1.9kb_GCGCGC}$::GFP])*.
(b) GABAergic (magenta) and cholinergic (yellow) MNs. Dashed box depicts imaged area. Fluorescence micrographs of *madd-4B (syb623[2xNLS::mScarlet::SL2::madd-4B])* and a cholinergic MN reporter *(otIs354[cho-1(fosmid)::SL2::YFP::H2B])* in control and *unc-30(e191)* animals, and *syb3561[2xNLS::mScarlet::SL2:: madd-4B$^{\Delta 506bp}$]*) animals. GABA MNs: *mScarlet+;YFP –;* cholinergic MNs: *mScarlet +;YFP+*. Images of day 1 adults. White arrowheads: GABAergic MNs.
(c) Quantification of number of GABAergic MNs of animal genotypes shown in (b). Unpaired t-test with Welch's correction. ****p<0.0001. Control: n=10, *unc-30(e191)*: n=10, Δ506bp mutant: n=8.
(d) Quantification of *madd-4B (2xNLS::mScarlet::SL2::madd-4B)* fluorescent intensity in individual GABAergic MNs. Two-way ANOVA followed by Sidak's multiple comparison test. ^{ns}p>0.05, *p<0.002, ****p<0.0001. Wild-type: n=10, *unc-30(e191):* n=10, Δ506bp mutant: n=8.
(e) Quantification of GABAergic MNs expressing *madd-4B(otEx5601[madd-4B$^{4.4kb}$::GFP])* in control and *unc-30(e191)* animals. Unpaired t-test with Welch's correction. ^{ns}p>0.05, ****p<0.0001. Control: n=9, *unc-30(e191)*: n=14.
(f) Quantification of GABAergic MNs expressing *otEx4948-9[madd-4B$^{1.9kb_TAATCC}$::GFP]* and *kasEx315-6[madd-4B$^{1.9kb_GCGCGC}$::GFP]s*. One-way ANOVA followed by Sidak's multiple comparison test. ***p<0.001, ****p<0.0001. Wild-type line1: n=10, wild-type line 2: n=10, *madd-4B$^{1.9kb_GCGCGC}$* line 1: n=10, *madd-4B$^{1.9kb_GCGCGC}$* line 2: n=10.
(g) Schematic of UNC-30 directly activating *madd-4B*.
*For panels c-f: Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values.

The single-cell resolution of our analysis indicates that *unc-30* controls *madd-4B* both in DD (e.g., DD2, DD3) and VD (e.g., VD3, VD4, VD5) neurons (**Figure 2.3d**). Corroborating this observation, we quantified *mScarlet::madd-4B* expression at larval

stage 1 (L1), a developmental stage at which only DD (not VD) neurons are present in the *C. elegans* nerve cord (**Figure 2.3.1a**). Again, we found a significant decrease in *madd-4B* expression in DD neurons of *unc-30(e191)* mutants (**Figure 2.3.1b**). In agreement with our endogenous transcriptional reporter (*mScarlet::madd-4B*), expression of a transgenic translational *madd-4B* reporter is also affected in *unc-30* animals at L1.[50] Altogether, we conclude that *unc-30* controls endogenous *madd-4B* transcription in GABAergic MNs, and this effect is observed both at early (L1) and late (L4) larval stages.

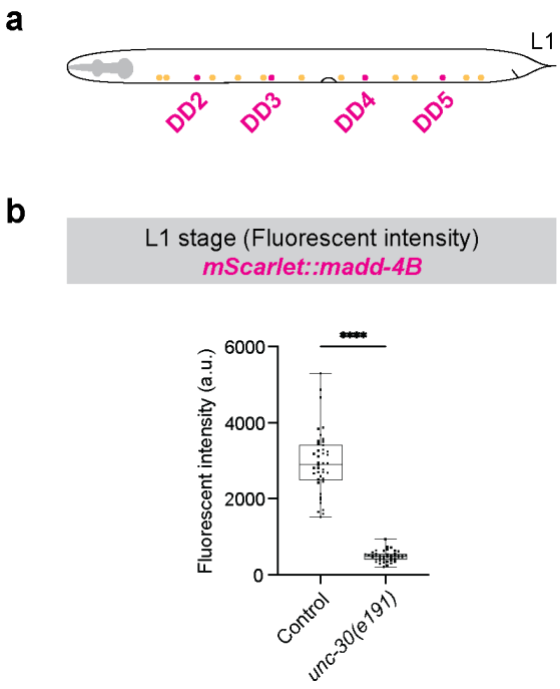

Figure 2.3.1: UNC-30 directly activates *madd-4B* in GABAergic motor neurons at larval stage 1.
(a) Schematic of GABAergic (magenta) and cholinergic (yellow) nerve cord MNs at L1. MNs depicted are embryonically born.

Figure 2.3.1 continued.

(b) Quantification of *madd-4B(syb623 [2xNLS::mScarlet::SL2::madd-4B])* fluorescent intensity in GABAergic MNs at L1 in control and *unc-30(191)* animals. Animals also carry a homozygous cholinergic Mn reporter *(otIs354[cho-1(fosmid)::SL2::YFP::H2B])*. Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black dots depict values. Unpaired t-test with Welch's correction. ****p<0.0001. Control: n=45 MNs, *unc-30(e191)*: n=45 MNs.

2.4.5 UNC-30 directly activates madd-4B transcription in GABAergic MNs

Because *madd-4B* expression is reduced in GABA MNs of *unc-30(e191)* animals (**Figure 2.3b-d**), we investigated whether *madd-4B* is a direct target of UNC-30. Leveraging an available dataset of chromatin immunoprecipitation followed by sequencing (ChIP-Seq)[51], we identified UNC-30 binding at four genomic regions (peaks I-IV): Peak I is upstream of *madd-4L*, whereas peaks II-IV surround the first exon of *madd-4B* (**Figure 2.3a**). Within peaks I, II, and IV, we identified a canonical UNC-30 binding site (TAATCC).[15,16] To test whether UNC-30 binding upstream of *madd-4B* is required for *madd-4B* expression, we employed CRISPR/Cas9 genome editing to delete a 506 bp-long region that spans peak II (Δ506bp, **Figure 2.3a**). This manipulation was conducted in animals carrying the endogenous *mScarlet::madd-4B* reporter. Similar to *unc-30(e191)* mutants, we observed a decrease in the number of GABAergic MNs expressing *mScarlet* in L4 stage animals homozygous for the 506bp deletion (**Figure 2.3b-c**), as well as a decrease in the levels of *mScarlet* expression in individual GABAergic MNs (**Figure 2.3d**).

ChIP-Seq data and our analysis of *mScarlet::madd-4B*$^{\Delta 506bp}$ animals strongly indicate that UNC-30 acts directly to activate *madd-4B* transcription. To further test direct transcriptional control, we examined transgenic animals carrying different transcriptional reporters of *madd-4B* (**Figure 2.3a**). First, we found that reporters containing DNA sequences either 4.4kb (*madd-4B*$^{4.4kb}$*::GFP*) or 1.9kb (*madd-4B*$^{1.9kb}$*::GFP*) upstream of *madd-4B* (both containing peak II) drive *GFP* expression in GABA MNs (**Figure 2.3a, e-f**), consistent with the endogenous *madd-4B::mScarlet* reporter (**Figure 2.3c**). Second, *madd-4B*$^{4.4kb}$*::GFP* reporter expression depends on *unc-30*, evidenced by a reduction in the number of GABA MNs expressing *GFP* in *unc-30(e191)* mutants (**Figure 2.3e**). Third, we found that mutation of the UNC-30 binding site II (wild type: TAATCC, mutated: GCGCGC) results in a significant decrease in the number of GABA MNs expressing *madd-4B*$^{1.9kb}$*::GFP* (**Figure 2.3f**). Altogether, we conclude that UNC-30 acts directly to activate *madd-4B* transcription in GABA MNs (**Figure 2.3g**).

2.4.6 UNC-30 represses madd-4L transcription in GABAergic MNs

The ChIP-Seq data also showed UNC-30 binding (peak I) in the *cis*-regulatory region upstream of exon 1 of *madd-4L* (**Figure 2.3a**). Because *madd-4L* is known to be specifically expressed in cholinergic MNs,[25,37] we hypothesized that UNC-30 binds directly upstream of *madd-4L* to repress its transcription in GABA MNs. Again, we employed CRISPR/Cas9 genome editing and generated an endogenous *mScarlet* reporter for *madd-4L* (*2x::NLS::mScarlet::SL2::madd-4L*), referred to as *mScarlet::madd-4L*

hereafter (**Figure 2.4a**). Supporting our hypothesis, we observed ectopic expression of *mScarlet::madd-4L* in GABA MNs of *unc-30(e191)* mutant animals both at L1 (**Figure 2.4.1)** and at L4 (**Figure 2.4b-d**). We found that up to 13 GABA MNs of the ventral nerve cord ectopically express *mScarlet::madd-4L* in *unc-30(e191)* mutants (**Figure 2.4b-d**).

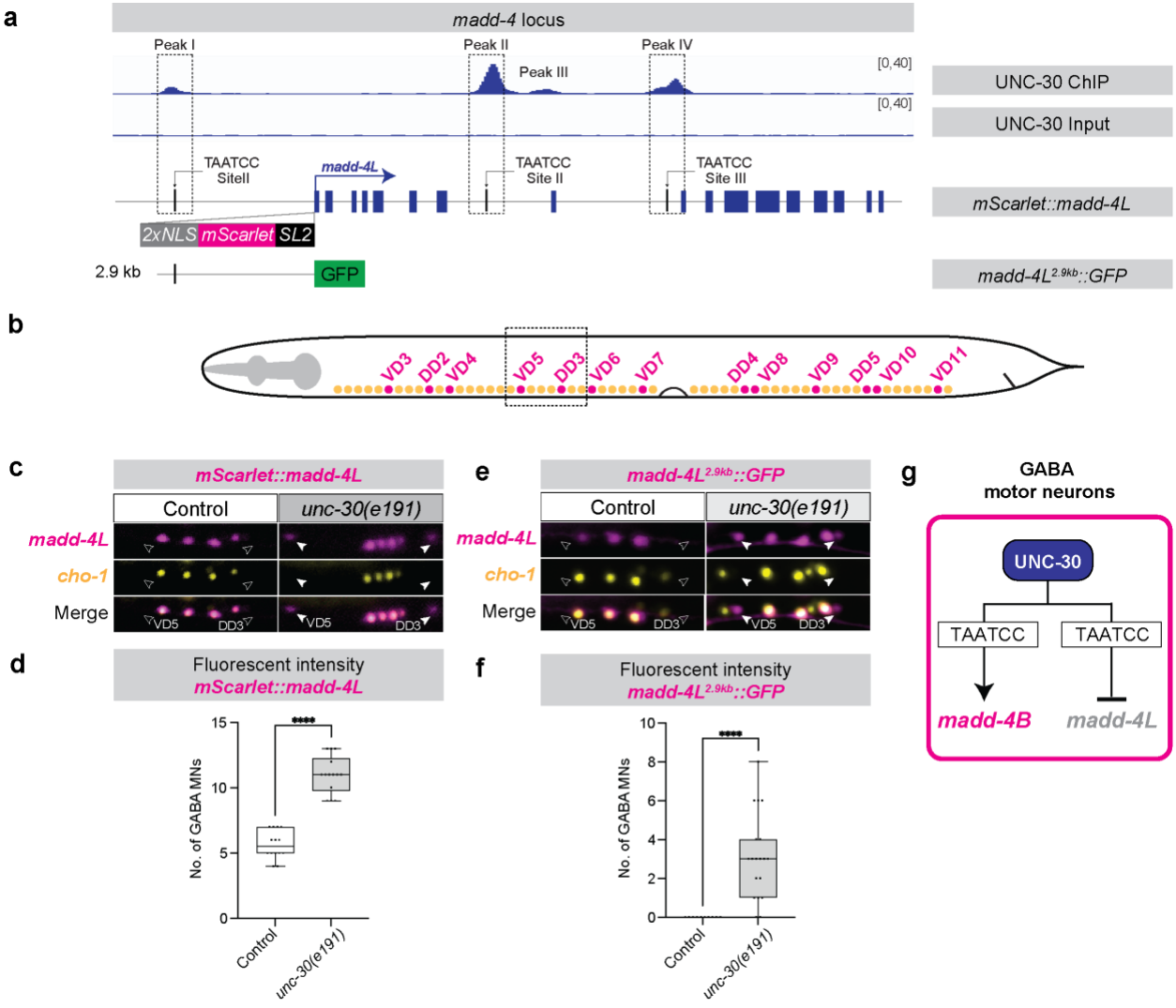

Figure 2.4: UNC-30 represses *madd-4L* in GABAergic motor neurons.
(a) UNC-30 ChIP-Seq and Input (negative control) tracks on *madd-4* locus. Depicted below: (1) endogenous (*syb624[2xNLS::mScarlet::SL2::madd-4L]*) and 2) transgenic (*sEx14990 [madd-4L(2.9kb prom)::GFP]*) *madd-4L* reporter.

Figure 2.4 continued.

(b) GABAergic (magenta) and cholinergic (yellow) MNs in *C. elegans*. Dashed box depicts imaged area shown in (c and e).
(c) Fluorescence micrographs of *madd-4L*(*syb624 [2xNLS::mScarlet::SL2::madd-4L]*) and a cholinergic motor neuron reporter *(otIs354[cho-1(fosmid)::SL2::YFP::H2B])* in control and *unc-30(e191)* animals. GABAergic MNs: *mScarlet+;YFP–*, cholinergic MNs: *mScarlet+;YFP+*. Images of day 1 adults. White arrowheads: GABAergic MNs.
(d) Quantification of GABAergic MNs expressing *madd-4L(syb624 [2xNLS::mScarlet::SL2::madd-4L])* as shown in (c). Unpaired t-test with Welch's correction. ****p<0.0001. Wild-type: n=13, *unc-30(e191)*: n=13.
(e) Fluorescence micrographs of *madd-L*(*sEx14990[madd-4L(2.9kb prom)::GFP])* and a cholinergic MN reporter*(otIs544 [cho-1(fosmid)::SL2::mCherry::H2B])* in control and *unc-30(e191)* animals. GABAergic MNs: *GFP+; mCherry -–*; cholinergic MNs: *GFP+;mCherry+*. Images of day 1 adults. White arrowheads: GABAergic MNs.
(f) Quantification of the number of GABAergic MNs expressing *madd-4L*(*sEx14990 [madd-4L(2.9kb prom)::GFP])* as shown in (e). Unpaired t-test with Welch's correction. ****p<0.0001. Wild-type: n=13, *unc-30(e191)*: n=13.
(g) Schematic: dual role of UNC-30 in controlling *madd-4* isoforms.
*For panels d and f: Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values.

UNC-30 binding (peak I) upstream of *madd-4L* suggests UNC-30 directly represses *madd-4L* in GABAergic MNs. In agreement with this notion, a transcriptional *madd-4L*$^{2.9kb}$*::GFP* reporter driving *GFP* under the control of a 2.9kb *cis*-regulatory region upstream of *madd-4L* (contains peak I) shows ectopic expression in GABA MNs of *unc-30* mutant animals at L4 (**Figure 2.4a, e-f**). We conclude that, in GABA MNs, UNC-30/PITX controls two isoforms of the same synapse organizer in opposite ways; it directly activates *madd-4B* and represses *madd-4L* (**Figure 2.4g**).

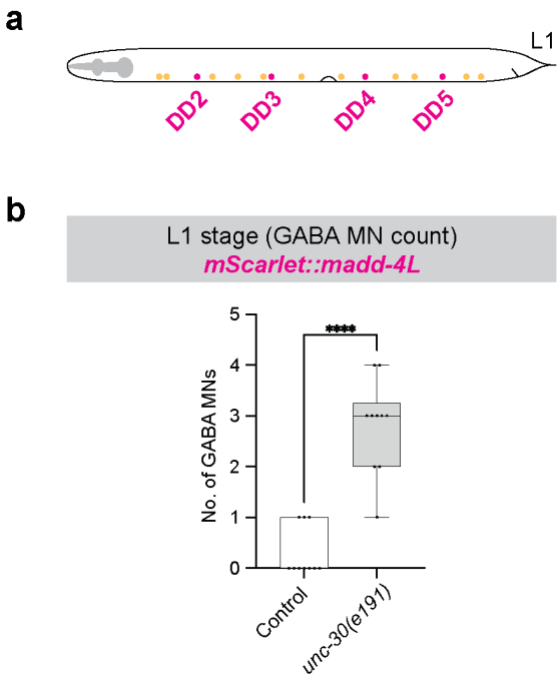

Figure 2.4.1: UNC-30 represses *madd-4L* in GABAergic DD motor neurons at larval stage 1.
(a) Schematic of GABAergic (magenta) and cholinergic (yellow) MNs at L1. MNs depicted are embryonically born.
(b) Quantification of GABAergic MNs expressing *madd-4L(syb624 [2xNLS::mScarlet::SL2::madd-4L])* at larval stage1 in control and *unc-30(191)* animals. Animals carry a cholinergic MN reporter *(otIs354 [cho-1(fosmid)::SL2::YFP::H2B])*. Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values. Unpaired t-test with Welch's correction. ****p<0.0001. Wild-type: n=45, *unc-30(e191)*: n=45.

2.4.7 UNC-30 is continuously required to maintain madd-4B expression in GABAergic MNs

The continuous expression of both *unc-30* and *madd-4B* in GABAergic MNs, from larval stages throughout adulthood, raises the question of whether UNC-30 is required continuously to activate *madd-4B* expression. We therefore generated an inducible *unc-*

30 allele, leveraging the auxin-inducible degradation (AID) system.[53,54] Using CRISPR/Cas9, we introduced the *mNG::3xFLAG::AID* cassette before the *unc-30* STOP codon (**Figure 2.5a**). The resulting *unc-30::mNG::3xFLAG::AID* allele *(syb2344)* serves as an endogenous fluorescent (mNG, mNeonGreen) reporter of the UNC-30 protein, which can be degraded upon auxin treatment due to the presence of the AID degron (**Figure 2.5b**). We generated double homozygous animals for *unc-30::mNG::3xFLAG::AID* and *ieSi57 (Peft-3::TIR1::mRuby),* the latter providing pansomatic expression of *TIR1* – an F-box protein that binds to AID in the presence of auxin, leading to proteasomal degradation of UNC-30::mNG::3xFLAG::AID. As proof-of-principle, we first assessed UNC-30::mNG::3xFLAG::AID levels in individual GABA MNs in ethanol-treated (control) or 4mM auxin-treated animal for 2 days, from L3 and to adult day 1 (**Figure 2.5b**). Compared to ethanol-treated animals, auxin-treated animals showed a robust reduction in the levels of UNC-30::mNG::3xFLAG::AID fluorescent intensity, indicating efficient depletion (**Figure 2.5c-e**). Auxin-treated animals exhibited a mild reduction in the total number of *mScarlet::madd-4B-expressing* MNs (**Figure 2.5f-g**), but a significant reduction in *mScarlet* fluorescent intensity levels in all individual GABA MNs (**Figure 2.5f-h, Figure 2.5.1**). We therefore conclude that UNC-30 is required during late larval and young adult stages to maintain *madd-4B* expression in GABAergic MNs (**Figure 2.5k**). UNC-30's continuous requirement is likely critical to maintaining $GABA_AR$ clustering throughout life.

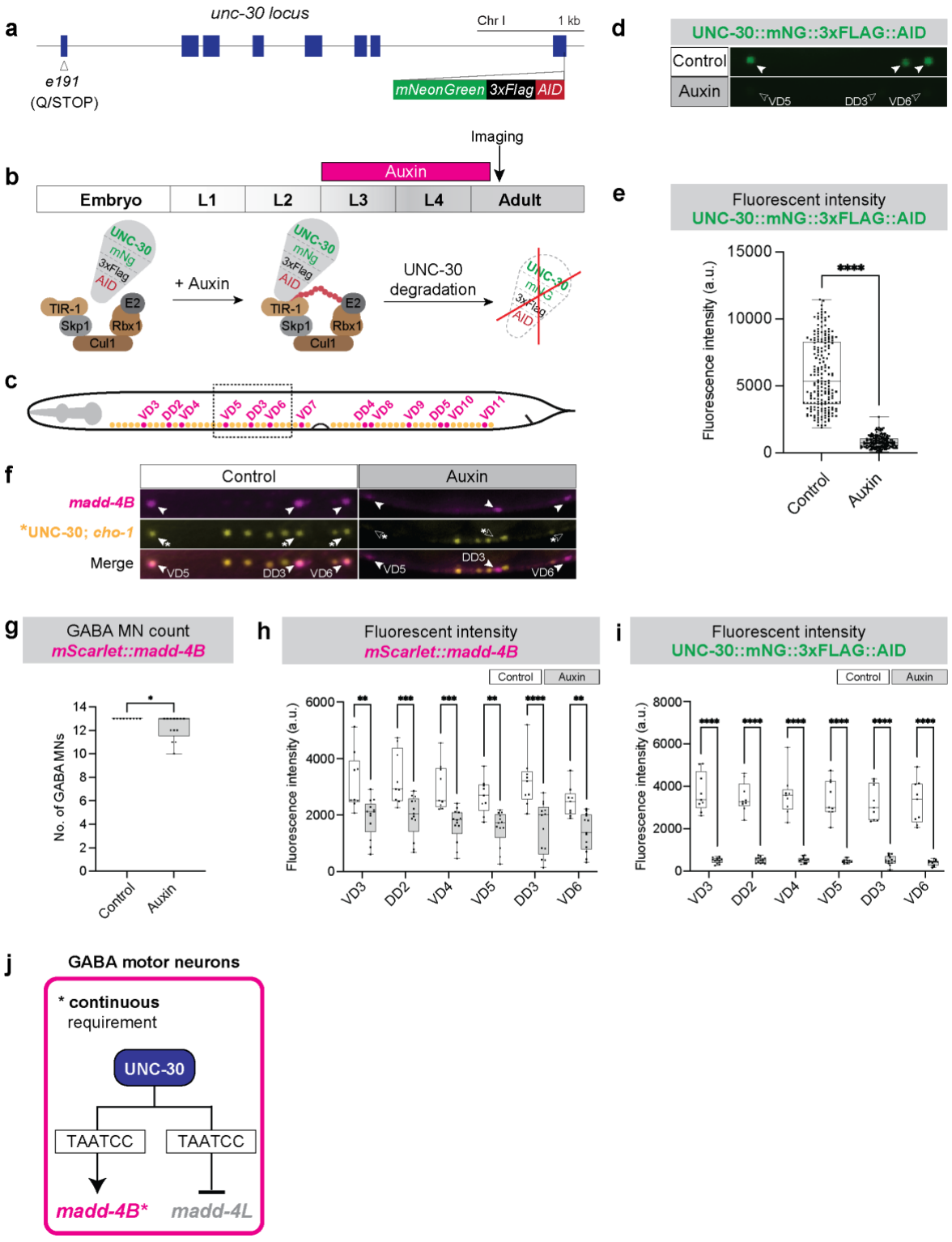

Figure 2.5: UNC-30 is required to maintain *madd-4B* expression in GABAergic motor neurons.

Figure 2.5 continued.

(a) Schematic of *unc-30* locus and *unc-30::mNG::3xFLAG::AID* allele (*syb2344*).
(b) Schematic of AID system. The E3 ligase complex: Skp1, Cul1, Rbx1, E2. Auxin treatment from L3 to adult day 1 stage. Imaging occurred at day 1.
(c) GABAergic (magenta) and cholinergic (yellow) MNs. Dashed box depicts imaged area shown in (d,f).
(d-e) Fluorescence micrographs of UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID])* in control (EtOH treated) and auxin treated animals. Animals express TIR1 pan-somatically (*ieSi57 [Peft-3::TIR1::mRuby]*). White arrowheads: GABAergic MNs. Quantification of UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID]*fluorescent intensity in GABAergic MNs. Unpaired t-test with Welch's correction. ****p<0.0001. Control: n=195, Auxin-treated=195.
(f) Fluorescence micrographs of *madd-4B (syb623[2xNLS::mScarlet::SL2::madd-4B]),* UNC-30 (*syb2344[UNC-30::mNG::3xFLAG::AID])*, and a cholinergic MN reporter *(otIs354[cho-1(fosmid)::SL2::YFP::H2B])* in control (EtOH treated) and auxin-treated animals. White arrowheads: GABAergic MNs. Green asterisk: GABA MNs expressing UNC-30::mNG::3xFLAG::AID.
(g) Quantification of the number of GABAergic MNs expressing *madd-4B (2xNLS::mScarlet::SL2::madd-4B)* as shown in (f). Unpaired t-test with Welch's correction. *p<0.01. Wild-type: n=9, *unc-30(e191)*: n=13.
(h-i) Quantification of (h) *madd-4B (2xNLS::mScarlet::SL2::madd-4B)* or (i) UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID])* fluorescent intensity in GABAergic MNs, as shown in (f). Two-way ANOVA followed by Sidak's multiple comparison test. **p<0.002, ***p<0.0002, ****p<0.0001. Control: n=9, Auxin-treated=13.
(j) UNC-30 is required to maintain *madd-4*..
*In panels e, h, and i: Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values.

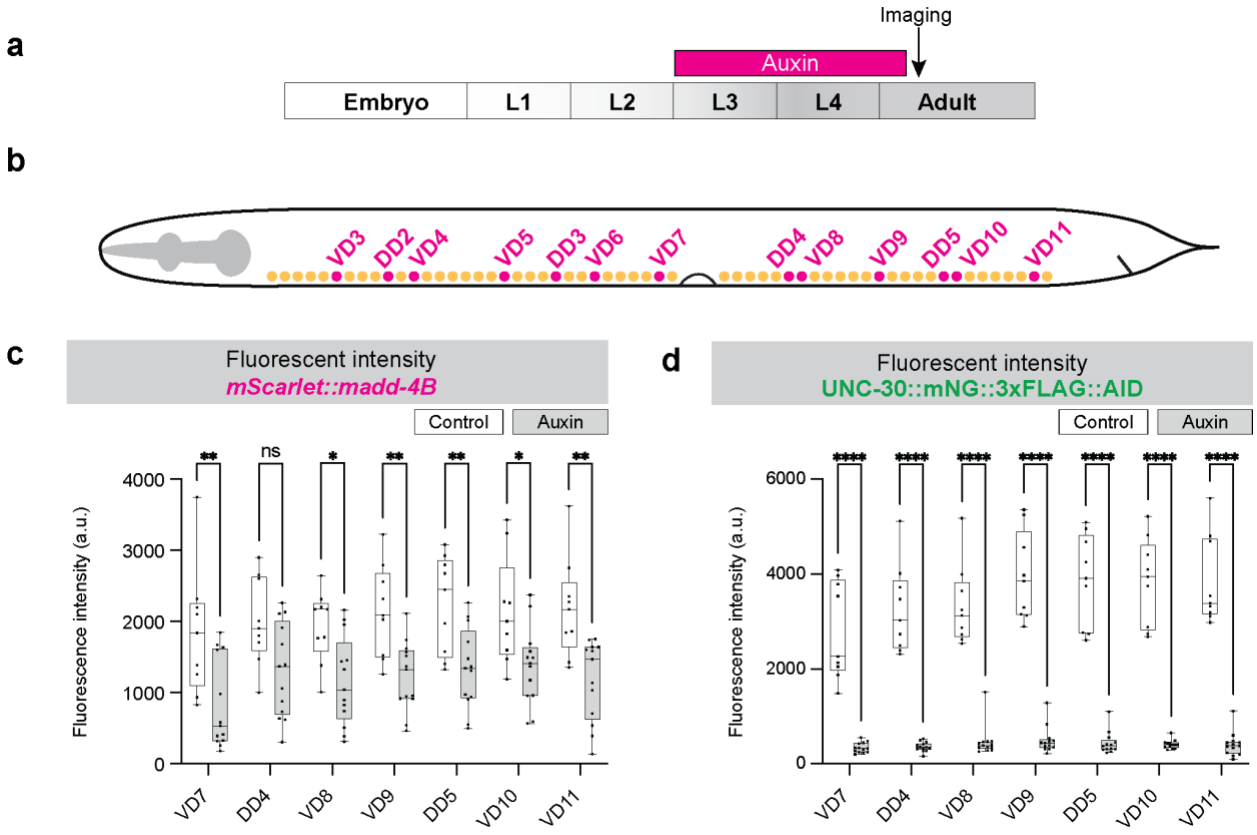

Figure 2.5.1: UNC-30 is required to maintain *madd-4B* expression in GABAergic motor neurons.

(a) Schematic of auxin treatment timeline.

(b) Schematic of GABAergic (magenta) and cholinergic (yellow) nerve cord MNs.

(c-d) Quantification of *madd-4B (syb623[2xNLS::mScarlet::SL2::madd-4B])* or UNC-30(*syb2344 [UNC-30::mNG::3xFLAG::AID])* fluorescent intensity in individual posteriorGABAergic MNs. Animals express TIR1 pan-somatically (*ieSi57 [Peft-3::TIR1::mRuby]*). Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values. Two-way ANOVA followed by Sidak's multiple comparison test. ****p<0.0001. Control: n=11, Auxin-treated=10.

2.4.8 UNC-30 is required to maintain the expression of GABA biosynthesis genes

Prompted by our *madd-4B* observations, we next asked whether UNC-30 is continuously required to maintain the expression of additional target genes. A previous study employing a constitutive null allele (*e191*) showed that UNC-30 activates the expression of two GABA identity genes during development, *unc-25*/GAD and *unc-47*/VGAT.[16] Mutating the UNC-30 binding site (TAATCC) in transgenic *unc-25* and *unc-47* reporter animals resulted in reduced reporter expression in GABA MNs, strongly suggesting UNC-30 regulates these targets via direct binding.[16] Consistent with these previous findings, analysis of the UNC-30 ChIP-Seq dataset showed UNC-30 binding in the *cis*-regulatory regions of *unc-25* and *unc-47* endogenous loci (**Figure 2.6c, f**).

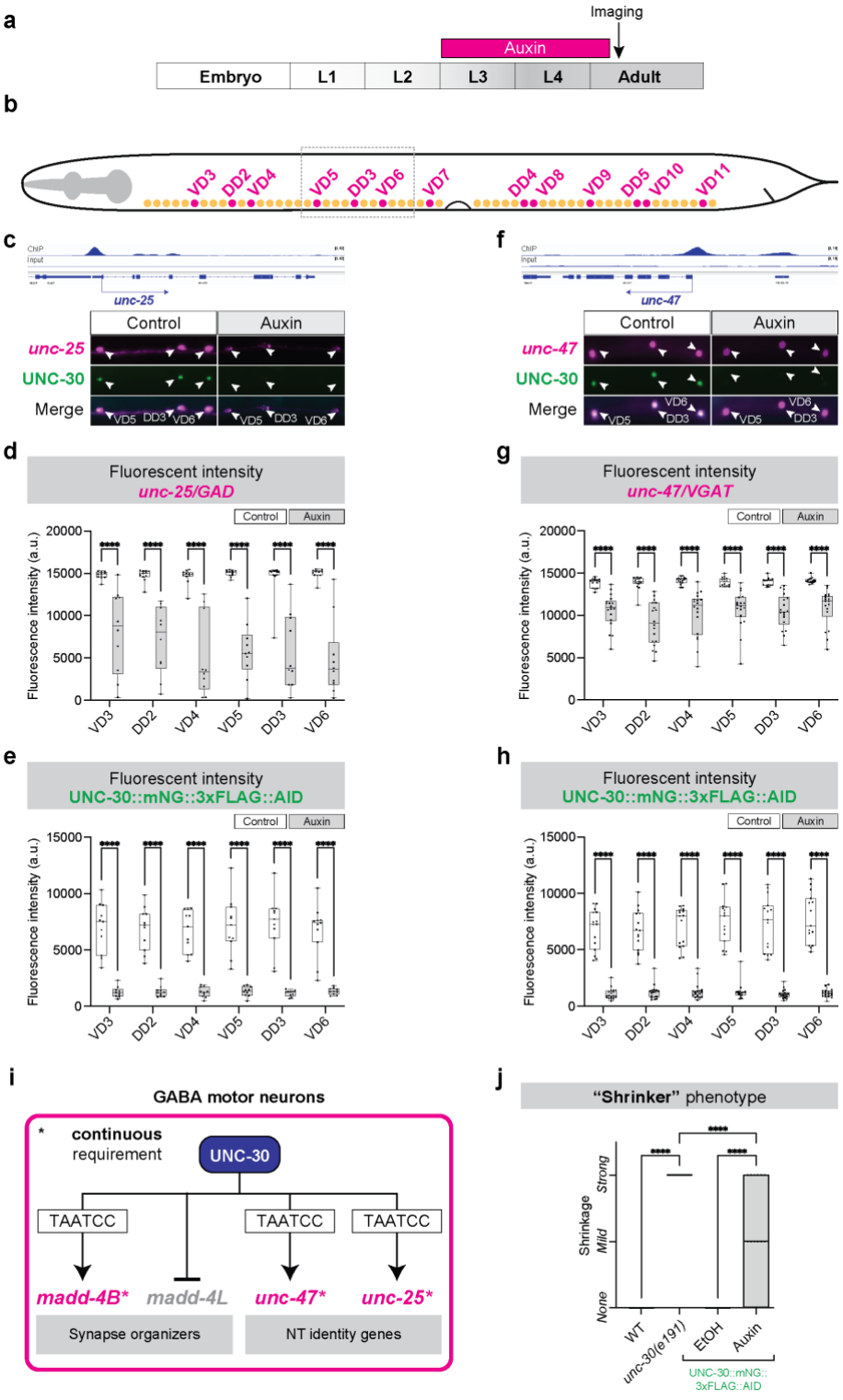

Figure 2.6: UNC-30 is required to maintain the expression of GABA biosynthesis genes.

(a) Auxin treatment timeline. Imaging occurred at day 1 adults.
(b) GABAergic (magenta) and cholinergic (yellow) MNs. Dashed box depicts imaged area shown in (c,f).
(c) Expression analysis of *unc-25(hpIs88 [unc-25p::mCherry)* and UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID])* in control (EtOH treated) and auxin-treated animals. Animals express TIR1 pan-somatically (*ieSi57 [Peft-3::TIR1::mRuby]*). White arrowheads: GABAergic MNs. UNC-30 ChIP-seq tracks on the *unc-25* and *unc-47* (f) loci.
(d-e) Quantification of (d) *unc-25(hpIs88 [unc-25p::mCherry)* or (e) UNC-30(*syb2344 [UNC-30::mNG::3xFLAG::AID])* fluorescent intensity in GABAergic MNs. Two-way ANOVA followed by Sidak's multiple comparison test. ****p<0.0001. Control: n=11, Auxin-treated=10.
(f) Expression analysis of *unc-47(otIs565 [unc-47(fosmid)::SL2::H2B::mChopti)* and UNC-30(*syb2344 [UNC-30::mNG::3xFLAG::AID])* in control (EtOH treated) and auxin-treated animals. Animals express TIR1 pan-somatically (*ieSi57 [Peft-3::TIR1::mRuby]*). White arrowheads: GABAergic MNs.
(g-h) Quantification of (g) *unc-47 (otIs565 [unc-47(fosmid)::SL2::H2B::mChopti))* or (h) UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID])* fluorescent intensity in GABAergic MNs. Two-way ANOVA followed by Sidak's multiple comparison test. ****p<0.0001. Control: n=15, Auxin-treated=18.
(i) UNC-30 is required to maintain *unc-25/GAD, unc-47/VGAT* and *madd-4S* expression in GABAergic MNs.
(j) Quantification of the "shrinker" phenotype upon response. One-way ANOVA followed by Sidak's multiple comparison test. ****p<0.0001. Wild-type: n=20, *unc-30(e191)*: n=20, Control: n=20, Auxin-treated: n=20.
*For panels d-e, g-h: Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values.

Whether UNC-30 is required at post-embryonic stages to maintain the expression of these critical determinants of GABAergic identity (e.g., *unc-25/GAD, unc-47/VGAT*) and function is not known. We again employed the AID system in late larval stages; this time assessing the effect of UNC-30 depletion on expression levels of *unc-25/GAD* and *unc-47/VGAT*. We observed a significant reduction in their expression levels in GABAergic MNs (**Figure 2.6a-h, Figure 2.6.1**), suggesting that UNC-30 is not only

required during early development to initiate expression of GABA biosynthesis genes but also to maintain their expression during late larval stages (**Figure 2.6i**).

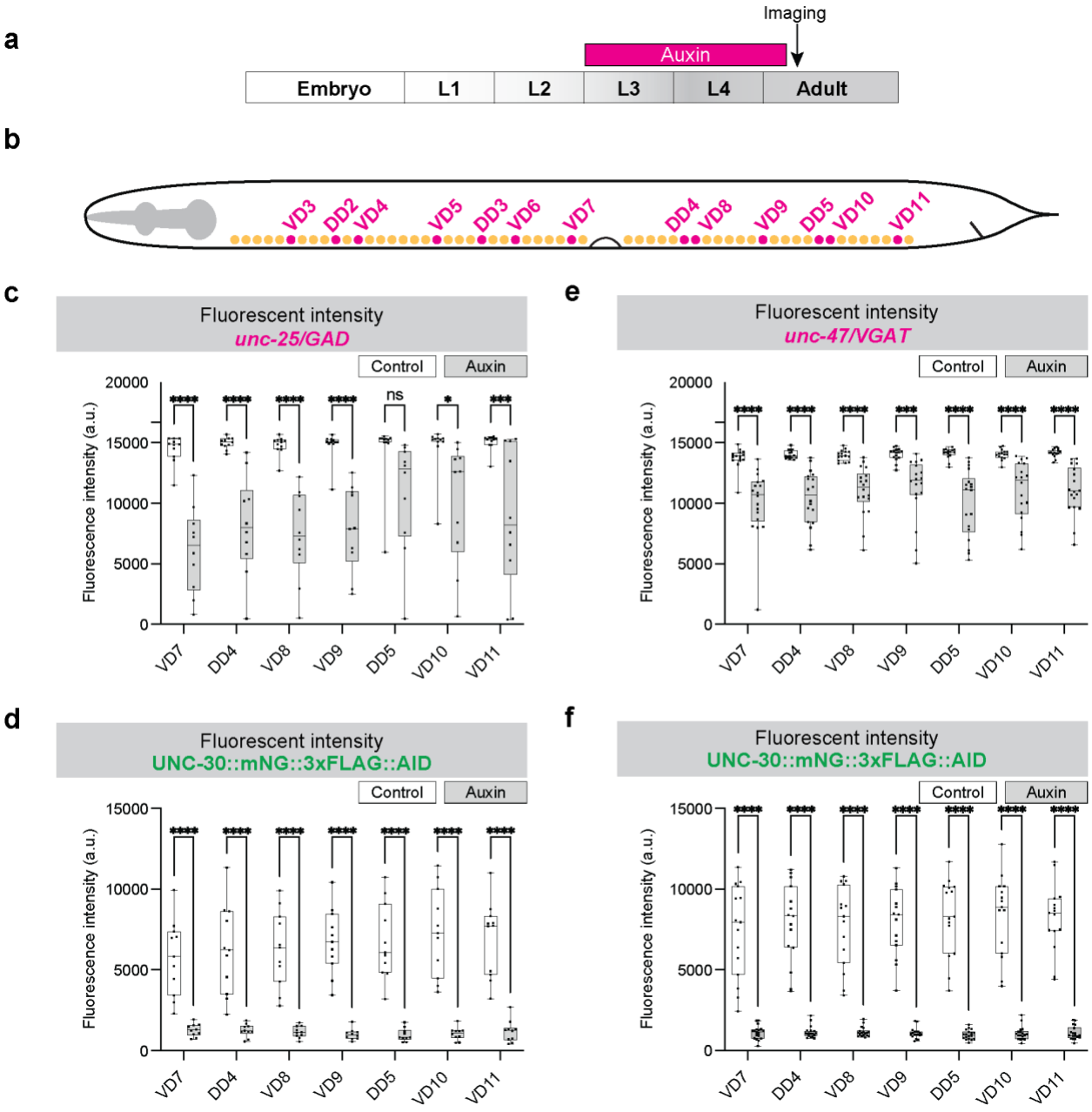

Figure 2.6.1: UNC-30 is required to maintain expression of GABA biosynthesis genes in MNs.

(a) Schematic of auxin treatment timeline.

(b) Schematic of GABAergic (magenta) and cholinergic (yellow) MNs.

Figure 2.6.1 continued.

(c-d) Quantification of *unc-25 (hpIs88 [unc-25p::mCherry)* or UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID])* fluorescent intensity in individual posterior GABAergic MNs. Animals express TIR1 pan-somatically (*ieSi57 [Peft-3::TIR1::mRuby]*). Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values. Two-way ANOVA followed by Sidak's multiple comparison test. ****p<0.0001. Control: n=11, Auxin-treated=10.
(e-f) Quantification of *unc-47 (otIs565 [unc-47(fosmid)::SL2::H2B::mChopti))* or UNC-30 (*syb2344 [UNC-30::mNG::3xFLAG::AID])* fluorescent intensity in posterior GABAergic MNs. Animals express TIR1 pan-somatically (*ieSi57 [Peft-3::TIR1::mRuby]*). Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values. Two-way ANOVA followed by Sidak's multiple comparison test. ****p<0.0001. Control: n=15, Auxin-treated=18.

2.4.9 UNC-30 is continuously required for normal touch response

Having established that UNC-30 is required at later stages to maintain expression of *unc-25/GAD*, *unc-47/VGAT,* and *madd-4B/Punctin*, we then asked: is UNC-30 also continuously required for normal animal behavior? Animals lacking unc-30 gene activity (homozygous null mutants) display a characteristic locomotory phenotype nicknamed "shrinker", [45,55] i.e., *unc-30* mutants hyper contract their body wall muscles in response to touch due to the lack of GABAergic MN inhibitory input to muscles. We indeed observed a striking and fully penetrant "shrinker" phenotype in *unc-30(e191)* mutants compared to control animals (**Figure 2.6j**). Importantly, auxin-mediated depletion of UNC-30 specifically at late larval/early adult stages also resulted in "shrinker" animals (**Figure 2.6j**). Because the auxin system does not fully eliminate UNC-30, as evidenced by quantification of UNC-30::mNG::3xFLAG::AID expression levels in individual GABAergic MNs (**Figure 2.5i**), the "shrinker" phenotype displays variable expressivity

(none, mild, strong) upon auxin treatment (**Figure 2.6j**). We note that in the control (ethanol) condition, we observed no shrinkers, suggesting that tagging the endogenous *unc-30* gene with the mNG::3xFLAG::AID cassette does not result in detectable hypomorphic effects on locomotory behavior (**Figure 2.6j**). We therefore conclude that UNC-30 is continuously required for normal touch response.

2.4.10 The dual role of UNC-30 in GABA MNs extends to other target genes

A handful of UNC-30 target genes are known to date, including *unc-25*/GAD, *unc-47*/VGAT*, pde-4/PDE4B, acy-1/ADCy9*, *oig-1, flp-11, flp-13,* and *ser-2* (**Table 2.3**).[15,16,47,51,56] A unifying theme emerging from these studies is that UNC-30 acts as a transcriptional activator. However, our findings on *madd-4L* suggest a repressive role for UNC-30 in GABA MNs (**Figure 2.4**). We therefore sought to identify new UNC-30 target genes to determine whether the duality in UNC-30 function (activator and repressor) is broadly employed.

Table 2.3: Summary of genetically validated UNC-30/PITX targets in GABAergic motor neurons

Gene	GABA MN expression (*)	UNC-30 binding (#)	*unc-30* dependency	Source
unc-25/GAD	Yes	Yes	Activated by UNC-30	[16]

Table 2.3 continued.

unc-47/VGAT	Yes	Yes	Activated by UNC-30	16
oig-1/one Ig	Yes	Yes	Activated by UNC-30	15,46,47,51,85
flp-13/neuropeptide	Yes	Yes	Activated by UNC-30	15,51,56
acr-14/AChR	Yes	Yes	Activated by UNC-30	15
ser-2/tyramine receptor	Yes	Yes	Activated by UNC-30	85
flp-11/neuropeptide	Yes	Yes	Activated by UNC-30	85
pde-4/PDE4B	Yes	Yes	Activated by UNC-30	51
acy-1/ADCY9	Yes	Yes	Activated by UNC-30	51
madd-4S/Punctin	Yes	Yes	Activated by UNC-30	This study, 50
mab-9/Tbx20	Yes	Yes	Activated by UNC-30	This study
aman-1/Man2b1	Yes	Yes	Activated by UNC-30	This study

Table 2.3 continued.

tsp-7/Cd63	Yes	Yes	Activated by UNC-30	This study
nhr-49/Hnf4a	Yes	Yes	Activated by UNC-30	This study
ilys-4	Yes	Yes	No effect	This study
nhr-40/NHR	Yes	Yes	No effect	This study
madd-4L/Punctin	No	Yes	Repressed by UNC-30	This study
unc-53/NAV1	No	Yes	Repressed by UNC-30	This study
glr-5/GRIK4	No	Yes	Repressed by UNC-30	This study
nhr-19/NHR	No	Yes	No effect	This study
slo-2/KCNT	No	Yes	No effect	This study
gpd-2/GAPDH	No	Yes	No effect	This study
del-1/SCNN1	No	Yes	No effect	This study
cat-1/SLAC18A	No	Yes	No effect	This study
lin-11/LHX1	No	Yes	No effect	This study
srb-16	No	No	No effect	This study

Table 2.3 continued.

unc-4/Uncx4.1	No	No	No effect	This study
ida-1/PTPRN2	No	Yes	No effect	This study

Notes for Table 2.3:

- Asterisk (*) indicates expression based on single-cell RNA-Seq data from[57,58].
- Hash (#) indicates UNC-30 binding based on ChIP-Seq data from [51].

First, we identified putative *unc-30* targets by searching for UNC-30 binding peaks in genes that are normally expressed in GABA MNs (**Table 2.3**) [57,58] In total, we tested six genes (*tsp-7/Cd63, aman-1/Man2b1, nhr-49/Hnf4a, mab-9/Tbx20, nhr-40/NHR, ilys-4*) by either generating new transgenic reporter animals (*nhr-49/Hnf4a, mab-9/Tbx20, nhr-40/NHR)*, or using available reporters (*tsp-7/Cd63, aman-1/Man2b1, ilys-4)*. Reporter expression for five of these genes (*tsp-7/Cd63, aman-1/Man2b1, nhr-49/Hnf4a, mab-9/Tbx20, nhr-40/NHR)* was significantly reduced in GABA MNs of *unc-30 (e191)* mutant animals (**Figure 2.7a-b, Table 2.3**). Because ChIP-Seq shows UNC-30 binding to all four of these genes (**Figure 2.7a-b**), it is likely that UNC-30 acts as a direct activator of *tsp-7/Cd63, aman-1/Man2b1, nhr-49/Hnf4a, nhr-40/NHR,* and *mab-9/Tbx20* transcription (**Figure 2.7d**).

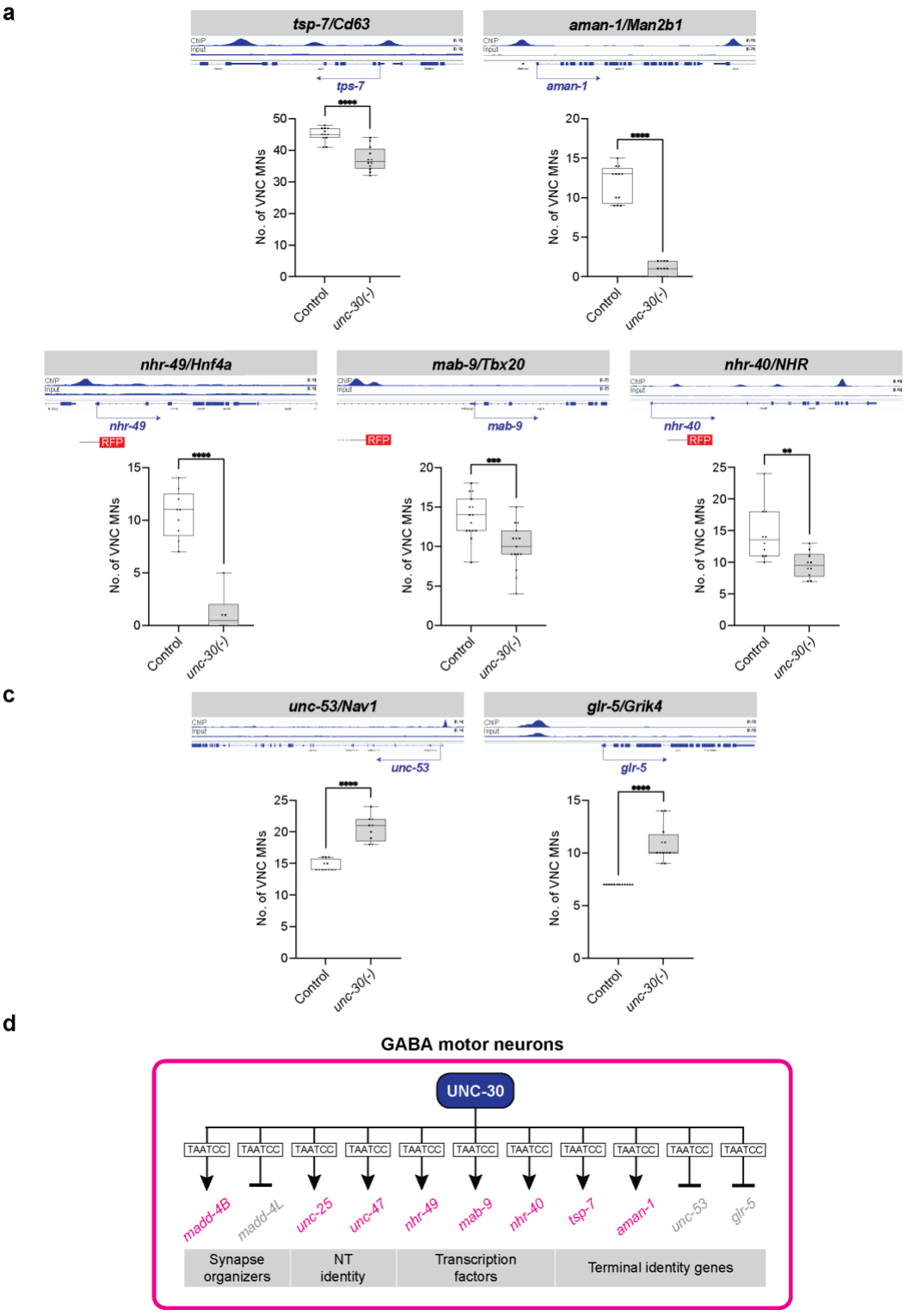

Figure 2.7: UNC-30 activates and represses different genes in GABAergic motor neurons.

Figure 2.7 continued.

(a-c) Quantification of the number of GABAergic nerve cord MNs cord expressing *wdEx351 [tsp-7::GFP], sEx11477 [aman-1::GFP]), kasEx220 [nhr-49 (-803 to +58bp from ATG)::RFP, kasEx232 [mab-9 (-5569 to -3768bp from ATG)::RFP, kasEx214 [nhr-40(+938 to +1846bp from ATG)::RFP], hdIs1 [unc-53p::GFP],* or *icIs270 [glr-5::GFP]* in control and *unc-30(191)* animals. Stage: L4. Box and whisker plots show median, lower, and upper quartiles – whiskers represent minimum and maximum. Black circles depict values. Unpaired t-test with Welch's correction. **p<0.002, ***p<0.0002, ****p<0.0001. Wild-type: n=10, *unc-30(e191)*: n=10.
(d) Summary of UNC-30 targets in GABAergic motor neurons.

Next, we aimed to identify genes that, like *madd-4L*, are repressed by UNC-30. We searched for UNC-30 binding peaks in genes that are not expressed in GABA MNs, but instead are normally expressed in cholinergic nerve cord MNs (**Table 2.3**). In total, we tested 11 genes, for which transgenic reporter animals were available. Two (*unc-53*/NAV1 and *glr-5*/GRIK4) of the 11 reporters showed ectopic expression in GABA MNs of *unc-30 (e191)* mutant animals (**Figure 2.7c**, **Table 2.3**).

Altogether, our work identified nine new UNC-30 target genes; six are activated (*madd-4B/Punctin, tsp-7/Cd63, aman-1/Man2b1, nhr-49/Hnf4a, mab-9/Tbx20, nhr-40/NHR)* and three are repressed (*madd-4L/Punctin, unc-53*/NAV1, *glr-5*/GRIK4) by UNC-30 (**Figure 2.7d**). This analysis significantly expands the known repertoire of UNC-30/PITX target genes in the *C. elegans* nervous system (**Table 2.3 –** summary of UNC-30 targets), consolidating its previously known activator role and uncovering a putative repressive function.

2.5 Discussion

Here, we describe a molecular mechanism that coordinates two spatially separated processes critical for the function of chemical synapses. That is NT biosynthesis in the presynaptic cell and NT receptor clustering in the postsynaptic cell. Using the *C. elegans* neuromuscular synapses as a model, we show that the terminal selector-type transcription factor UNC-30/PITX is required continuously to maintain the expression of GABA identity genes (e.g., *unc-25/GAD, unc-47/VGAT*) in presynaptic GABAergic MNs, thereby ensuring GABA synthesis and release. In postsynaptic target muscle cells, UNC-30 acts non-cell autonomously to control the clustering of $GABA_AR$s – the most prominent inhibitory NT receptors in animal nervous systems.[38,39] Mechanistically, we propose that UNC-30 directly regulates the production of MADD-4B, a secreted synapse organizer. Hence, UNC-30 coordinates $GABA_AR$ clustering on postsynaptic muscle cells with the acquisition and maintenance of GABAergic identity of presynaptic cells (**Figure 2.1c**, model 3), essentially safeguarding GABA neurotransmission. Further, this work advances our understanding of PITX transcription factor function in the nervous system by uncovering: a) a dual role (activator and repressor) for UNC-30 in GABA MNs, and b) multiple additional UNC-30 target genes. Altogether, our findings on UNC-30/PITX may help us understand the molecular mechanisms underlying the Axenfeld-Rieger syndrome and other genetic conditions caused by PITX gene mutations.[40-42]

2.5.1 Transcriptional coordination of two spatially separated processes: NT biosynthesis in the presynaptic cell and postsynaptic NT receptor clustering

To enable synaptic output, the presynaptic neuron must synthesize, package, and release a specific NT, whereas the postsynaptic neuron must present cognate NT receptors. Whether and how these distinct and spatially separated processes are coordinated remains poorly understood. Here, we show that the conserved transcription factor UNC-30/PITX coordinates the process of GABA biosynthesis in *C. elegans* MNs with $GABA_AR$ clustering in postsynaptic target muscle cells. Through inducible protein depletion (**Figure 2.6**), we found that UNC-30 is required in late larval and adult stages to maintain expression of GABA biosynthesis genes (e.g., *unc-25/GAD, unc-47/VGAT*), consolidating its role as a terminal selector of GABA MN identity.[16,17].Further, UNC-30 acts directly to activate and maintain transcription of *madd-4B/Punctin,* a secreted synapse organizer necessary for $GABA_A$ receptor clustering on target muscle cells.[25] Because UNC-30/PITX is continuously required in GABA MNs, this simple coregulatory strategy of *unc-25/GAD, unc-47/VGAT*, and *madd-4B/Punctin* by a terminal selector may ensure that key features of a functional synapse will continue to appear together throughout life (**Figure 2.1c**), ensuring continuous GABA neurotransmission. Hence, the presynaptic neuron will continue to synthesize and release GABA (ensured by continuous *unc-25/GAD* and *unc-47/VGAT* expression) and the postsynaptic neuron will constantly have the means to receive GABA via cognate receptor clustering (ensured by continuous m*add-4B/Punctin* expression). Because UNC-30 orthologs are expressed in planarian,[59,60] fly,[61] zebrafish,[62] and mouse nervous systems,[63,64] the coregulatory principle described here may be broadly applicable across species.

2.5.2 Terminal selectors control synaptic connectivity

The only other known example of a terminal selector that operates in an analogous manner is UNC-3, the sole *C. elegans* ortholog of the COE (Collier/Olf/EBF) family of proteins.[65] In nerve cord cholinergic MNs, UNC-3 acts as a terminal selector, directly regulating scores of effector genes (e.g., ACh biosynthesis proteins, ion channels, neuropeptides).[66,67] Like *unc-30*, *unc-3* is not expressed in *C. elegans* muscles. Yet, it is required for AChR clustering on muscle cells.[37] In cholinergic MNs, UNC-3 not only directly activates *madd-4B* (whose function in these cells is discussed in next section), but also *madd-4L*, which is required for AChR clustering.[37] On the other hand, we find that UNC-30 activates *madd-4B* but represses *madd-4L* in GABA MNs, thereby ensuring expression of the appropriate *madd-4* isoform (*madd-4B*). Altogether, NT receptor clustering in *C. elegans* neuromuscular synapses is achieved by two different terminal selectors regulating, in distinct ways, the two isoforms of the same synapse-organizing molecule. UNC-3 activates both *madd-4B* and *madd-4L* in cholinergic MNs, whereas UNC-30 activates *madd-4B* but represses *madd-4L* in GABA MNs.

Besides NT receptor clustering, additional synaptic connectivity defects have been reported in MNs of *unc-3* and *unc-30* mutant animals.[37,46,47,68,69] Specifically, cholinergic MN input onto GABA MNs is disrupted in *unc-3* mutants[68]. In this case, UNC-3 controls *nrx-1*/neurexin, a synapse organizer necessary for AChR localization onto dendrites of GABA MNs.[69] Presynaptic specializations of cholinergic MNs onto muscle cells are also affected in *unc-3* mutants,[37] but the underlying mechanism remains unclear. On the other

hand, UNC-30 has been implicated in *C. elegans* synaptic remodeling, as it is necessary to prevent premature synapse rewiring of DD neurons and aberrant synapse rewiring of VD neurons.[46,47] This is achieved by UNC-30 directly regulating OIG-1, a single Ig domain protein that functions as a synaptic organizer.[46,47] Consistent with a recent review,[6],this work and the aforementioned studies provide strong evidence for expanding the definition of terminal selector genes. That is, terminal selectors not only regulate effector genes critical for NT biosynthesis and neuronal signaling (e.g., ion channels, neuropeptides), but also control synaptic connectivity via the regulation of distinct synapse-organizing molecules.

It is tempting to speculate that mammalian terminal selectors may operate in an analogous manner. For example, the terminal selector of mouse spinal MNs, Isl1,[70] may control the transcription of agrin, a MN-derived synapse organizer necessary for AChR clustering on mouse skeletal muscles.[71] Curiously, although the function of MADD-4/Punctin in *C. elegans* is reminiscent of mammalian agrin in mice, *agr-1 (*agrin ortholog) is not involved in *C. elegans* neuromuscular synapse formation.[72]

2.5.3 Neuron type-specific regulation of synapse organizers

Synapse organizers are cell adhesion or secreted molecules that control synapse formation and/or maintenance.[4,73] Their adhesive and signaling properties mediate uni- or bidirectional signaling, enabling pre- and/or postsynaptic differentiation.[74] Understanding the spatiotemporal regulation of synapse organizers is important because

synapses must be built at the right place and time. However, we know very little about the transcriptional mechanisms that control synapse organizer expression, in part because these molecules usually have multiple isoforms (e.g., neurexins, neuroligins, leukocyte common antigen-related receptor protein-tyrosine phosphatases [LAR-PTPRs], Agrin, MADD-4/Punctin).[75-77] Multiple isoforms can be produced via either alternative RNA splicing or promoter usage. To date, substantial research has focused on the regulation of alternative splicing of synapse organizers (e.g., neurexin isoforms),[78] leaving the transcriptional mechanisms underlying alternative promoter usage poorly understood. Our work uncovered a transcriptional mechanism for the spatial (neuron type-specific) regulation of two different isoforms (produced via alternative promoter usage) of the same synapse organizer (MADD-4/Punctin) in *C. elegans* neuromuscular synapses.

MADD-4L is only produced by cholinergic MNs.[25,37] Upon secretion, it promotes clustering of the levamisole-sensitive heteromeric ACh receptor (L-AChR) by an extracellular scaffold composed of LEV-10 (Levamisole resistant-10), LEV-9 and OIG-4 (One ImmunoGlobulin domain-4).[25,30-32] On the other hand, MADD-4B is produced by both cholinergic and GABAergic MNs. At GABAergic neuromuscular synapses, MADD-4B promotes $GABA_AR$ clustering on muscle cells through binding to NLG-1/neuroligin and activation of UNC-40/DCC signaling.[26-28] At cholinergic neuromuscular synapses, MADD-4B inhibits the attraction of $GABA_A$ receptors by MADD-4L.[25] Hence, spatial (neuron type-specific) regulation of MADD-4 isoform expression is crucial for the formation and function of excitatory (ACh) and inhibitory (GABA) synapses in *C. elegans*. Our previous work identified UNC-3 as a critical activator of both *madd-4* isoforms in

cholinergic MNs.[37] Here, we show in GABA MNs that UNC-30 controls the two *madd-4* isoforms in opposite ways; it provides direct and positive input to the *madd-4B* promoter and negative input to the *madd-4L* promoter, thereby ensuring proper $GABA_A$ receptor clustering on target muscle cells.

2.5.4 Advancing our understanding of PITX gene function in the nervous system

In humans, *PITX* gene mutations cause various congenital defects and cancer[42]. *Pitx* genes belong to the PAIRED (PRD) class of highly conserved homeobox genes; vertebrates have three *Pitx* genes and invertebrates have one. In mice, *Pitx* genes play critical roles in the development of the nervous system, craniofacial structures, and limbs.[42] *Pitx2* and *Pitx3* are expressed in discrete cell populations of the mouse midbrain and spinal cord, and their expression persists into adult stages.[63,64,79] *Pitx3* is essential for the survival of dopaminergic neurons of the substantia nigra, a key cellular substrate of Parkinson's disease.[64] Importantly, human mutations in *PITX2* or *PITX3* affect eye development.[42,80] *PITX2* mutations cause Axenfeld-Rieger syndrome, a primary eye disorder that also affects the craniofacial and cardiovascular systems, whereas PITX3 mutations are associated with congenital cataracts.[40,41]

Mechanistically, functional assays showed that human *PITX2* and *PITX3* gene mutations result in reduced transcriptional activity.[81-83] However, the transcriptional targets of PITX proteins remain poorly defined and whether they act as transcriptional activators and/or repressors is not well defined. Our study contributes to these knowledge

gaps in three ways. First, we identify nine new UNC-30 target genes (*madd-4B/PunctinB, madd-4L/PunctinL, mab-9/Tbx20, nhr-49/Hnf4a, nhr-40/NHR, tsp-7/Cd63, aman-1/Man2b1, unc-53*/NAV1, *glr-5*/GRIK4), significantly expanding the list of PITX targets in the nervous system (**Table 2.3**). Second, consistent with its previously described direct mode of activation of genes involved in GABA biosynthesis and neuronal rewiring,[16,46,47,51] our mutational analysis and available ChIP-Seq data indicate that UNC-30 primarily acts directly to control the expression of these nine genes. Last, we found that six genes are activated (*madd-4B, tsp-7, aman-1, mab-9, nhr-40, nhr-49*) and three are repressed (*madd-4L, unc-53, glr-5*) by UNC-30. Because it binds directly to the *cis*-regulatory region of most of these genes, we propose that, in GABA MNs, UNC-30 acts as a direct activator and direct repressor of distinct sets of genes. Intriguingly, a similar dual role for UNC-30 has recently been described in *C. elegans* glia.[84]

2.5.5 Limitations of this work

Future studies are needed to dissect the molecular mechanism underlying the dual role of UNC-30 in GABA MNs. It is likely that cooperation with distinct transcription factors shifts its transcriptional activity from an activator to a repressor. Candidates include LIN-39/HOX, a known transcriptional activator in GABA MNs,[85] and UNC-55/NR2F, a known transcriptional repressor in these cells.[51,56] Another limitation relates to the maintenance of $GABA_AR$ clustering. Although we showed that UNC-30 is required to maintain *madd-4B* transcription in late larval/early adult stages, it remains unknown whether inducible UNC-30 depletion at these stages affects the maintenance of $GABA_AR$

clustering. Last, our work is focused on neuromuscular synapses. Notably, Punctin (ADAMTSL3) and other secreted synapse organizers (e.g., cerebellins, pentraxins, Sema3F, BDNF) are expressed in the mammalian brain.[35,36,86,87] Hence, similar coregulatory strategies to the one described here may operate in neuron-neuron or neuron-glia synapses in the central nervous system.

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CHAPTER 3

DISCUSSION AND FUTURE DIRECTIONS

3.1 Discussion

The proper performance of the nervous system relies on effective communication between various types of cells. For this, every neuron must acquire its unique identity, which ultimately leads to the establishment of functional synapses. Terminal selectors – transcription factors that are expressed throughout the life of an organism – control neuron terminal identity by regulating the expression of terminal identity genes, encompassing those responsible for synthesizing, packaging, and releasing specific neurotransmitters (e.g., neuropeptides, ion channels, enzymes).[1] To date, terminal selectors have been identified across a variety of neuron types in worms (*C. elegans*), fruit flies (*D. melanogaster*), cnidarians (*N. vectensis*), marine chordates (*C. intestinalis*), and mice (*M. musculus*), highlighting a deeply conserved role for these transcription factors in neuron terminal identity.[2] While the essential roles of terminal selectors in controlling neuron terminal identity are well-attested, there is still limited understanding of whether they also govern other crucial aspects of neuronal development, such as the establishment and maintenance of functional synapses.

Synapse differentiation is primarily mediated by synaptic organizers, cell adhesion or secreted molecules that facilitate the recruitment of presynaptic and postsynaptic

elements.[3,4] Understanding the spatiotemporal regulation of synaptic organizers is vital, as synapses must be established at the right locations, between the right cell types, and at the right time. However, we know very little about the transcriptional programs (i.e., mechanisms) controlling the expression of the synaptic organizers is limited.

Using UNC-30/PITX, the terminal selector of GABAergic motor neurons, this thesis provides evidence supporting an expanded definition of terminal selectors. We demonstrate that UNC-30 not only directly controls the expression of GABA terminal identity genes (*unc-25*/GAD, *unc-47*/VGAT, and *unc-46*/LAMP) but also the expression of synaptic organizers (*madd-4*/Punctin) in GABAergic motor neurons, thereby mediating the establishment of functional synapses. In addition, our study reveals that UNC-30/PITX is required for the maintenance of functional synapses, as it continuously controls the expression of GABA terminal identity genes and synaptic organizers throughout the lifespan of *C. elegans*. Given that defects in PITX (UNC-30 ortholog) and synaptic organizers have been linked to human disorders such as Alzheimer's disease and Axenfeld–Rieger syndrome, our findings offer invaluable insight into the field of medicine.[5–9]

3.1.1 Transcriptional programs control the establishment of motor neuron identity and neuromuscular synapses

To date, transcriptional programs have been identified to govern neuron terminal identity and differentiation.[10] Mechanistically, terminal selectors, present in particular

presynaptic neurons, directly bind to the cis-regulatory regions of neuron type-specific terminal identity genes (i.e., genes that code for biosynthesis of a specific neurotransmitter) and regulate their transcription via a consensus binding site. For instance, in mice, the transcription factor EBF2 is expressed in motor neurons of the hypaxial and medial columns of the developing spinal cord and is required for the proper differentiation of a subset of these neurons.[11] Notably, its role is conserved across species, as it compensates for the loss of UNC-3 (COE, Collier/Olf/EBF ortholog) in the nematode *C. elegans*. In this nematode, the terminal transcription factor UNC-3 controls the terminal identity of cholinergic motor neurons.[12–14] It directly binds to the COE sequence of genes that code for acetylcholine biosynthesis (*unc-17*/VAChT, *cho-1*/ChT, *cha-1*/ChAT), activating their expression. UNC-3 is simultaneously required to prevent the expression of genes normally expressed in other neuron types (e.g., GABA terminal identity genes), altogether, safeguarding cholinergic motor neuron identity and enabling proper synaptic output.

To receive input from the presynaptic neuron, in neuromuscular synapses, the muscle must present the corresponding neurotransmitter receptors. Despite not being expressed in the muscle, recent evidence reveals that UNC-3 plays a role in controlling the clustering of acetylcholine receptors at the postsynaptic side of cholinergic neuromuscular synapses.[15] Mechanistically, UNC-3 directly activates *madd-4*/Punctin, a synaptic organizer synthesized in the presynaptic neuron, which is subsequently secreted and localized at the postsynaptic side to facilitate the clustering of neurotransmitter receptors.[4,16,17] Altogether, this highlights the existence of a transcriptional program that coordinates two spatially separated processes: neurotransmitter biosynthesis in the

presynaptic neuron and neurotransmitter receptor clustering in the postsynaptic cell. Until now, it has remained unclear whether other neuron types coordinate these two processes or if other terminal selectors function in a similar manner.

In this thesis, we present evidence demonstrating that the terminal selector UNC-30/PITX coordinates GABA biosynthesis in GABAergic motor neurons and GABA neurotransmitter receptor clustering in the muscle of *C. elegans*. Mechanistically, UNC-30 directly binds to the consensus binding site (TAATCC) of genes that code for GABA biosynthesis (*unc-25*/GAD, *unc-47*/VGAT, and *unc-46*/LAMP), activating their expression.[18–20] Even though UNC-30 is not expressed in the muscle, UNC-30 regulates GABA receptor clustering on the postsynaptic side of GABAergic neuromuscular synapses by directly controlling the expression of synaptic organizer *madd-4*/Punctin.

One might wonder how the same synaptic organizer facilitates acetylcholine and GABA receptor clustering in different neuron types of *C. elegans*. Studies show that, through alternative promoter usage, *madd-4/*Punctin generates two isoforms: a long isoform (*madd-4L*) and a short isoform (*madd-4B*).[4] These isoforms are expressed in a neuron-type-specific manner, and each has a distinct function. Cholinergic motor neurons produce both *madd-4L* and *madd-4B*.[15,17] Upon secretion, *madd-4L* mediates the clustering of the levamisole-sensitive heteromeric acetylcholine receptor through an extracellular scaffold composed of LEV-10 (levamisole resistant-10), LEV-9, and OIG-4 (one immunoglobulin domain-4). Simultaneously, in cholinergic motor neurons, *madd-4B* interacts with *madd-4L* to inhibit GABA receptor clustering at cholinergic neuromuscular synapses. On the other hand, GABAergic motor neurons exclusively

produce *madd-4B*, which mediates the clustering of GABA receptor type A receptors through interacting with other synaptic organizers (e.g. NLG-1/neuroligin) and activating UNC-40/DCC signaling.

How do GABAergic motor neurons avoid *madd-4L* expression and prevent acetylcholine receptor clustering at GABAergic neuromuscular synapses? In this thesis, we demonstrate that UNC-30 represses *madd-4L,* revealing a novel dual role for UNC-30 as both an activator and a repressor. This newly identified function extends to seven other genes: UNC-30 activates five genes (*mab-9*/Tbx20, *nhr-49*/Hnf4a, *nhr-40*/NHR, *tsp-7*/Cd63, *aman-1*/Man2b1) while repressing two others, which are typically expressed in cholinergic motor neurons (*unc-53*/NAV1, *glr-5*/GRIK4). Moreover, our mutational analysis and available ChIP-Seq data suggest that UNC-30 acts directly to control the expression of these seven genes.[18] Together, these instances illustrate a transcriptional program that coordinates the establishment of neuron terminal identity and the functionality of neuromuscular synapses by controlling both neurotransmitter biosynthesis in the presynaptic neuron and neurotransmitter receptor clustering in the postsynaptic cell. It would be intriguing to investigate whether this mechanistic model extends across species and if the function of these terminal selectors is conserved.

3.1.2 Transcriptional programs control the maintenance of motor neuron identity and neuromuscular synapses

In the former section, we discuss the significance of establishing neuron terminal identity and the functionality of neuromuscular synapses, along with the transcriptional programs that govern these processes. However, equally important is the ability of these neurons to maintain their identity and synapse functionality throughout the life of an organism.

For instance, the terminal selectors UNC-3 and UNC-30 play pivotal roles in establishing cholinergic and GABAergic motor neuron terminal identity, respectively, and neurotransmitter receptor clustering.[1,2,10,12,19–23] These terminal selectors are expressed throughout the lifespan of *C. elegans,* and existing evidence indicates that UNC-3 is continuously required to maintain the expression of cholinergic terminal identity genes.[10,22] However, it remained unclear whether UNC-30 is continuously required to maintain the expression of GABAergic terminal identity genes. Additionally, the continuous requirement of these terminal selectors in maintaining neurotransmitter receptor clustering is not yet established.

In this thesis, we provide evidence showcasing that the terminal selector UNC-30/PITX not only activates but also maintains the expression of GABA terminal identity genes (*unc-25*/GAD and *unc-47*/VGAT) in GABAergic motor neurons. Furthermore, our findings indicate that UNC-30 is required to maintain the expression of the synaptic organizer *madd-4B*/Punctin, strongly suggesting that this terminal selector is continuously essential for maintaining proper GABA receptor clustering on the postsynaptic side of GABAergic neuromuscular synapses. An intriguing avenue for

further exploration would be to investigate whether UNC-3 is continuously required to maintain the expression of both isoforms of this synaptic organizer (*madd-4L* and *madd-4B*) in cholinergic motor neurons and assess if the continuous maintenance of MADD-4 is indeed crucial to sustaining proper neurotransmitter receptor clusters on the postsynaptic side of neuromuscular synapses.

3.1.3 PITX in regulating GABA neuron identity, synapse assembly, and implications in diseases

In a healthy nervous system, neurons send and receive information through electrochemical signaling, involving various neurotransmitters and their corresponding receptors (respectively). Disruptions in this process (i.e., neuronal communication) can occur in multiple ways. For instance, the presynaptic neuron or the postsynaptic side may exhibit abnormal levels of neurotransmitters or their receptors (respectively). Another possibility is the failure of neurons to differentiate. Collectively, these scenarios are linked to several pathological changes in the brain and neuronal diseases.[5,8,9,24,25]

Importantly, the inhibitory neurotransmitter GABA plays a crucial role in basic brain function, and its dysregulation, along with that of GABA neurotransmitter receptors, can lead to health complications. For example, patients with Alzheimer's disease (AD) or Parkinson's disease (PD) exhibit abnormal levels of the GABA neurotransmitter (AD), GABA neurotransmitter receptor (PD), or both (PD).[26,27] This

raises the question: How are levels of GABA, and its neurotransmitter receptor, regulated?

The bicoid-related subclass of the homeodomain transcription factor family, PITX, is conserved across nearly all members of the animal kingdom, with vertebrates possessing three *Pitx* paralogs (*Pitx1-3*) and non-vertebrates having just one *Pitx* gene. PITX2 emerges as a crucial player in the development of GABAergic neurons. Studies in mice reveal PITX2 as a direct regulator of *Gad1* – a gene that codes for an enzyme that mediates the major physiological supply of GABA in mammals by catalyzing the decarboxylation of GABA – implicating PITX2 as a regulator of GABAergic differentiation during mammalian development.[28]

In this thesis, we characterize the PITX ortholog in *C. elegans*, UNC-30, and its role in GABAergic motor neuron development. UNC-30 acts as a terminal selector not only directly activating the expression of GABA biosynthesis genes (*unc-25*/GAD and *unc-47*/VGAT) but also playing a crucial role in maintaining their expression. Importantly, our data reveals that UNC-30's control over the expression of these genes is not a simple binary (on or off) mechanism but involves more intricate and subtle regulatory patterns. We observe varying reductions in the expression levels of GABA biosynthesis genes (unc-25/GAD and *unc-47*/VGAT), with the extent of reduction differing based on the neuron type. This proposed mechanistic model extends to UNC-30's control over the expression levels of the synaptic organizer *madd-4B*/Punctin, which, in turn, mediate GABA receptor clustering. This finding implicates UNC-30 in potentially controlling GABA receptor levels on the postsynaptic side. Importantly, its role is

conserved across species, as PITX2 compensates for the loss of UNC-30 in the nematode *C. elegan*. However, the scope of this understanding is limited, as the study primarily focused on the loss (on and off) of GABA biosynthesis gene expression. It would be interesting to investigate whether PITX2 influences GABA biosynthesis gene expression levels and whether it additionally regulates the expression of genes that code for synaptic organizers, thereby exerting control over GABA receptor clustering.

Collectively, these findings emphasize the significance of characterizing the molecular mechanisms governing GABAergic synapse differentiation. A comprehensive understanding of the intricacies of this transcriptional program, specifically the role of UNC-30/PITX, becomes particularly crucial when considering its potential implications for advancing disease diagnosis, treatment, and prevention.

3.2 Future directions

This section marks the starting point for contemplating what comes next — a forward-looking journey into new territories. As we characterize molecular mechanisms governing the development of a functional nervous system, we're also intrigued by the potential applications of the intricate details we discover. Our curiosity extends beyond neurons, wondering how these insights might reach into other types of cells.

3.2.1 Uncovering the mechanism underlying the dual role of UNC-30/PITX: activating genes while repressing other genes in GABAergic motor neurons

Characterizing the molecular mechanisms governing genetic regulation during nervous system development holds the key to understanding complex processes such as synaptic differentiation (both at the presynaptic and postsynaptic sides). This thesis sheds light on the distinctive function of UNC-30/PITX, a transcription factor with a dual role — simultaneously activating specific genes while repressing others — in the context of GABAergic motor neurons. This terminal selector is essential for shaping the identity of GABAergic neurons, as it directly activates the expression of genes required for GABA biosynthesis and GABA receptor clustering, while also repressing the expression genes normally expressed in cholinergic motor neurons (i.e., genes involved in acetylcholine biosynthesis and acetylcholine receptor clustering). Altogether, these findings contribute to the intricate web of molecular mechanisms that mediate synapse formation and functionality. This raises the question: How does a transcription factor activate one set of genes while concurrently repressing another in the same cell type? Importantly, in the context of UNC-30, we observe how this transcription factor exerts a dual regulatory role within the same gene, activating its short isoform and repressing its long isoform, ultimately safeguarding synaptic functionality. As we embark on the exploration into UNC-30/PITX's dual regulatory function (discussed below), we delve into the nuanced molecular events underpinning the development of GABAergic motor neurons.

3.2.1.1 Cofactors function with transcriptional programs in the context of motor neuron identity

In *C. elegans*, the terminal selector UNC-3 (COE) plays a critical role in safeguarding the identity of cholinergic motor neurons. Mechanistically, it not only directly activates genes essential for acetylcholine biosynthesis but also represses genes typically expressed in GABAergic motor neurons. Recent studies have uncovered additional players influencing UNC-3's dual role — deciding which genes are activated or repressed.[12] The mid-body Hox proteins LIN-39 (homolog of Scr/Dfd/Hox4-5) and MAB-5 (homolog of Antp/Hox6-8) collaborate with UNC-3 in cholinergic motor neurons, acting as direct activators for several terminal identity genes.[12] Mechanistically, evidence also suggests Hox proteins act as rate-limiting factors crucial for maintaining cholinergic motor neuron identity. In the absence of UNC-3, LIN-39 no longer binds to the cis-regulatory region of cholinergic identity genes, altering its genetic target – now activating genes normally expressed in other neuron types – and compromising cholinergic motor neuron identity. Preliminary data indicate that LIN-39 and MAB-5 also collaborate with UNC-30 in GABAergic motor neurons to regulate terminal identity gene expression. While the molecular mechanism behind UNC-30's dual role remains unknown, this preliminary evidence suggests the involvement of additional players in determining UNC-30-dependent activation or repression of specific genes. Uncovering this mechanism will elucidate the extent of UNC-30's function in safeguarding GABAergic motor neuron identity.

3.2.1.2 Insights into the role of coactivators and corepressors driving UNC-30/PITX function

In this thesis and former sections, we highlight the novel dual function of the terminal selector UNC-30 (COE) in safeguarding the identity of GABAergic motor neurons. Mechanistically, it not only directly activates genes essential for GABA biosynthesis but also represses genes typically expressed in cholinergic motor neurons. How does a transcription factor activate one set of genes while concurrently repressing another in the same cell type? Here I discuss candidates for additional players influencing UNC-30's dual role — deciding which genes are activated or repressed (**Figure 3.1**).

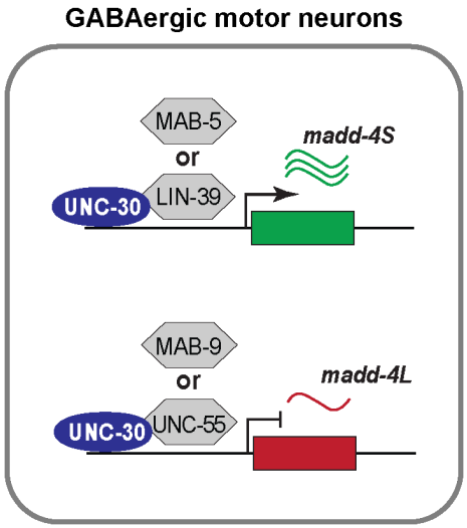

Figure 3.1: Model of cofactors dictating UNC-30's dual role.
(Top) Coactivators mediate UNC-30's role of activating (green) gene expression. (Bottom) Corepressors mediate UNC-30's role of inhibiting (red) gene expression.

Preliminary data indicate that Hox proteins LIN-39 (homolog of Scr/Dfd/Hox4-5) and MAB-5 (homolog of Antp/Hox6-8) collaborate with UNC-30 in GABAergic motor

neurons to regulate terminal identity gene expression in *C. elegans*.[29] For instance, GABAergic motor neurons lose expression of GABA biosynthesis genes in animals lacking LIN-39, indicating LIN-39 as a potential coactivator. Through a literature search, we also identified conserved transcription factors known to act as corepressors (MAB-9/Tbx20, UNC-55/COUP) of gene expression in nerve cord motor neurons of *C. elegans*.[18,30–33] However, whether these corepressors act together with UNC-30/PITX to repress cholinergic terminal identity genes and *madd-4L*/Punctin is unknown. In addition, the molecular mechanism between these additional players (coactivators and corepressors) and UNC-30-dependent activation or repression of specific genes remains unknown, representing an area of great interest for future studies.

3.2.1.3 Bioinformatic analysis offers insights into potential mechanisms underlying UNC-30/PITX function

In the context of neurodevelopmental processes, transcription factors, often referred to as terminal selectors, and coregulators play a pivotal role in shaping neuron identity and postsynaptic differentiation. Traditionally, the understanding has been that these molecular players operate in concert, with terminal selectors acting as the main drivers and coregulators fine-tuning their activity. However, recent advances in bioinformatic and biochemical analyses have unveiled a deeper complexity, revealing how the DNA sequence itself influences protein binding and subsequent functional outcomes, including the activation or repression of genes. For instance, Bicoid is a morphogen crucial for the development of the Drosophila embryo - it activates the transcription of multiple target

genes in different positions along the anterior–posterior.[34] Studies suggest that the number and affinity of Bicoid binding sites significantly impact its regulatory role on its target genes.[35,36] Mechanistically, cooperative DNA binding by Bicoid further characterizes its function.[36,37] Moreover, Bicoid exhibits dual functionality as both a gene activator and a repressor.[34,35] For example, it represses the translation of Caudal, a homeodomain transcription factor pivotal for activating genes essential in abdomen formation.[34,35,38] These findings have implications not only for Drosophila development but also for studies involving gene regulation.

In this thesis, we uncover the dual role of UNC-30/PITX, a member of the bicoid-related subclass within the homeodomain transcription factor family. Our studies support UNC-30's direct activation of genes via a consensus binding site (TAATCC). However, whether UNC-30 exerts its repressive role directly or indirectly, remains unclear. If direct, it's uncertain if UNC-30 directly binds its consensus binding site or another site for repression. In addition, it would be intriguing to explore whether the number and affinity of these binding sites influence its regulatory function. Altogether, understanding UNC-30's specific binding motifs and cooperative dynamics would offer insights into its pivotal role in gene expression regulation and neuronal development.

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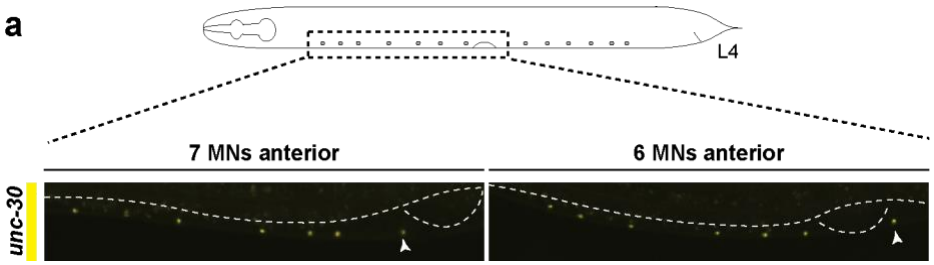
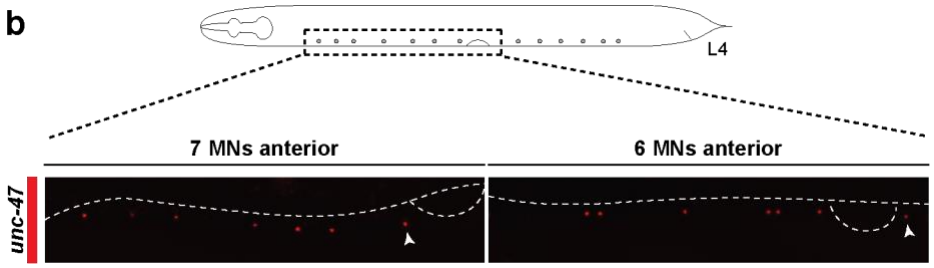
APPENDIX A

FLUORESCENT REPORTER ANALYSIS HIGHLIGHTS GABAERGIC MOTOR NEURON MIGRATION VARIABILITY



Temperature	% of worms displaying 7 MNs anterior to vulva	% of worms displaying 6 MNS anterior vulva
15º	68%	32%
20º	73%	27%
25º	64%	36%



Temperature	% of worms displaying 7 MNs anterior to vulva	% of worms displaying 6 MNS anterior vulva
15º	95%	5%
20º	100%	0%
25º	86%	14%

Figure A.1: GABAergic motor neuron VD7 migrates anterior or posterior to the vulva.
(a-b) Percentage of animals with GABAergic motor neuron VD7 migration anterior or posterior to the vulva. Animals were raised at the described temperatures and analyzed at L4 stage. (a) Animals analyzed are homozygous for *syb2344 [unc-30::mNG::3xFlag::AID]*. (b) Animals analyzed are homozygous for *otIs564 [unc-47(fosmid)::SL2::H2B::mChopti + pha-1(+)]*. n=20.

APPENDIX B

TEMPORAL MODULARITY IN UNC-30/PITX FUNCTION IN GABAERGIC MOTOR NEURONS

The data in this appendix is from Li et al., *Elife*, in which I am the secondary author. I am the main contributor to the work included in this section.

Relevant Publication

Li, Y., Osuma, A., Correa, E., Okebalama, M.A., Dao, P., Gaylord, O., Aburas, J., Islam, P., Brown, A.E. and Kratsios, P., 2020. "**Establishment and maintenance of motor neuron identity via temporal modularity in terminal selector function.**" Elife, 9, p.e59464.

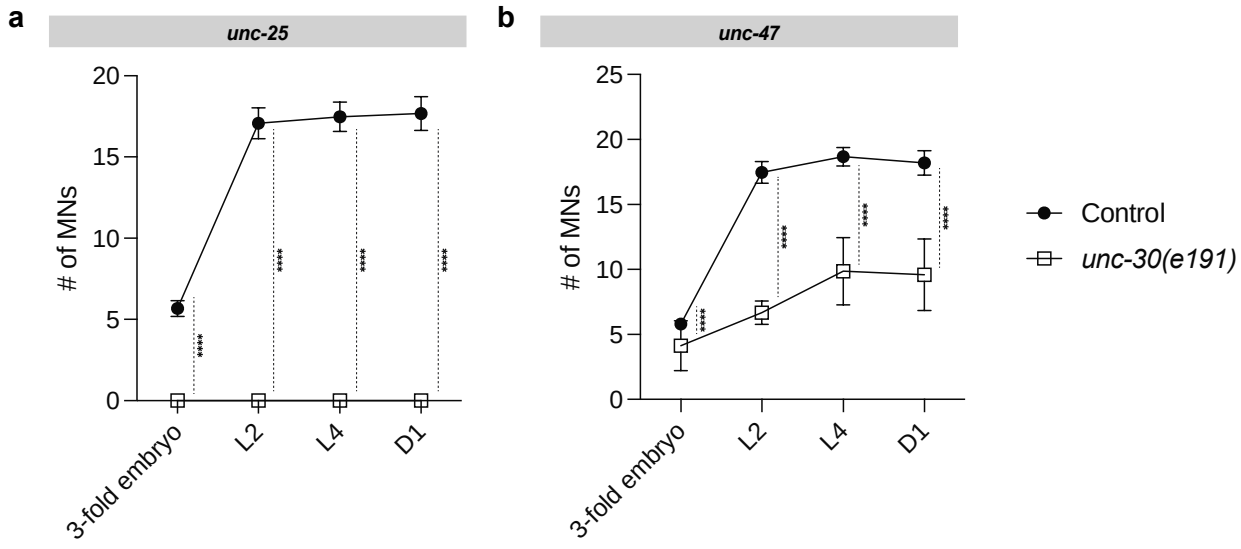


Figure B.1: UNC-30 is required for both initiation and maintenance of *unc-25*/GAD and *unc-47*/VGAT.

(a-b) Quantification of the expression of GABAergic terminal identity gene reporters (unc-25/GAD, unc-47/VGAT) in control and *unc-30(e191)* animals at 4 different developmental stages: 3-fold embryo, L2, L4, and day 1 adults. Data points show mean, whiskers show SD. Unpaired t-test with Welch's correction. *** $p < 0.001$. n=15. (a) Animals analyzed are homozygous for *otIs514 [unc-25p::unc-25(partial)::GFP::unc-54 3'UTR + pha-1(+)].* (b) Animals analyzed are homozygous for *otIs564 [unc-47(fosmid)::SL2::H2B::mChopti + pha-1(+)].*

APPENDIX C

A PRELIMINARY INSIGHT OF HOX FUNCTION IN REGULATING GABA TERMINAL IDENTITY GENES

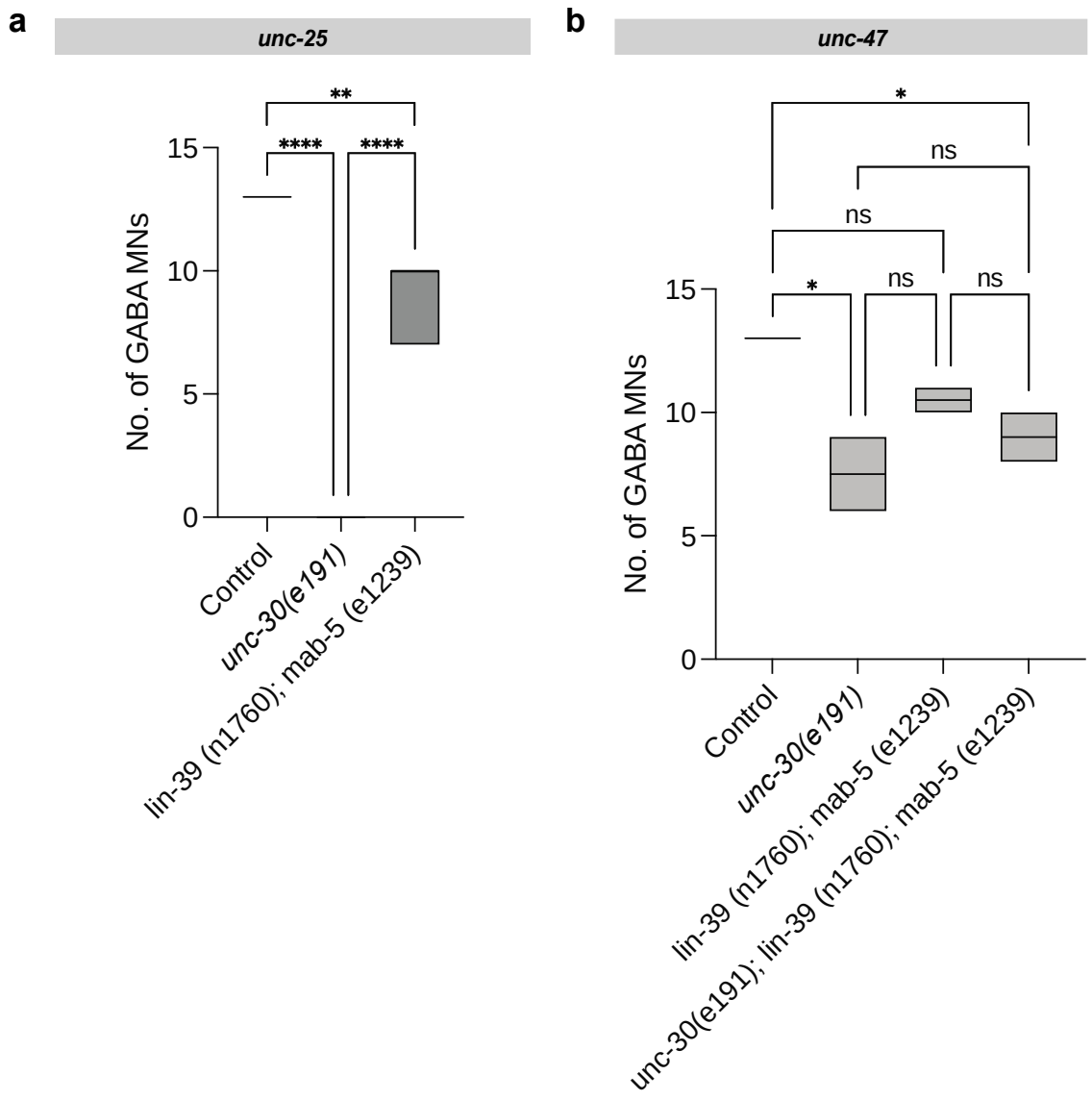


Figure C.1: Hox genes are required to establish GABA terminal identity.
(a-b) Quantification of the expression of GABAergic terminal identity gene reporters (unc-25/GAD, unc-47/VGAT) in control and mutant animals at L4 stage. Unpaired t-test with Welch's correction. *p < 0.01, **p < 0.001, ****p < 0.0001. n=15. (a) Animals analyzed are homozygous for *otIs514 [unc-25p::unc-25(partial)::GFP::unc-54 3'UTR + pha-1(+)]*. (b) Animals analyzed are homozygous for *otIs564 [unc-47(fosmid)::SL2::H2B::mChopti + pha-1(+)]*.