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WormBook

Neurogenesis in *Caenorhabditis elegans*

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Animals rely on their nervous systems to process sensory inputs, integrate these with internal signals, and produce behavioral outputs. This is enabled by the highly specialized morphologies and functions of neurons. Neuronal cells share multiple structural and physiological features, but they also come in a large diversity of types or classes that give the nervous system its broad range of functions and plasticity. This diversity, first recognized over a century ago, spurred classification efforts based on morphology, function, and molecular criteria. *Caenorhabditis elegans*, with its precisely mapped nervous system at the anatomical level, an extensive molecular description of most of its neurons, and its genetic amenability, has been a prime model for understanding how neurons develop and diversify at a mechanistic level. Here, we review the gene regulatory mechanisms driving neurogenesis and the diversification of neuron classes and subclasses in *C. elegans*. We discuss our current understanding of the specification of neuronal progenitors and their differentiation in terms of the transcription factors involved and ensuing changes in gene expression and chromatin landscape. The central theme that has emerged is that the identity of a neuron is defined by modules of gene batteries that are under control of parallel yet interconnected regulatory mechanisms. We focus on how, to achieve these terminal identities, cells integrate information along their developmental lineages. Moreover, we discuss how neurons are diversified postembryonically in a time-, genetic sex-, and activity-dependent manner. Finally, we discuss how the understanding of neuronal development can provide insights into the evolution of neuronal diversity.

Keywords: neurogenesis; *Caenorhabditis elegans*; gene regulation; neurodevelopment; transcription factors; WormBook

Introduction

The complex functionalities of animal nervous systems rely on a vast diversity of neurons, which receive sensory inputs, integrate different internal and external signals, and produce cognitive and behavioral outputs. Neurons are highly specialized cells, with unique morphological and physiological properties and an outstanding capacity for intercellular communication. While they share several common functional and molecular properties, they present in a multitude of different types or classes. Cellular diversity within nervous systems was recognized more than 100 years ago when the remarkable range of differences in neuronal morphologies was revealed (Ramón y Cajal (1889-1904)), and prompted multiple efforts toward neuronal classification, initially based on morphology but later including functional and molecular criteria. As Sydney Brenner envisioned, *Caenorhabditis elegans* has provided a unique opportunity to understand how a complete nervous system develops, thanks to the knowledge of the total number of neurons in the system—302 in the adult hermaphrodite and 387 in the adult male—and their precise lineage origins. The complete reconstruction of the nervous system based on electron microscopy (EM) enabled a classification based on morphology and connectivity, uncovering 118 different classes in the hermaphrodite, with additional accurate predictions of whether they constituted sensory neurons, interneurons, or motor neurons (White *et al.* 1986; Cook *et al.* 2019). As discussed below, more recent molecular and functional classifications converge to the same number of *classes* and have helped to refine the number of *subclasses* (Table 1—Box of terms).

In this WormBook chapter, we summarize our understanding of how different neuron classes and subclasses are generated during development. The process of *neurogenesis* requires first that a cell be specified as a *neuronal progenitor* (Table 1—Box of terms), as opposed to e.g. a muscle or epidermal cell progenitor, and that it further differentiates into a particular neuron class and subclass. These steps require specific changes in gene expression and chromatin landscape that ultimately result in a cell expressing the necessary sets of genes to morphologically and functionally become a specific neuron class. *Caenorhabditis elegans* has been instrumental for understanding how the unique gene expression profiles that define the many neuron classes are determined during development. The possibility of analyzing gene expression with single-cell resolution in the well-defined *C. elegans* nervous system, based on countless transgenic studies and more modern single-cell

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Table 1. Box of terms.

Neuron types, classes, or identities
In this review, we refer to the 118 neuron types, classes, or identities of *C. elegans* hermaphrodite. These identities were initially proposed on the basis of anatomical characteristics such as position, morphology, neurite projection patterns, and synaptic connectivity (White *et al.* 1986). More recently, transcriptome information from scRNA-seq data confirmed this classification at the molecular level and expanded it to additional *neuron subclasses*, which differentiate neurons in the same class that differ in expression of specific set of genes and/or functions (Taylor *et al.* 2021).

Blastomere/blast cell
A blastomere or blast cell is the name given to cells that are produced by cell division of the zygote after fertilization. In *C. elegans*, a set of 6 so-called founder blastomeres give rise to the different cells of the embryo. These are AB, MS, E, C, D, and P_4, and they are briefly discussed in the text and reviewed in detail in Liu and Murray (2023).

Neurogenesis, neuronal specification, and neuronal differentiation
Neurogenesis is the overall process by which new neurons are formed during either embryonic or postembryonic development. Neuronal specification refers to the developmental process that drive precursors toward a neural fate, and neuronal differentiation refers to the acquisition of terminal neuronal features.

Neural induction
This is the process in vertebrate embryos by which the BMP, Hh, NGF, and other signaling pathways restrict competent ectodermal cells to adopt neuronal rather than nonneuronal cell fates (reviewed in Hemmati-Brivanlou and Melton 1997; Stern 2006).

Neural progenitor/precursor/neuroblast
A neuronal progenitor is a mitotic cell that will give rise to neurons. In this chapter, we use the terms neural progenitor, neural precursor, and neuroblast interchangeably, although we note that in the vertebrate literature, a neuroblast is postmitotic.

Progressive determination model
This is a model of sequential steps of neurogenesis that was proposed on the basis of genetic and molecular studies of the development of the *Drosophila* peripheral nervous system (Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1994).

Proneural transcription factors
This term, for historical reasons, refers to a specific subfamily of bHLH transcription factors that are both necessary and sufficient for neurogenesis in many animal species (reviewed in Bertrand *et al.* 2002).

Gene battery
Initially, this term was used for each of the sets of genes, expressed differently in individual cell types, that provide specific phenotypes (Morgan 1934). In this review, we have refined the definition, using the term to refer to a set of genes coexpressed because their *cis*-regulatory regions respond to common *trans*-acting factors (Britten and Davidson 1969). A particular cell type requires the operation of different and parallel gene batteries.

Regulatory program/gene regulatory network
Our review focuses mainly on transcriptional regulators of gene expression. Thus, we refer to gene regulatory networks (GRNs) or regulatory programs that provide the functional interconnections between target genes and transcription factors (TFs) leading to spatial and temporal cell-specific patterns of gene activity. Gene regulation is not a linear one-to-one process but rather occurs in the context of complex networks of interactions between multiple genes and multiple TFs that can be mapped onto graphic GRN diagrams. GRNs are composed of 2 types of modules, gene modules, which are defined as sets of genes bound by similar TFs (termed gene batteries), and TF modules, which are sets of TFs that share similar target genes.

Effector gene
A gene that defines the functional properties of a cell and is thus the endpoint of a gene regulatory network.

Terminal selector
In *C. elegans* neuron specification GRN, the set of TFs that directly control expression of neuronal effector gene batteries are termed *terminal selectors*. Terminal selectors act in combinations through binding to genomic enhancers and constituting the core of gene regulatory networks (GRNs) in neuronal terminal differentiation. The combinations of transcription factor binding sites of terminal selectors form enhancer codes that are characteristic for the identity of cell types. The set of terminal selectors acting in a particular neuron type is termed a *terminal selector collective*. As TFs are pleiotropic and expressed in different cell types, what provides neuron type specificity is the intersection of the different TF expression patterns that provide specific combinations of terminal selectors acting together on the genomic enhancers.

Neuronal genome
In addition to ubiquitous genes, a differentiated neuron is thought to express many hundreds if not thousands of genes that define its functional properties (neuronal gene batteries). These genes code, for example, for ion channels, G protein-coupled receptors, neurotransmitter-synthesizing enzymes, transporters and receptors, neuropeptides and their receptors, cell adhesion molecules, motor proteins, signaling molecules, and many others. These genes have been termed *neuronal terminal differentiation genes* or *neuron class effector genes* and collectively are known as the neuronal genome (Hobert 2013). The neuronal genome can be divided into different, partially overlapping gene batteries.

Panneuronal gene battery
Genes expressed in all neurons, coding for the neuronal machinery necessary to carry out all generic neuronal processes, are referred individually as *panneuronal effector genes* or collectively as the *panneuronal genome*.

Ciliome gene battery
The subset of neuronal effector genes coding for cilium components are known as *ciliome effector genes*. Ciliome genes can be divided into 2 types of gene batteries according to their expression pattern: structural cilia components expressed in all ciliated neurons comprise the *core ciliome gene battery*, while ciliome genes expressed in a neuron-type specific manner are part of the *neuron-type effector gene battery*.

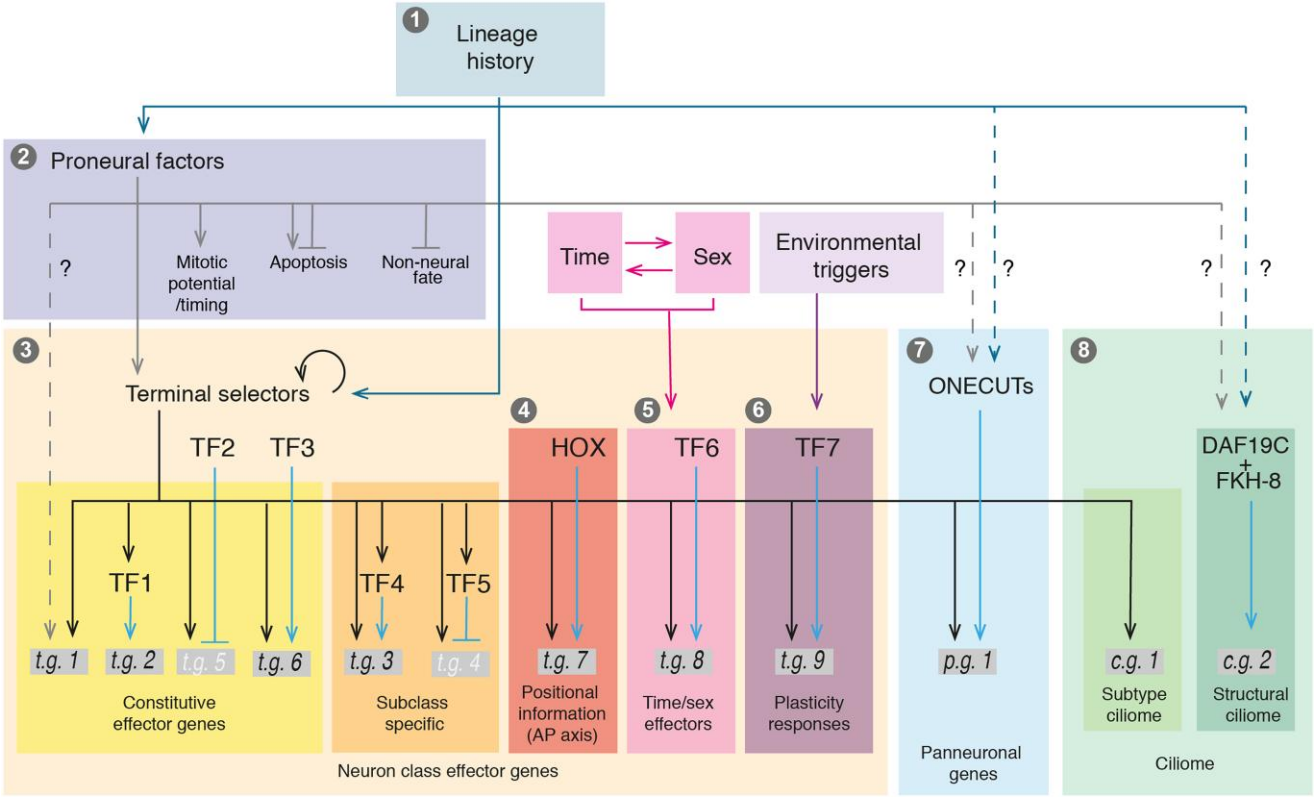

Fig. 1. Regulatory framework for *C. elegans* neurogenesis. Summary of the main regulatory interactions controlling neurogenesis and neuron diversification that should serve as a roadmap for this chapter. Development of each neuron class requires integration of regulatory information from different developmental timepoints into parallel, yet interconnected regulatory modules that coexist in the cell. (1) Lineage history determines the set of TFs expressed in the neuron and its progenitors, the signalling events it receives over time, and the particular chromatin landscape of the postmitotic neuron (see *Lineage-based mechanisms of neuronal diversification*). (2) bHLH proneural factors have evolutionary conserved roles in the specification of neuronal progenitors (see *Diverse functions of bHLH proneural TFs*). (3) Terminal selector TFs act in the postmitotic neuron to directly activate broad neuron class effector gene batteries (see *Rich sets of effector genes distinguish different neuron classes* and *Principles of neuron class specification by terminal selectors*). (4) Terminal selectors act together with HOX TFs to diversify some neuron subclasses along the A–P axis (see *Neuronal diversification across body axes*). (5) Neuron class transcriptomes are also shaped by postembryonic time or genetic sex (see *Neuronal diversification over developmental time and across sexes*). (6) Mature neurons can modify their transcriptome in response to environmental stimuli (see *Environmental effects on neuron gene expression*). (7 and 8) At least 2 regulatory modules run in parallel to those specifying neuron class properties: panneuronal effector genes, those shared by all neuron classes are under direct control of CUT HD TFs (7) (see *Panneuronal features define neurons as a tissue type*) while different TFs activate the ciliome components expressed by all sensory ciliated neurons (8) (see *Rich sets of effector genes distinguish different neuron classes*). *t.g.*, *neuron type effector gene*; *p.g.*, *panneuronal effector gene*; *c.g.*, *ciliome effector gene*.

sequencing approaches, has revealed shared *batteries of panneuronal genes* that define neurons as a distinct tissue, as well as class-specific *gene batteries* (Table 1—Box of terms). Moreover, the extensive genetic toolbox of *C. elegans* has allowed the elucidation of regulatory programs that drive *neuronal differentiation* (Table 1—Box of terms) and has revealed key principles.

Here, we specifically focus on the *gene regulatory networks* (Table 1—Box of terms) that drive neuronal development and diversification, primarily at the transcriptional level. The *C. elegans* genome codes for almost 900 different transcription factors (TFs), which can be classified in families according to their DNA-binding domain. Over 100 TFs from different families have been implicated in different steps of nervous system development. An overarching principle that has emerged is that the identity of a neuron can be defined by a number of *effector gene batteries* (Table 1—Box of terms), each under control of parallel yet interconnected regulatory mechanisms. Figure 1 provides a summary of our current understanding of neuron class gene regulatory networks and should serve as a guiding thread throughout the different sections of this chapter.

We first introduce the complexity of the *C. elegans* nervous system and its lineage origins. We provide a molecular description of the core gene batteries that define neurons and describe in broad terms how these separable batteries are regulated. Secondly, we describe the upstream mechanisms that specify neuronal progenitors, examining *C. elegans* neurogenesis within the framework of a *progressive determination model* (Table 1—Box of terms) that has guided the understanding of neurogenesis across different animals. We describe similarities with and differences from flies and vertebrates, paying close attention to the roles of the main classes of conserved TFs involved. We then delve deep into the intricacies of neuronal diversification, summarizing a wealth of work from which we are learning how cells integrate regulatory information along the lineage, but also postembryonic developmental time, genetic sex, and environmental stimuli, to give rise to the 302 distinct neurons (387 in the male) that make up our favorite nervous system. To conclude, we discuss how our knowledge of neuronal diversification during development can illuminate our understanding of how neuronal diversity may evolve.

Lineage origin and molecular makeup of *C. elegans* neurons

Classes and lineage origins of *C. elegans* neurons

To achieve complex functions, nervous systems require a sophisticated degree of division of labor, giving rise to the most intricate cellular diversity found in any organ. The 118 morphologically different

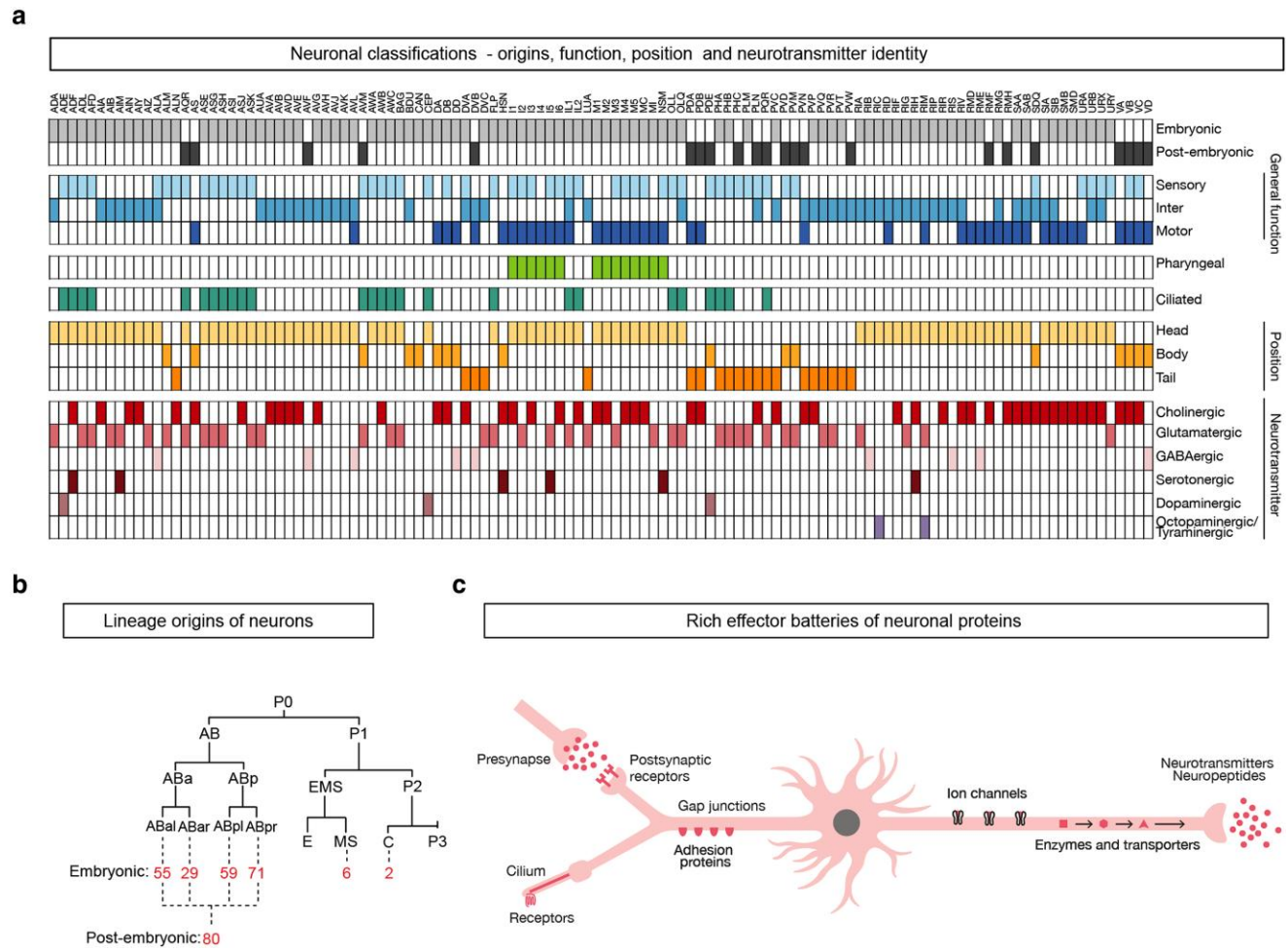

Fig. 2. Developmental origin of neurons and neuronal diversity. a) Matrix of the 118 morphologically different neuron classes in hermaphrodite *C. elegans* classified according to different criteria including embryonic/postembryonic lineage, function, and neurotransmitter identity. For simplicity, large effector gene categories, such as neuropeptides or innexins, have not been included but references to their expression patterns are provided in the text. b) Lineage origins of all 302 embryonically and postembryonically generated hermaphrodite neurons. c) A cartoon illustration of a generic neuron depicting different sets of proteins required for specific neuronal functions, which include panneuronal factors such as synaptic components, cilium proteins (ciliome) present in ciliated sensory neurons, and different sets of neuron class-specific effector proteins such as adhesion molecules, enzymes, ion channels, receptors, or neuropeptides.

classes in the *C. elegans* hermaphrodite can be grouped in different manners that reflect broader, shared functional features defined ad hoc (Fig. 2a) (Wormatlas.org; Serrano-Saiz *et al.* 2013; Pereira *et al.* 2015; Gendrel *et al.* 2016). For example, there are 39 classes of sensory neurons, 41 of interneurons, 24 of motor neurons, and 14 of pharyngeal neurons that form the enteric nervous system of the foregut. Sensory neurons can be further subdivided according to the modality they sense (chemosensory, mechanosensory, thermosensory, etc.) or whether they have a sensory cilium (26/39 classes). Similarly, interneuron and motor neuron classes can be grouped on the basis of other functional properties, such as their specific behavioral outputs. Neurons can also be divided into 2 broad groups depending on the organs they form part of: 14 enteric neuron classes are embedded in the pharynx (foregut) and are synaptically connected to the other 104 somatic neuron classes only through the RIP class of neurons. Another broad classification groups neurons according to the neurotransmitter they produce. Although a few neuron classes produce more than 1 neurotransmitter, they can be roughly classified as follows: 52 cholinergic (ACh), 39 glutamatergic (Glu), 9 GABAergic (GABA), 3 dopaminergic (Dop), 6 serotonergic (5HT), 1 octopaminergic, 1 tyraminergic, and 13 without known classic neurotransmitter identity. Every neuron can be defined by a combination of these (and countless other) features, e.g. AWB is a ciliated, cholinergic, chemosensory neuron, while VD12 is a posterior GABAergic motor neuron that innervates dorsal–ventral (D–V) muscles. Males have an additional set of 93 neurons that fall into 27 classes (reviewed in Tekieli *et al.* 2021). Importantly, all these different functional criteria for neuron classification have molecular bases.

In animals with well-defined germ layers, neurons and glia are ectodermal in origin and are distinguished early in development from other nonneural ectodermal cells (e.g. epidermal cells) as well as from cells from mesodermal (e.g. muscle cells) and endodermal (e.g. intestinal cells) origin. In *C. elegans*, germ layers are less well defined, and 3 of the 5 somatic founder *blastomeres* (Table 1—Box of terms) (AB, MS, and C) do not give rise exclusively to cells from 1 germ layer type; the exceptions are the D lineage that produces exclusively mesodermal body wall muscles and the E lineage that produces exclusively endodermal gut cells (Sulston *et al.* 1983; Liu and Murray 2023). Nevertheless, the AB lineage gives rise to the vast majority of the 222 embryonically born neurons (Fig. 2b) (Sulston *et al.* 1983). Neurons arise nonclonally from this blastomere, which also gives rise to nonneural ectodermal derivatives such as hypodermis and a few mesodermal

derivatives such as muscles. In addition, 2 neurons arise from the C blastomere (DVC and PVR), which also gives rise to hypodermal cells and muscles, and 6 pharyngeal neurons arise from the MS blastomere (I3, I4, I6, M1, M4, and M5), which otherwise gives rise to mesodermal derivatives. Postembryonically, several ectodermal *blast cells* act as *neural progenitors* (Table 1—Box of terms) and give rise to an additional 80 neurons in the hermaphrodite (Fig. 2b) (Sulston and Horvitz 1977; Sulston 1983). Surprisingly, these blast cells display varying degrees of differentiation, raising interesting questions about cell fate plasticity during postembryonic neural development (Lambert *et al.* 2021). Of note, many neurons are derived from terminal divisions in which the sister cell undergoes apoptosis (Sulston and Horvitz 1977; Sulston *et al.* 1983). Several genes including *pig-1*, *ham-1*, *cnt-2*, *ces-1*, and *ces-2* have been implicated in the asymmetric division of these neural progenitors and the neuronal vs cell death decision (Ellis and Horvitz 1991; Sagasti *et al.* 1999; Singhvi *et al.* 2011; Feng *et al.* 2013; Wei *et al.* 2017).

The precise knowledge of the lineage history of every cell has allowed gene expression analyses at the single-cell level along individual lineage trajectories and has facilitated dissection of the role of key conserved regulators of early *neuronal specification* (Table 1—Box of terms). Moreover, the fact that we have a complete view of terminal fates produced from each branch provides additional information by suggesting the point at which key neuronal lineage specification events may be occurring. For example, a blast cell that only gives rise to neurons and glia may commit to these fates earlier than a cell that produces a neuron and a muscle cell in its terminal division. Study of the lineage also indicates that several neurons seem to reach the same terminal identity through different lineage paths, a phenomenon known as developmental convergence. The mechanisms for convergence are still largely unexplored, but we do come back to this concept as a means of generating neuronal diversity.

Panneuronal features define neurons as a tissue type

Neurons are highly specialized for sensing, integrating, and distributing information throughout an organism. In order to carry out these functions, all neuron classes share a number of features: at the cellular architecture level, they have axons and dendrites; at the organelle level, the chemical synapse is the most characteristic feature, converting electrical excitation into chemical signals and vice versa; and at the molecular level, they share the expression of numerous genes coding for the neuronal machinery necessary to carry out all generic neuronal processes (Fig. 2c) (Arendt 2020). Such genes are termed *panneuronal effector genes* (Table 1—Box of terms) and include proteins involved in synaptic vesicle biology (*unc-64/syntaxin* and *ric-4/SNAP25* or the GTPase *rab-3*), dense core vesicle biogenesis (such as the calcium-dependent secretion activator *unc-31*/CAPS or the GTPase *ric-19*), neuropeptide-processing enzymes (such as the peptidases *egl-3* and *egl-21*), and molecular motors (such as the kinesin-like protein *unc-104*) (Hobert 2013). In addition to their broad neuronal expression, some panneuronal genes are also expressed in nonneuronal tissues, for example SNB-1/Vamp or TBB-1/tubulin are ubiquitously transcribed, and UNC-18 displays intestinal expression (Stefanakis *et al.* 2015).

Panneuronal gene expression has recently been found to be directly controlled by the CUT subfamily of homeodomain (HD) TFs (Fig. 1) (Leyva-Díaz and Hobert 2022). These include *ceh-44* CUX HD and *ceh-48* ONECUT HD, which are expressed panneuronally, and 4 additional ONECUT HDs that are ubiquitously expressed. CEH-48 binds the regulatory regions of panneuronal genes, and CRISPR-engineered mutations of several of those sites produced a reduction in panneuronal gene expression and corresponding behavioral defects. Surprisingly, the phenotypes of these *cis*-regulatory mutations are not reproduced in *ceh-48* or *ceh-44* single or double mutants. However, mutation of the ubiquitous *ceh-38* by itself, which has the highest neuronal expression of the 6 CUT TFs, shows partial defects in panneuronal effector gene expression. These defects are further enhanced in the sextuple CUT HD mutant (*ceh-38*, *ceh-44*, *ceh-48*, *ceh-41*, *ceh-21*, and *ceh-39*). The CUT sextuple mutant phenotype can be rescued by expression of any of the CUT TF members. Thus, it seems that CUT TFs act redundantly in a dosage-dependent manner to control panneuronal gene expression (Leyva-Díaz and Hobert 2022). CUT sextuple mutants also show defects in some ubiquitously expressed genes, in neurons, and in nonneuronal tissues, expanding the role of these TFs.

Rich sets of effector genes distinguish different neuron classes

The distinctive morphological and functional features of every neuron class are determined by the expression and action of specific sets of proteins encoded by specific *neuron class effector genes* (Table 1—Box of terms), in addition to the panneuronal effector genes. Knowledge of these class-specific effector gene batteries provides (1) a global first approximation to a neuron's specialized function, (2) a rich set of features for neuronal classification, and (3) an entry point for dissecting the regulatory mechanisms that drive neuronal diversity. Transcriptional profiling, as a proxy for expression of those defining effector proteins, has become an essential tool for neuronal classification across all animals, especially since the advent of single-cell RNA sequencing (scRNA-seq). We should all be reminded, however, that differences in transcriptome alone cannot explain cellular diversity in its entirety (e.g. Lago-Baldaia *et al.* 2023), as posttranscriptional and posttranslational mechanisms can have large contributions to both gene expression and function.

In *C. elegans*, our knowledge of neuron class-specific gene batteries began with the analysis of many hundreds of reporters for genes expressed in different subsets of unambiguously identified neurons. This collection of reporters, amassed by the worm community over 3 decades, had already led to a very rich gene expression atlas for different neuron types long before the widespread use of scRNA-seq. Neuronal classification based on reporter gene expression is in strong agreement with the anatomical classification based on EM, i.e. members of anatomical classes share reporter expression patterns that unambiguously distinguish them from other classes (Hobert *et al.* 2016). A noteworthy application of the knowledge gained from this vast collection of reporters is the development of the NeuroPal strain, which contains 41 different reporter fluorophore fusions that in combination enable unambiguous identification of all neuron classes within individual worms (Yemini *et al.* 2021).

More recently, scRNA-seq of all L4 hermaphrodite neurons has substantially expanded the molecular description of *C. elegans* neurons (Taylor *et al.* 2021). The single-cell transcriptomes additionally confirm the subclassification of 10 classes, including the 2 known left–right (L–R) asymmetric pairs of sensory neurons (ASE and AWC), D–V asymmetries in radially symmetric classes (IL2 and RMD), and anterior–posterior (A–P) asymmetries across various motor neurons. In contrast, 2 classes of GABAergic motor neurons that are known to have molecular differences, the DDs and VDs, could not be separated by the initial scRNA-seq effort and required more focused and deeper scRNA-seq of motor neurons to identify known and new subclasses (Smith *et al.* 2024). These differences are due to technical parameters, i.e. number

of sequenced cells per class, number of detected genes per cell, and the exclusive focus on the L4 stage. Therefore, while the current data sets capture most of the neuronal heterogeneity, we cannot exclude the possibility that additional differences among classes and subclasses remain unknown.

What are the effector genes that are differentially expressed across neurons and contribute to their distinct identities and functions? The distinguishing components of the *neuronal genome* (Table 1—Box of terms) have already been comprehensively catalogued (Hobert 2013) and include, for example, the enzymes and factors required for biosynthesis, transport into vesicles, and recycling of the major neurotransmitters (ACh, Glu, GABA, Dop, and 5HT), as well as their receptors. Other groups of key factors for defining specific neuronal properties are the genes coding for factors involved in the assembly and function of sensory cilia (*the ciliome*, Table 1—Box of terms), the components of the gap junctions or electrical synapses (innexins), neuropeptides and their receptors, and channels that regulate the electrophysiological properties of each neuron type (Fig. 2c). The expression of some of these groups of genes is particularly diverse among neuron classes. For example, 98/118 neuron classes express different combinations of the 14 innexin genes expressed in the nervous system (Bhattacharya *et al.* 2019), and every one of the 118 neuron classes expresses a unique combination of neuropeptides, ranging from around 6 to 60 different peptides/cell (Ripoll-Sánchez *et al.* 2023). Different neuron classes are also distinguished by their combinations of ionotropic neurotransmitter receptors, expressing an average of ~20/cell (Taylor *et al.* 2021).

Another defining category of neuronal effector genes is the complement of surface molecules that mediate cell recognition, axon guidance, adhesion, and synapse formation. Work in worms, flies, and mice has supported a view in which a combinatorial code of cell adhesion molecules (e.g. immunoglobulin superfamily, Leucine-Rich Repeat family, cadherins, and neurexins) in different neuron classes defines the connectivity patterns among neurons. The elaboration and organization of the nervous system depend heavily on these classes of molecules but will not be covered here as there have been a few recent reviews on this topic (Chisholm *et al.* 2016; Mizumoto *et al.* 2023; Rapti 2023). It is important to note that given that the expression of genes involved in nervous system wiring could be transient, the current profiling of L4 neurons likely misses dynamically expressed genes that contribute to the initial layout of the nervous system. Analysis of available embryonic and L2 scRNA-seq data sets (Cao *et al.* 2017; Warner *et al.* 2019; Packer *et al.* 2019; Durham *et al.* 2021) has already begun to highlight temporally regulated genes important for the acquisition of neuron morphology and connectivity (e.g. Godini *et al.* 2022), although scRNA-seq of late embryos and other larval stages will probably still be necessary to cover all relevant developmental timepoints.

Transcriptional activation of neuron class-specific effector genes by terminal selectors

The expression of terminal gene batteries is by and large under transcriptional control (although other regulators are also important, and we introduce some of these below). Supporting this notion, decades of work in various organisms, but most systematically and deeply in *C. elegans*, have contributed to the idea that every neuron class expresses a unique combination of terminal TFs that together define the transcriptome and, thus, the functional properties of each class (Hobert and Kratsios 2019; Sousa and Flames 2022). The use of *C. elegans* has been instrumental for dissecting the regulatory logic that controls expression of neuron class-specific effector genes. Two complementary approaches have been critical in reaching the current level of understanding. First, forward genetic screens allowed the identification of TFs that, when mutated, produced behavioral phenotypes, often similar to laser ablation of specific neuron classes (reviewed in Hobert 2010). Secondly, the genetic amenability of *C. elegans*, together with its compact genome, allowed a systematic dissection of the *cis*-regulatory sequences that are necessary and/or sufficient to produce neuron class-specific expression of various effector/reporter genes. Together, these approaches revealed that combinations of key TFs are necessary for defining neuron class identity and act, for the most part, by directly activating the expression of broad batteries of terminal effector genes. This was in contrast to an alternative scenario in which these key TFs activated the effector genes indirectly, solely via intermediate, gene-specific TFs. Accordingly, these TFs have been termed *terminal selectors* (Table 1—Box of terms—and Fig. 1) (Hobert 2008) as they directly select the complement of genes that define specific terminal identities. Some by now classic examples of TFs acting as terminal selectors include UNC-86 and MEC-3 selecting touch receptor neuron (TRN) identity, TTX-3 and CEH-10 for AIY interneuron fate, CHE-1 for ASE chemosensory neurons, AST-1 for CEP, ADE, and PDE dopaminergic neurons, and UNC-3 for cholinergic motor neurons (Duggan *et al.* 1998; Wenick and Hobert 2004; Etchberger *et al.* 2007; Flames and Hobert 2009; Kratsios *et al.* 2011). Currently, at least 1 terminal selector has been described for 117 of the 118 neuron classes, the exception being the RIM motor neuron (Table 2) (Reilly *et al.* 2022).

A preponderance of HD TFs act as terminal selectors

The *C. elegans* genome codes for 102 HD TFs. These constitute ~10% of all TFs, yet they represent the majority (~70%) of known neuronal terminal selectors (Reilly *et al.* 2022). The pivotal role for HD TFs as identity specifiers is also well established in other organisms including cnidarians, *Drosophila*, zebrafish, and mammals, suggesting that it could constitute an ancestral function (Zeisel *et al.* 2018; Sugino *et al.* 2019; Tournière *et al.* 2020; Leung *et al.* 2022; Xu *et al.* 2024). In *C. elegans*, each neuron type expresses a median of 7 different HD TFs, excluding ubiquitous or panneuronal members. HD expression is sufficient to distinguish all *C. elegans* neuron classes (Reilly *et al.* 2020), and in several instances, transcriptionally similar neurons tend to express similar HD combinations (Reilly *et al.* 2022). Based on these data, it has been suggested that specific HD TF codes, acting as terminal selectors, define specific neuron class fates. However, for most neurons, only 1 or 2 HD TFs have been validated as terminal selectors and often the same TF is reused in several neurons. Future work should therefore be aimed at improving our understanding of how HD codes work at the mechanistic level. Several TFs belonging to other families also play important roles as detailed below. In the section on neuronal diversification, we go deeper into how combinations of terminal selectors achieve neuron class-specific gene expression and how these combinations are generated during development.

The ciliome—a neuron-specific program not fully under terminal selector control

Mutational analysis of terminal selectors in multiple different contexts has revealed that while most neuron-specific genes fail to be normally expressed, there are at least 2 gene batteries that are typically unaffected: (1) panneuronal genes, which as explained above are primarily regulated by the CUT TFs, and (2) genes coding for structural cilia components, which are shared by all sensory ciliated neurons (26/118 classes) (Lewis and Hodgkin 1977; Flames

Table 2. Terminal selector TFs (adapted from Reilly *et al.* 2022, with additions from Feng *et al.* 2020 and Feng *et al.* 2022

	Neuron class	Neurotransmitter	HD terminal selector	Subfeature selector	Non-HD TS
Sensory neuron	ADA	Glutamatergic	*unc-86, ceh-20, unc-62*		
	ADE	Dopaminergic	*ceh-43, ceh-20, unc-62*		*ast-1*
	ADF	Serotonergic			*lag-1*
	ADL	Glutamatergic	*lin-11*		*hlh-4*
	AFD	Glutamatergic	*ttx-1, ceh-14*		
	ALA	GABAergic	*ceh-17, ceh-14*		
	ALN	Cholinergic	*unc-86*		
	ALM	Glutamatergic	*unc-86, mec-3*		
	AVM	Glutamatergic	*unc-86, mec-3, lin-39*		
	AQR	Glutamatergic	*unc-86; lin-39, ceh-20, unc-62*		*egl-13*
	ASE	Glutamatergic	*ceh-36*	*cog-1, lim-6*	*che-1*
	ASG	Glutamatergic	*ceh-37, lin-11*		
	ASH	Glutamatergic	*unc-42*		
	ASI				*unc-3*
	ASJ	Cholinergic			*sptf-1*
	ASK	Glutamatergic	*ttx-3*	*mls-2*	
	AWA		*egl-5*		*odr-7*
	AWB	Cholinergic	*lim-4*		*sox-2*
	AWC	Glutamatergic	*ceh-36*	*nsy-7*	*sox-2*
	BAG	Glutamatergic	*ceh-37*		*ets-5, egl-13*
	CEP	Dopaminergic	*ceh-43, ceh-20, unc-62*		
	FLP	Glutamatergic	*unc-86, mec-3, alr-1*		
	IL1	Glutamatergic	*ceh-32*		*sox-2*
	IL2	Cholinergic	*unc-86*	*unc-39*	*sox-2, cfi-1*
	OLL	Glutamatergic	*vab-3, ceh-32*		*sox-2, eor-1*
	OLQ	Glutamatergic	*vab-3*		
	PDE	Dopaminergic	*ceh-43, ceh-20, unc-62*	*lin-39*	
	PHA	Glutamatergic	*ceh-14*		
	PHB	Glutamatergic	*ceh-14, egl-5*		
	PHC	Glutamatergic	*ceh-14, unc-86, nob-1, php-3*		
	PLM	Glutamatergic	*unc-86, mec-3, egl-5*		
	PLN	Cholinergic	*unc-86, nob-1, php-3*		
	PQR	Glutamatergic	*unc-86; mab-5*		
	PVD	Glutamatergic	*unc-86, mec-3, lin-39*		
	PVM	Glutamatergic	*unc-86, mec-3*		
	URA	Cholinergic	*unc-86*		*sox-2, cfi-1*
	URB	Cholinergic	*unc-86*		*sox-2*
	URX	Cholinergic	*unc-86*		*egl-13*
	URY	Glutamatergic	*vab-3, ceh-32*		
Interneuron	AIA	Cholinergic	*ttx-3, unc-39*		
	AIB	Glutamatergic	*unc-42, unc-62, ceh-20*		
	AIM	Glutamatergic	*unc-86, ceh-14, mls-2*		
	AIN	Cholinergic	*tab-1*		
	AIY	Cholinergic	*ttx-3, ceh-10*	*ceh-23*	
	AIZ	Glutamatergic	*unc-86*		
	AUA	Glutamatergic	*ceh-6*		
	AVA	Cholinergic	*unc-42*		*unc-3*
	AVB	Cholinergic	*unc-42*		*unc-3*
	AVD	Cholinergic	*unc-42, tab-1*		*unc-3, cfi-1*
	AVE	Cholinergic	*unc-42*		*unc-3*
	AVF	GABAergic	*unc-4*		
	AVG	Cholinergic	*lin-11*		*ast-1*
	AVH		*unc-42*		*hlh-34*
	AVJ		*lin-11, mls-2, unc-30*		
	AVK		*unc-42*		
	BDU		*unc-86*	*ceh-14*	*pag-3*
	CAN		*ceh-10*		
	DVA	Cholinergic			*unc-3*
	DVC	Glutamatergic	*ceh-14*		
	LUA	Glutamatergic	*egl-5*		*cfi-1*
	PVC	Cholinergic	*ceh-14; egl-5*		*unc-3*
	PVN	Cholinergic	*ceh-14, ceh-9*		*unc-3*
	PVP	Cholinergic	*lin-11, unc-30*		
	PVQ	Glutamatergic	*vab-15, zag-1*		*pag-3*
	PVR	Glutamatergic	*unc-86, ceh-14, ceh-31*		
	PVT		*ceh-14, lim-6*		
	PVW		*ceh-14*		
	RIA	Glutamatergic	*ceh-8, ceh-32*		
	RIB	GABAergic	*ttx-1*		*aptf-1*
	RIC	Octopaminergic	*unc-62*		
	RIF	Cholinergic	*lin-11*		

(continued)

Table 2. (continued)

	Neuron class	Neurotransmitter	HD terminal selector	Subfeature selector	Non-HD TS
	RIG	Glutamatergic	*lim-6*		
	RIH	Cholinergic	*unc-86*		
	RIM	Tyraminergic			
	RIP		*ttx-1*		
	RIR	Cholinergic	*unc-86*		
	RIS	GABAergic	*lim-6*		*nhr-67*
	RIV	Cholinergic	*unc-42*		
	SAA	Cholinergic	*unc-42*		*sox-3*
	SDQ	Cholinergic	*lin-39*, *mab-5*		
Motor neuron	AS	Cholinergic	*lin-39*	*mab-5*	*unc-3*, *mab-9*, *unc-55*
	AVL	GABAergic	*lim-6*		*nhr-67*
	DA	Cholinergic	*lin-39*	*unc-4*, *mab-5*, *egl-5*	*mab-9*, *cfi-1*
	DB	Cholinergic	*lin-39*	*vab-7*	*mab-9*, *cfi-1*
	DD	GABAergic	*unc-30*		*elt-1*
	VD	GABAergic	*unc-30*	*irx-1*	*elt-1*, *unc-55*
	DVB	GABAergic	*lim-6*, *egl-5*		
	HSN	Serotonergic	*unc-86*, *egl-5*, *zag-1*		*ast-1*, *sem-4*, *egl-18*
	PDA	Cholinergic	*egl-5*, *ceh-6*		*unc-3*
	PDB	Cholinergic	*egl-5*		*unc-3*
	RID		*lim-4*		
	RMD	Cholinergic	*unc-42*	*ceh-32*	
	RME	GABAergic	*ceh-32*		*nhr-67*
	RMF	Cholinergic	*unc-42*, *lim-4*		
	RMG		*unc-86*, *ceh-13*		
	RMH	Cholinergic	*unc-42*, *lim-4*		
	SAB	Cholinergic	*unc-4*		*unc-3*
	SIA	Cholinergic	*ceh-24*		
	SIB	Cholinergic	*ceh-24*, *unc-42*		
	SMB	Cholinergic	*lim-4*		
	SMD	Cholinergic	*unc-42*, *lim-4*, *ceh-24*		
	VA	Cholinergic	*lin-39*	*unc-4*, *mab-5*, *egl-5*	*unc-3*, *bnc-1*, *cfi-1*
	VB	Cholinergic	*lin-39*	*vab-7*, *ceh-12*	*unc-3*, *bnc-1*, *cfi-1*
	VC	Cholinergic	*lin-39*		
Enteric	I1	Cholinergic	*ceh-34*, *unc-86*		
	I2	Glutamatergic	*ceh-34*, *ceh-14*		
	I3	Cholinergic	*ceh-34*, *ceh-2*, *ceh-7*, *pros-1*		
	I4		*ceh-34*		
	I5	Glutamatergic	*ceh-34*		
	I6	Cholinergic	*ceh-34*		
	NSM	Serotonergic	*ceh-34*, *ttx-3*, *unc-86*		
	M1	Cholinergic	*ceh-34*		
	M2	Cholinergic	*ceh-34*		
	M3	Glutamatergic	*ceh-34*, *ceh-2*		
	M4	Cholinergic	*ceh-34*, *ceh-28*, *zag-1*		
	M5	Cholinergic	*ceh-34*, *vab-15*		
	MI	Glutamatergic	*ceh-34*, *ceh-45*		
	MC	Cholinergic	*ceh-34*		

and Hobert 2009; Masoudi *et al.* 2018). These 2 groups of genes are directly controlled by parallel regulatory routines (Fig. 1).

Cilia are complex, evolutionarily conserved, eukaryotic structures composed of hundreds of proteins that are required for their assembly, structure, and function and are collectively known as the ciliome. *Caenorhabditis elegans* lacks motile cilia and, unlike many other animals, has only 1 cell type possessing ciliary structures, sensory neurons (26 of the 118 neuron classes). All sensory ciliated neurons are characterized by coexpression of the Forkhead TF FKH-8 (Brocal-Ruiz *et al.* 2023) and a specific isoform of the unique member of the RFX TF family in *C. elegans*, DAF-19C [for "Cilia" according to published terminology (Senti and Swoboda 2008) but named DAF-19 isoform d in WormBase]. These TFs interact physically and act as terminal selectors of the structural ciliome components that are expressed in all ciliated sensory neurons (Swoboda *et al.* 2000; Efimenko *et al.* 2005; Brocal-Ruiz *et al.* 2023). The role of RFX TFs as direct master regulators of ciliome gene expression was originally described in *C. elegans* (Swoboda *et al.* 2000) and next expanded to other invertebrate and vertebrate animals, including humans (reviewed in Choksi *et al.* 2014). There is also a neuron class-specific component of the ciliome, including specific receptors, adhesion proteins, and others that contribute to specific cilium morphologies and functions. Such class-specific components are unaffected in DAF-19 and FKH-8 mutants and are instead under direct control of neuron class terminal selectors (Fig. 1) (Howell and Hobert 2017).

Beyond the panneuronal and cilia transcriptomes, it remains possible that other gene batteries are controlled by parallel programs that are not or not fully under terminal selector control. For example, broad gene batteries that control morphology and/or connectivity have been relatively poorly studied compared to other functional sets of genes. Future work is needed to place these regulatory routines in a comprehensive model for the establishment of specific neuron class identities.

Specification of neuronal progenitors

As with the specification of all cell types during development, the generation of a neuron can be considered a process of progressive cell fate commitment. Based on early genetic and molecular

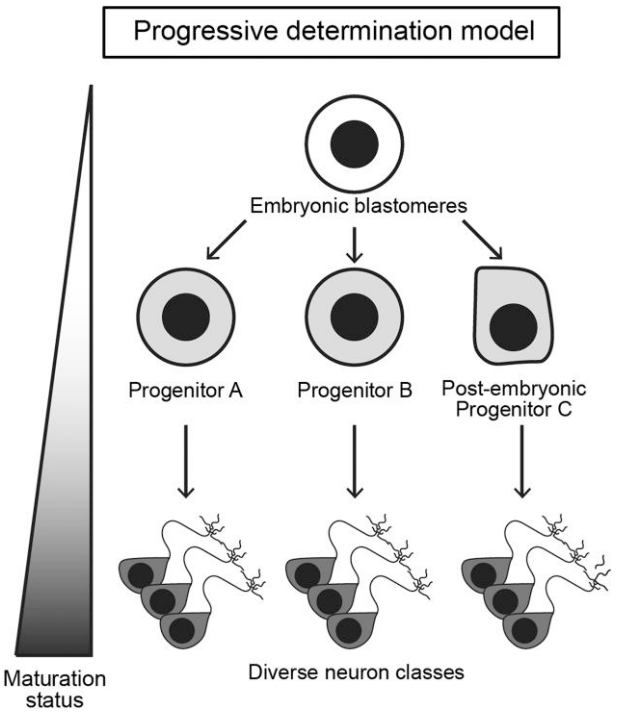

Fig. 3. Progressive determination model of neuronal development. A cartoon illustration of the progressive determination model of neural development. Key steps relevant to *C. elegans* include the specification of neural progenitors and the differentiation of postmitotic cells into neurons.

studies of the development of the *Drosophila* peripheral nervous system, a progressive determination model of neural development was proposed that includes the following key steps (also summarized in Fig. 3): (1) the specification of cells with neurogenic potential; (2) the singling out and commitment of individual neural progenitors; (3) the regulation of neural progenitor cell divisions; and (4) the differentiation of individual immature postmitotic cells into neurons (Ghysen and Dambly-Chaudiere 1989; Jan and Jan 1994). This model provides a useful framework in which to consider the generation of a neuron and compare neurogenesis between systems. While it is tempting to map steps of neurogenesis as defined in other organisms onto the *C. elegans* lineage, these steps occur at different times in different branches as we discuss further below. We think that a more useful comparison is to discuss the conserved TFs and signaling pathways that play a role in the various steps of neurogenesis across different animals. We pay close attention to the basic helix–loop–helix (bHLH) family of *proneural TFs* (Table 1—Box of terms) as the role of these factors is the most comparable across different species. Of note, while some TF classes seem enriched for certain functions during neurogenesis, many TFs are pleiotropic and play different roles in different contexts (e.g. in progenitors vs postmitotic neurons). We highlight the similarities and novelties in their actions in *C. elegans*, with a focus on the lineage origins of neurons.

Are the steps of the progressive model identifiable in *C. elegans*?

As mentioned previously, the differentiation of postmitotic neural precursors into neurons requires the action of the CUT HD TFs and terminal selectors to regulate panneuronal and class-specific effector gene batteries, respectively. This last step of the progressive model is therefore identifiable in *C. elegans* and likely conserved given the important roles of both CUT HD TFs and HD terminal selector TFs in neuronal differentiation across the animal kingdom (Zeisel *et al.* 2018; Sugino *et al.* 2019; Tournière *et al.* 2020; Leung *et al.* 2022; Xu *et al.* 2024). A key aim moving forward is to understand how the earlier steps of neural specification are regulated and the links between these and later differentiation events.

Nervous system specification in many animals begins with the specification of the ectodermal germ layer (reviewed in Sasai and De Robertis 1997; Kiecker *et al.* 2016; Crews 2019). In vertebrates, this is shortly followed by a process known as *neural induction* (Table 1—Box of terms), which specifies a particular region of the embryo as competent to produce neural rather than nonneural ectodermal derivatives, such as skin (reviewed in Hemmati-Brivanlou and Melton 1997; Stern 2006). Given the nonclonal origin of many tissues in *C. elegans*, including neurons, it is not completely clear from a lineage perspective how to apply the concept of germ layers (Sulston and Horvitz 1977; Chalfie *et al.* 1983; Sulston *et al.* 1983). For example, some embryonic blastomeres make predominantly neurons and glia, while others make what could be considered as neural vs nonneural ectoderm decisions, or even neural vs mesoderm decisions at the terminal division. There is also no evidence that the key signaling pathways implicated in neural induction in vertebrates (BMP, Hh, NGF, Notch, and others; reviewed in Hemmati-Brivanlou and Melton 1997; Stern 2006) are involved in inducing neural fates in *C. elegans*. A series of Notch signaling events that pattern the early embryonic lineages of *C. elegans* (reviewed in Priess 2005) could be reminiscent of the key role of the Notch pathway in neural development in flies and vertebrates. However, these inductive events affect both neural and nonneural cell types and neural cells also arise from non-Notch-induced lineages.

One key gene family that is upregulated during vertebrate neural induction consists of the Sox TFs, which play crucial roles in the specification and maintenance of neural progenitors (reviewed in Pevny and Placzek 2005). Although there is little evidence for neural induction in other invertebrates such as *Drosophila*, Sox family genes do play a key role in the specification of neural progenitors in this system (Crémazy *et al.* 2000; Buescher *et al.* 2002). A second key event in the specification of neural progenitors across the animal kingdom is the expression of proneural bHLH TFs (reviewed in Dambly-Chaudière and Vervoort 1998; Bertrand *et al.* 2002; Maurange and Gould 2005). These are a subgroup of a larger family of bHLH TFs that often act in cell type specification, e.g. MyoD in muscle (Crews 1998). First identified in the *Drosophila* peripheral nervous system, these factors are both necessary and sufficient (in some specific contexts) for neural precursor specification in *Drosophila* and vertebrates (reviewed in Dambly-Chaudière and Vervoort 1998). They also play important roles in the regulation of neural progenitor divisions and can regulate various aspects of neuronal differentiation, including the acquisition of panneuronal and subtype-specific features. Other key regulators of neural precursor specification in both *Drosophila* and vertebrates include the HOX cluster HD TFs (reviewed in Maurange and Gould 2005; Philippidou and Dasen 2013). Below, we focus on the roles of Sox TFs, HOX cluster TFs, and proneural bHLH TFs in neural specification in *C. elegans*. Even though the mechanisms of early blastomere diversification in *C. elegans* are such that specification of ectoderm and induction of neuronal ectoderm are unlikely to occur in the same way as in other animals, exploration of all these TF families has revealed that they are all involved in some aspect of neural specification. We highlight similarities and differences with other model systems where appropriate.

Roles of Sox TFs

Sox proteins are a group of TFs characterized by the presence of an HMG box-type DNA-binding domain. The genome of *C. elegans* contains 2 SoxB genes, *sox-2* and *sox-3*, and 1 SoxC gene, *sem-2* (Bowles *et al.* 2000). Sox TFs are deeply conserved and present in all Metazoa, and members of the SoxB and SoxC groups are important regulators of neural development in many species. Sox TFs are key regulators of neural progenitors in both the embryo and adult neural stem cells (reviewed in Pevny and Placzek 2005; Reiprich and Wegner 2015). In vertebrates, SoxB genes such as Sox2 are expressed in the neural plate in response to neural-inducing factors where they specify a neural ectodermal fate. At later stages, the SoxB genes are expressed in many neurogenic regions both during juvenile development and in the adult central nervous system. Their role at later stages appears to be in maintaining the undifferentiated state of neural progenitors.

Surprisingly, and in contrast to the early and broad neurogenic defects observed upon removal of *Drosophila* and vertebrate SoxB genes, *sox-2* and *sox-3* are mostly dispensable for embryonic neurogenesis in *C. elegans* (Vidal *et al.* 2015). Moreover, 4D lineage analysis of fosmid-based reporter transgenes demonstrates that *sox-2* is expressed in a limited subset of neuroblasts rather than at earlier stages in neuronal lineages. *Sox-3* is expressed even later, exclusively in postmitotic neurons (Vidal *et al.* 2015). Analysis of *sox-2* maternal/zygotic null mutants and *sox-2; sox-3* double mutants reveals that both genes are dispensable for general neurogenesis, as panneuronal markers are unaffected in these mutant backgrounds (Vidal *et al.* 2015). Instead, they appear to function in 2 more discrete aspects of neural development. Firstly, they play a role in the terminal differentiation of specific postmitotic neurons. Sustained expression throughout adulthood of *sox-2* and *sox-3* is observed in several sensory neurons, interneurons, and motor neurons, and the subtype identity of several of these neurons is affected in the respective mutant backgrounds (Vidal *et al.* 2015). Secondly, *sox-2* acts to maintain the developmental potential of postembryonic blast cells. For example, genetic mosaic analysis reveals that *sox-2* is required for the K blast cell to generate the DVB neuron and for the V5 blast cell to generate the PDE and PVD neurons (Vidal *et al.* 2015). *sox-2* is also required for the direct transdifferentiation of the rectal epithelial cell Y to the PDA neuron, where it acts together with the POU homeobox gene *ceh-6*, the Sal1-type TF *sem-4*, and the Hox gene *egl-5* (Kagias *et al.* 2012). Several of the rectal epithelial blast cells affected in *sox-2* mutants also express these genes and so they may act together to maintain blast cell potency, in a fashion analogous to the role of SoxB genes in maintaining neural stem cell potency in vertebrates.

SoxC genes in vertebrates play an important role at later stages of neurogenesis in the embryonic and adult nervous system, where they act downstream of SoxB genes to promote neuronal differentiation in postmitotic neurons (reviewed in Reiprich and Wegner 2015). Again, in contrast to this, *C. elegans sem-2* is expressed early in development both in neuronal and nonneuronal progenitors and has a very restricted expression pattern in postmitotic neurons. It is not required for broad neuronal specification, as evidenced by the lack of disruption to panneuronal markers in maternal/zygotic *sem-2* mutants but does affect the subtype identity of a very limited number of neurons (Vidal *et al.* 2015). Altogether, it seems that the roles of Sox genes in early neural specification are not conserved in *C. elegans*, although there may be some similarities of their roles in maintaining the neurogenic potential of postembryonic neuroblasts.

Roles of HOX cluster TFs

HOX cluster genes are a specific subfamily of HD TFs that are well known for their roles in A–P body patterning and segmentation across metazoans. *Caenorhabditis elegans* has 6 clear homologs of the HOX cluster genes: *ceh-13*, *lin-39*, *mab-5*, *egl-5*, *nob-1*, and *php-3*. These are all present on chromosome III, though not in a contiguous cluster as in other animals. However, to make the distinction from other HD TFs clear, we will still refer to them as HOX cluster genes. Studies in several model organisms have revealed that, in addition to their roles in A–P patterning, HOX cluster genes also play fundamental roles during the early steps of neuronal specification, influencing both the timing and number of cell divisions of several embryonic neural and neural stem cell lineages (Bello *et al.* 2003; Maurange and Gould 2005; Gouti and Gavalas 2008). In *C. elegans*, there are also 2 phases of HOX cluster gene importance: in the early specification of neuronal precursors and the A–P patterning of postembryonic neuronal lineages, but also later during the acquisition of specific neuronal features (recently reviewed in Smith and Kratsios 2024).

With regard to the functions in neuroblast specification, single and double mutant analyses have indicated that HOX cluster genes influence both the timing and number of cell divisions of several embryonic neuroblasts in a manner analogous to observations in *Drosophila* and vertebrate neural stem cells. For example, in *ceh-13* null mutants, the neuroblasts that generate the DA, DD, and SAB motor neurons display delayed divisions and in *nob-1; php-3* double mutants, there are delayed divisions in the neuroblasts that generate the PVQ interneurons, the PHB sensory neurons, and the HSN motor neurons, and the neuroblast that generates the PLM and ALN sensory neurons fails to divide entirely (Murray *et al.* 2022). It is not clear what fate these neuroblasts acquire in these mutant backgrounds. Studies investigating the postembryonic neuroblast lineages have revealed that they also play a role in establishing neuronal lineage patterning along the A–P axis (reviewed in Smith and Kratsios 2024). Several studies have revealed that *lin-39*, *mab-5*, and *egl-5* are expressed in the P neuroblasts in an A–P sequence, and mutations in these factors result in lineages that are altered in a manner resembling homeotic transformations (reviewed in Smith and Kratsios 2024). Similar conclusions were made from studies concerning the roles of *mab-5* and *egl-5* in postembryonic specification of the male rays and in neuroblast migration in the Q lineage (reviewed in Smith and Kratsios 2024). It is also worth noting that HOX cluster gene expression in *C. elegans* is established in a lineage-specific manner rather than being based strictly on position (Cowing and Kenyon 1996). HOX cluster genes also regulate the migration of Q neuroblasts and the HSN neurons (Salser and Kenyon 1992; Baum *et al.* 1999). Altogether, in *C. elegans*, the HOX cluster genes play important roles in early neuroblast specification and in imparting A–P positional information on neuroblast fate in a manner remarkably like that seen in other animals. The later function of HOX cluster genes as terminal selectors in postmitotic neurons is described in the section on neuronal diversification below. Possibly highlighting similarities in the late functions of HOX cluster genes, there is also a late phase of HOX cluster gene function in postmitotic neurons in vertebrates (reviewed in Feng *et al.* 2021).

Diverse functions of bHLH proneural TFs

Proneural genes belong to the bHLH class of TFs and the *C. elegans* genome encodes orthologs for all proneural bHLH subclasses (Ledent and Vervoort 2001; Ledent *et al.* 2002; Simionato *et al.* 2007). Over the last few years, the expression and function of

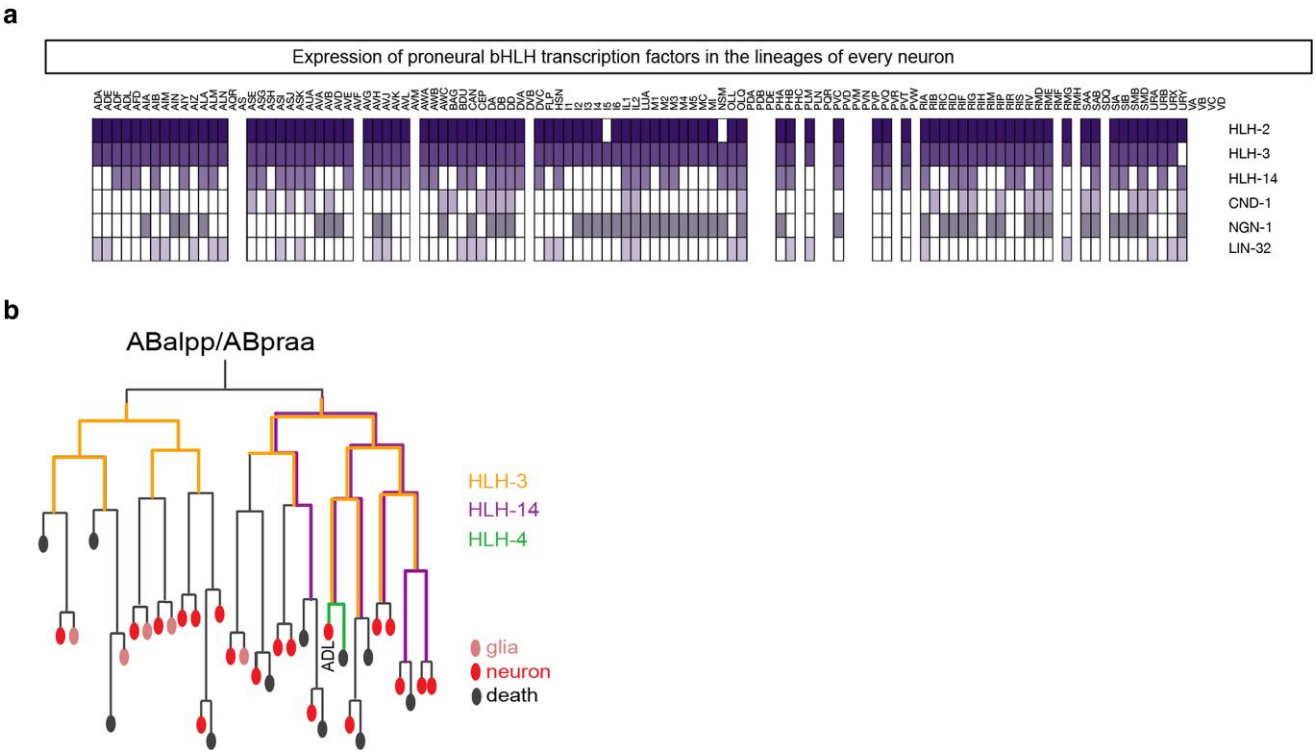

Fig. 4. Expression of bHLH TFs in neuronal lineages. a) Summary of proneural gene expression at any point in the lineage of the indicated embryonic neurons (sources: WormAtlas, Sulston and Horvitz 1977; Sulston *et al.* 1983; Reilly *et al.* 2022; Masoudi *et al.* 2023). b) An illustrative example of sequential bHLH expression in the ABalpp/ABpraa lineage. *hlh-3* is expressed both early and broadly and has no known role in this lineage. *hlh-14* is expressed in a more restricted manner and is required for neural precursor specification. *hlh-4* is only expressed in the ADL neurons where it acts as a terminal selector and not as proneural gene. See the main text for more details.

C. elegans proneural genes has been extensively analyzed, shedding additional light on how these genes control various aspects of neuronal development at the molecular level. The single-cell precision the worm allows has revealed several key aspects that have been more difficult to assess in other model systems. Proneural genes were first identified in *Drosophila* for their role in the development of the sensory organs of the peripheral nervous system (reviewed in Dambly-Chaudière and Vervoort 1998). Loss-of-function and gain-of-function experiments have revealed they are necessary and sufficient for neuronal differentiation in a wide variety of animal systems and in this classically defined role often specify neural vs nonneural ectoderm (reviewed in Bertrand *et al.* 2002).

There are 2 main families of proneural bHLHs: (1) the Achaete–Scute family, which includes the 4 *Drosophila* AS-C genes and the vertebrate *MASH* genes, and (2) the Atonal family, which includes the vertebrate MATH genes, *atonal* in *Drosophila* and the vertebrate Neurogenin and NeuroD genes (reviewed in Hassan and Bellen 2000; Baker and Brown 2018). The *C. elegans* proneural genes include the following: the AS-C homologs *hlh-3*, *hlh-4*, *hlh-6*, *hlh-14*, and *hlh-19*; the Atonal homolog *lin-32*; the Neurogenin homolog *ngn-1*; and the NeuroD homolog *cnd-1* (Ledent and Vervoort 2001; Ledent *et al.* 2002; Simionato *et al.* 2007). As discussed below, many but not all these genes have been shown to regulate neural vs nonneural decisions and the specification of neural progenitors.

The discovery in *Drosophila* that *achaete–scute* controls the development of the external sensory organ precursors and that *atonal* controls the development of the internal chordotonal organs led to the realization that proneural genes not only regulate the specification of neuronal precursors but may also play important roles in the differentiation of different classes of neurons (reviewed in Guillemot 2007). This led to the "coupling hypothesis" in which bHLH proteins can activate both generic neuronal properties and neuron class-specific features (Brunet and Ghysen 1999). *Caenorhabditis elegans* proneural bHLHs have been shown to play a pivotal role in these later steps of neuronal differentiation, mediated via the control of downstream terminal selectors (Fig. 1). Below, we discuss *C. elegans* proneural bHLH TFs in more detail, focusing on specific examples that reveal their roles in neural precursor specification, neural differentiation, and subtype specification. We also highlight some novel functions revealed from *C. elegans* studies.

Expression patterns of C. elegans *bHLH proneural genes*

Until recently, the description of the expression patterns of *C. elegans* proneural genes (like all other genes) relied on the use of multicopy promoter fusion arrays, which frequently lack key regulatory elements. The advent of fosmid recombineering, CRISPR-based endogenous gene tagging, and scRNA-seq has facilitated more comprehensive and accurate analyses (Masoudi *et al.* 2018; Packer *et al.* 2019; Ma *et al.* 2021; Masoudi *et al.* 2021, 2023) from which several key themes have emerged (Fig. 4). Firstly, there is broad expression of proneural genes in early lineages followed by more cell-specific expression at later embryonic stages. Examples of this include *hlh-3*, *cnd-1*, *ngn-1*, and *lin-32*. At least 1 proneural gene is expressed at some point in the lineage of every terminal postmitotic neuron (Fig. 4a). Secondly, many lineages express multiple proneural bHLHs, either concomitantly or in sequential waves or both (Fig. 4b). Thirdly, the vast majority of proneural gene expression is transient and not postmitotic, which is also a common feature in other animal nervous systems. Lastly, there are a few homologs of proneural factors, such as *hlh-6*, whose expression and function lie outside the nervous system (Smit *et al.* 2008; Sallee *et al.* 2017; Littleford *et al.* 2021). Intriguingly, *hlh-6* is expressed

in the secretory pharyngeal gland cells where it acts as a terminal selector. *hlh-6* is an AS-C homolog, the most evolutionarily ancient proneural group, and it has been speculated that secretory gland cells are evolutionary ancestors of neurons (Moroz 2021). It is highly likely that the expression of proneural genes is dependent on the integration of previous lineage information and current state. However, the exact molecular mechanisms involved are currently unclear and certainly warrant detailed future investigation.

Classic proneural function of C. elegans *bHLH TFs—neuroblast specification*

A role for *C. elegans* proneural bHLH TFs in specifying neural precursors was first described for *lin-32*, the Atonal homolog, during the postembryonic development of the sensory rays in the male tail (Zhao and Emmons 1995). Each of the 9 pairs of rays is generated from a neural precursor that undergoes a stereotyped division pattern to generate 2 ray neurons (RnA and RnB) and a ray structural glial cell (RnSt). This is conceptually similar to the development of the sensory organ precursors in *Drosophila*. In *lin-32* mutants, the ray neuroblasts fail to divide and are transformed into hypodermal cells, a nonneural ectodermal cell fate, revealing this to be a classic neural vs nonneural decision (Zhao and Emmons 1995). Moreover, as with fly and vertebrate proneural genes, ectopic expression of *lin-32* generates ectopic neurons, such as additional rays likely from the seam cells. This strongly indicates that *lin-32* is necessary and sufficient (in some specific contexts) to generate ray neural precursors and is also reminiscent of the overexpression of Atonal or AS-C genes in *Drosophila*. Similar observations have been made for other postembryonic neuroblasts, such as the Q neuroblast, the V5 postdeirid neuroblast, and the anterior daughter of B (Zhao and Emmons 1995; Tekieli *et al.* 2021). This classic proneural function is not restricted to postembryonic lineages as *lin-32* is also expressed and required in the ABplaaaaa/ABarpapaa neuroblasts that give rise respectively to the left and right URAD, CEPD, and URX neurons (Rojo Romanos, Pladevall-Morera, *et al.* 2017; Masoudi *et al.* 2021).

Several other *C. elegans* proneural genes have also been shown to regulate the specification of neuroblasts including (1) the AS-C homolog *hlh-14*, which is required for the embryonic specification of the PVQ/HSN/PHB neuroblasts, the ABalpppp/ABpraaap neuroblasts, the DVC neuroblast, and the PVR neuron (Frank *et al.* 2003; Poole *et al.* 2011); (2) the NeuroD homolog *cnd-1*, which is required for the specification of the neuroblasts that give rise to the embryonic DA, DB, and DD ventral nerve cord motor neurons (Hallam *et al.* 2000); and (3) the Neurogenin homolog *ngn-1*, which is required for the L–R asymmetric specification of the MI neuron. MI is transformed into its bilateral homolog, the e3D epithelial cell, in *ngn-1* mutants (Nakano *et al.* 2010). In the majority of cases, not only are the cells transformed to nonneural ectoderm fates, but they prematurely exit the cell cycle. This suggests that proneural genes in *C. elegans* also regulate neural precursor divisions. In vertebrates, proneural genes can either drive differentiating precursors to exit the cell cycle or to maintain the proliferative capacity of neural stem cells (reviewed in Bertrand *et al.* 2002; Guillemot and Hassan 2017). The loss of divisions in *C. elegans* proneural gene mutants is more reminiscent of the latter. The molecular targets of proneural genes that regulate cell divisions are currently unclear.

An extreme example of a neural/nonneural fate decision worth highlighting is the specification of the I4 neuron, which is dependent on the AS-C gene homolog *hlh-3* (Luo and Horvitz 2017). This pharyngeal neuron is derived from the MS lineage, a largely mesodermal lineage, and its sister cell is pm5, a pharyngeal muscle cell. In *hlh-3* mutants, I4 adopts the fate of pm5, which leads to 2 important conclusions. Firstly, proneural genes in worms are required for neural/mesodermal cell fate decisions. There is a tempting analogy that can be made here to the bipotent neuromesodermal progenitors that reside in the posterior growth zone of vertebrates and give rise to both mesodermal and neural tissue (Tzouanacou *et al.* 2009; Henrique *et al.* 2015), and in the chick express the AS-C gene *Cash4* (Henrique *et al.* 1997). Secondly, together with the data described above, worm proneural genes can act at several different developmental stages and in different cellular contexts to regulate neuroblast specification. At 1 extreme is the specification of proliferating early embryonic neuroblasts, such as ABalppp/ABpraaa that will divide several times to generate only neurons and glia. At the other is the specification of a single neuron postmitotically at the terminal division, such as the I4 and PVR neurons.

It is somewhat surprising that, despite the broad expression of several proneural genes described above, the neuroblast specification defects observed in mutants are relatively cell specific. In some cases, such as *cnd-1*, the effects of null alleles have not been fully described. In others, such as *hlh-3* and *ngn-1*, studies with putative null alleles have revealed that they are largely dispensable for general neuroblast specification (Doonan *et al.* 2008; Luo and Horvitz 2017; Lloret-Fernández *et al.* 2018; Christensen *et al.* 2020). One possibility is of course redundancy; as Fig. 4 illustrates, many lineages express multiple proneural bHLHs and a thorough analysis of double and triple mutants has not been undertaken. Another possibility is that in many lineages, the defects could be more specific, affecting later aspects of neuronal differentiation in the absence of earlier neural precursor defects. These 2 possibilities are not mutually exclusive of course, but there is now a good deal of evidence for the latter, as we discuss in more detail in the next section.

Differentiation functions of bHLH TFs—panneuronal and neuron class specification

As described above, in vertebrates and flies, proneural genes regulate the specification of neuroblast identity but can also regulate a variety of aspects of neuronal differentiation. The same is true in *C. elegans*. A common theme has emerged indicating that, although proneural gene expression is transient and fades rapidly in postmitotic neurons, proneural genes influence postmitotic neuronal differentiation through the regulation of panneuronal genes and the terminal selectors for specific neuron classes.

Although the initial analysis of the Atonal ortholog *lin-32* during male ray development revealed its classic proneural function in the specification of neural precursor identity, subsequent studies suggested that it also plays a role in regulating later aspects of neuronal differentiation. In hypomorphic alleles of *lin-32*, analysis of molecular markers for the 2 neurons (RnA and RnB) and the glial support cell (RnSt) that arise from an individual ray neuroblast has revealed that they are independently affected (Portman and Emmons 2000). This suggests that *lin-32* is also required to specify later aspects of ray lineage development. Similar defects in terminal neuron specification have also been observed in hypomorphic alleles or RNAi knockdown of several other proneural genes in which the neuroblast specification described above is only partially affected, including *hlh-14* (Frank *et al.* 2003) and *cnd-1* (Hallam *et al.* 2000). Initial indications were that they do so via the regulation of terminal selector expression. *cnd-1* mutants for example not only display a loss of embryonic DA, DB, and DD motor neurons but there are also misspecifications of GABAergic vs cholinergic neurotransmitter fate and

defects in the morphology and connectivity of remaining motor neurons. Importantly, the expression of *unc-3*, *unc-4*, and *unc-30*, known terminal selectors for these motor neurons, is also affected (Hallam *et al.* 2000). Although it cannot be completely ruled out that these effects are due to misspecification of the neuroblast itself, these experiments were among the first indications that proneural genes may play additional roles in later neuronal differentiation events in *C. elegans*.

Prompted by previous reports that *lin-32* can affect the expression of the terminal selector *unc-86* in the postembryonic Q and V5 neuroblasts (Baumeister *et al.* 1996), a comprehensive analysis of *lin-32* embryonic expression and *lin-32* null mutants was undertaken (Masoudi *et al.* 2021). This revealed that in many lineages in which *lin-32* is expressed, *lin-32* null mutants show no obvious cell division defects or transformations to hypodermal fates. Instead, panneuronal markers are lost. Making use of the NeuroPAL transgene strain, which contains a large number of additional neuron class-specific markers (Yemini *et al.* 2021), it is clear that *lin-32* also affects the class identity of a number of neurons in which it is transiently expressed. It does so, at least in part, through the regulation of the corresponding terminal selectors, the expression of which is lost in these neurons in the absence of *lin-32*. Although both panneuronal and neuronal class features are affected, it is known that terminal selector mutants do not usually show defects in panneuronal gene expression (Stefanakis *et al.* 2015). It is therefore likely that *lin-32* independently regulates these 2 aspects of neuronal differentiation, perhaps acting upstream of the CUT HD TFs that regulate panneuronal effector gene expression (Fig. 1) (Leyva-Díaz and Hobert 2022).

Proneural proteins of the AS-C and Atonal families are Class II bHLH proteins that heterodimerize with broadly expressed Class I bHLH proteins of the Daughterless/E protein family to regulate target gene expression (Massari and Murre 2000; Grove *et al.* 2009). The sole Class I ortholog in *C. elegans* is *hlh-2* and as such it coregulates many of the neuroblast specification events described above (Zhao and Emmons 1995; Krause *et al.* 1997; Portman and Emmons 2000; Frank *et al.* 2003; Nakano *et al.* 2010; Poole *et al.* 2011; Luo and Horvitz 2017). To begin to address the question of redundancy in relation to the absence of early neural precursor specification defects in *lin-32* and other proneural bHLH mutants, a recent study has analyzed the maternal and zygotic removal of *hlh-2*, in which all interactions with Class II bHLHs should be compromised (Masoudi *et al.* 2023). Surprisingly however, although many neuroblasts are misspecified in maternal and zygotic *hlh-2* (*hlh-2*$^{m/z}$) mutants, adopting either hypodermal cell fates or undergoing apoptosis, 122/221 embryonically born neurons are still generated (as assessed by nuclear morphology) (Masoudi *et al.* 2023). Many of these postmitotic neurons display differentiation defects, however, losing panneuronal gene expression, neuron class-specific gene expression, or both, without a concomitant switch in cell fate to hypodermal cells (Masoudi *et al.* 2023). This again suggests that these 2 aspects of neuronal differentiation can be genetically separated and in addition provides strong support to the conclusion that the role of proneural genes in many lineages is to promote terminal neuronal differentiation rather than neuronal precursor specification. As described earlier, there is a preponderance of HD TFs among known terminal selectors and therefore another emerging theme, conserved with vertebrates, is the regulation of HD TFs by proneural genes (reviewed in Guillemot 2007). How specificity is achieved is currently unclear, although it is known that proneural factors can have different E-box-binding site preferences and activate different subsets of target genes (reviewed in Powell and Jarman 2008; Castro and Guillemot 2011). Elegant domain swap experiments have revealed that the specificity of action resides in the basic DNA-binding domain and the HLH dimerization domain (Chien *et al.* 1996; Jarman and Ahmed 1998; Nakada *et al.* 2004). It will be important going forward to determine the direct molecular links between proneural gene expression and both panneuronal and subtype-specific effector gene batteries and their regulators.

Novel functions of bHLH TFs revealed from C. elegans *studies*

In addition to the previously known roles of bHLH proneural factors in neuron specification and differentiation, their study in *C. elegans* has revealed additional functions in nervous system development:

1) *Acting as a terminal selector*: there is only one well-described case in which a *C. elegans* ortholog of a proneural gene acts directly and specifically to regulate neuronal subtype specification and maintenance without initiating TF cascades or panneuronal features. The expression of *hlh-4* is distinct in comparison to other proneural genes as it is only postmitotic and maintained throughout the life of the animal in the ADL chemosensory neurons (Masoudi *et al.* 2018). Consistent with this maintained expression, *hlh-4* has been shown to act as a terminal selector, directly regulating the class-specific features of ADL (Masoudi *et al.* 2018).
2) *Robustness of neuronal differentiation*: as already stated, proneural genes can regulate later aspects of neuronal differentiation via regulation of terminal selector genes. One set of experiments not only suggests that this is direct but also indicates that robust expression of terminal selectors may require the input of multiple proneural genes. The AIY interneuron pair is specified by the terminal selector TTX-3 (Hobert *et al.* 1997; Wenick and Hobert 2004), and a mechanism to ensure robust *ttx-3* transcription that requires redundant activity of at least 3 proneural genes has been described (Filippopoulou *et al.* 2021). In *ngn-1* null mutants, the expression of *ttx-3* and the differentiation of the AIY are partially affected, but there is an additive effect on both when *hlh-3* and/or *hlh-16* are also removed. The effect on *ttx-3* expression is likely direct and at the transcriptional level through putative binding sites for these proneural bHLHs in the *cis*-regulatory regions of *ttx-3* (Filippopoulou *et al.* 2021).
3) *Convergent use of different proneural factors*: the adoption of identical terminal selectors in the left and right members of bilateral neuronal pairs is, in some cases, driven by the convergent activity of 2 different proneural genes. Rather remarkably, *lin-32* is expressed asymmetrically in several L–R and radially symmetric neuron classes (Masoudi *et al.* 2021). For example, *lin-32* is expressed in AVHL and CANL but not in AVHR or CANR. The expression of the terminal selectors of these neurons (*hlh-34*/*unc-42* and *ceh-10*/*ceh-43*, respectively) is only affected in the subclass (in this case left and not right) in which *lin-32* is expressed (Masoudi *et al.* 2021). On the contralateral side, a different proneural gene *hlh-14* is expressed and it regulates expression of the same terminal selectors in AVHR and CANR (Masoudi *et al.* 2021). This convergent function of 2 different proneural genes, *lin-32* an Atonal homolog and *hlh-14* an AS-C homolog, is perhaps surprising given the specificity in E-box binding and bHLH

domain swap experiments described above. Whether they can substitute for one another in *C. elegans* is currently unclear.

4) *Unequal cleavage*: many neuroblasts not only divide asymmetrically to self-renew and generate a neuron but also generate daughter cells of different sizes through unequal cleavage. A recent study has revealed that proneural genes have a rather remarkable role in this process in both neuroblasts and neuroblast precursors (Zhu *et al.* 2014; Mullan *et al.* 2024). In the C-lineage, the 2 divisions that lead to the generation of the DVC neuroblast (which generate DVC and a cell death) are dramatically unequal in size—the neuronal daughter of each division is half the size of the non-neuronal daughter. *hlh-14* regulates the second of these unequal cleavages. This suggests that proneural bHLH TFs can concomitantly regulate the fate and size of neuroblasts through control of unequal cleavage (Mullan *et al.* 2024). How they may do this at the molecular level is unknown.
5) *Cell death*: the *hlh-2*$^{m/z}$ analysis described above demonstrates that some neural precursors undergo ectopic cell death in these mutants. This suggests that proneural genes can antagonize cell death pathways in certain lineages. Consistent with this, in AIY and SMDD, *ngn-1* suppresses the expression of the key proapoptotic gene *egl-1* (Filippopoulou *et al.* 2021). However, in other lineages, *hlh-3* is known to activate *egl-1* (Thellmann *et al.* 2003). The role of proneural proteins in regulating cell death requires further examination.

Sequential action of bHLHs

In vertebrates and flies, cascades of neuronal differentiation genes are activated as a consequence of proneural protein activity (reviewed in Bertrand *et al.* 2002; Guillemot 2007). This includes HD TFs but also other bHLH family members such as Neurogenin and NeuroD. Expression analysis of bHLH genes in the ADL lineage reveals a cascade of sequentially acting proneural bHLHs (Fig. 4b). First, the expression of *hlh-3* and *hlh-14* is initiated in the ABalppp/praaa neural precursor daughters (Poole *et al.* 2011; Masoudi *et al.* 2018). In the absence of *hlh-14*, the ABalpppp/praaap neural progenitors are not specified (Poole *et al.* 2011). Shortly after this, the expression of *hlh-2* is initiated and is also required for neural precursor specification in the lineage (Masoudi *et al.* 2023). Lastly, the expression of *hlh-4* is initiated in ADL where, as described above, it acts as the ADL terminal selector and is maintained throughout the life of the animal (Masoudi *et al.* 2018). *hlh-2* expression is also maintained in ADL, and this expression is dependent on *hlh-4*. Thus, inferring from genetic and expression data, it is likely that initially an HLH-14/HLH-2 dimer regulates neural progenitor specification and then HLH-4/HLH-2 dimer regulates neuronal differentiation and class specification. Other examples include the HSN lineage in which the neuroblast is specified by *hlh-14* and *hlh-2*, and the class-specific features are regulated by *hlh-3*. It is noteworthy that the closest ortholog of *hlh-3* in flies is *asense*, an AS-C gene that is expressed at later stages of neuronal development, and it has been suggested that this regulates neuronal differentiation rather than neural progenitor specification (Jarman *et al.* 1993).

The role of repression in neural specification

In vertebrates, early neural inductive events rely heavily on transcriptional repression, and it has been suggested that neuronal fate may be the ground state of many, if not all cells (reviewed in Hemmati-Brivanlou and Melton 1997; Stern 2006), meaning that neuronal fate is thought to be the default fate in the absence of specific repression. The identification of the REST/NRSF TF, a repressor that binds directly to neuronally expressed genes in nonneuronal tissue, has given weight to this argument (reviewed in Schoenherr and Anderson 1995). However, detailed analysis of the *cis*-regulatory control mechanisms of panneuronally expressed genes in *C. elegans* reveals little evidence for repressive control in nonneuronal cells (Stefanakis *et al.* 2015).

Proneural genes undergo repression in flies and vertebrates via a process known as lateral inhibition (reviewed in Bertrand *et al.* 2002). Activation of Notch ligand expression by proneural factors leads to the activation of the Notch pathway in neighboring cells and expression of Notch target genes of the hairy/HES family of bHLHs. These factors then repress proneural gene expression. In *C. elegans*, mutations in *lin-22*, a bHLH TF with similarities to the *hairy/E(spl)* family of proteins, cause extra neuronal nuclei (Horvitz *et al.* 1983). In *lin-22* mutants, the anterior seam cells V1–V4 undergo homeotic transformations generating postdeirid or ray neurons, the production of which is normally restricted to the posterior V5 and V6 seam cells (Wrischnik and Kenyon 1997). This ectopic neuronal specification depends on *lin-32*, strongly suggesting that *lin-22* can repress proneural gene expression in a manner perhaps analogous to the role of *hairy/E(spl)* genes in Notch-responding cells during lateral inhibition. However, there is no evidence that *lin-22* is a target of Notch signaling in *C. elegans* and the homology of LIN-22 to the *hairy/HES* family is restricted to the bHLH domain. LIN-22 also lacks other characteristic HES family domains, including that for interaction with the corepressor Groucho (Wrischnik and Kenyon 1997). Moreover, although Notch signaling does indeed regulate several aspects of lineage specification in *C. elegans* embryos, these patterning events are not restricted to neuronal/nonneuronal lineages and the targets of Notch signaling are a family of bHLH proteins highly divergent from the *hairy/E(spl)* family (Neves and Priess 2005).

Other repressive interactions are evident in *C. elegans*. These include repression of nonneuronal ectodermal fates (i.e. hypodermis and glia) in neuronal cells and the converse. *lin-26* mutants were originally isolated because the vulva fails to form, and subsequent observation revealed this to be result of the transformation of ventral epidermal cells into neurons (Horvitz *et al.* 1983). LIN-26 is a zinc-finger TF that is expressed in most if not all hypodermal and glial cells (Labouesse *et al.* 1994, 1996). In the absence of *lin-26*, these cells are either transformed into neurons or undergo cell death. This strongly suggests that *lin-26* is required either to specify or to maintain nonneuronal ectodermal cell fates and repress neuronally expressed genes. The molecular mechanisms of LIN-26 function are currently unclear.

Conversely, *ztf-11*, a MyT1 family TF, is required postembryonically to repress nonneuronal ectodermal cell fates in neuronal cells (Lee, Taylor, *et al.* 2019). *ztf-11* is expressed in all embryonic neuronal lineages and in postembryonic ectodermal neuroblasts such as Q and V5. However, it is rapidly downregulated in postmitotic neurons in a manner very similar to that in most proneural TFs. Although largely dispensable for embryonic neurogenesis, loss of *ztf-11* prevents postembryonic neural specification from a number of postembryonic neuroblasts in which it is expressed, including Q and V5, but also from the rectal epithelial cells K and Y, which produce neurons via transdifferentiation (Jarriault *et al.* 2008; Lee, Taylor, et al. 2019; Riva *et al.* 2022). Importantly, ZTF-11 acts in a positive feedback loop with proneural genes; it is regulated by proneural genes but can also induce proneural gene expression. However, unlike its vertebrate MyT1 homologs, which have been shown to promote neurogenesis by counteracting the lateral inhibition mechanisms described above, it does not

function via repression of *lin-22*. Instead, it appears to function with the MuvB corepressor complex to suppress the expression of nonneuronal genes in neurons. Interactions between *ztf-11* and *lin-26* have not been investigated. Despite the mechanistic differences, it is intriguing that both *lin-22* and *ztf-11* share similar roles in promoting nonneuronal and neuronal fates, respectively, and that they both do so via the active repression of alternative fates and, either directly or indirectly, via the regulation of proneural genes.

Glial specification and diversity

Glial cells are a crucial component of the nervous system, contributing in many ways to its development and function. The nervous system of *C. elegans* contains 56 glial cells in the hermaphrodite and 90 in the male (Sulston and Horvitz 1977; Molina-García *et al.* 2020). The majority are found in sense organs, within which the dendrites of various sensory neurons are either embedded in a sheath (sh) glia or pass through a channel formed of a sh and a socket (so) glia (Ward *et al.* 1975). Twenty-six socket glia and 24 sheath glia arise embryonically from the AB blastomere, and in addition, there are 6 mesodermally derived GLR glia that arise from the MS blastomere. Six socket glia and 2 sheath glia arise postembryonically from the V5 and T seam cells. In the male, an additional 36 glia arise postembryonically from male-specific divisions of the V5, V6, and T seam cells, the B and Y rectal epithelial cells, and the ventral P10 and P11 cells (Sulston and Horvitz 1977). These glia form part of the male-specific copulatory sensory organs including the rays, the spicules, the hook, and the postcloacal sensillum. Two glia, the PHso cells, transdifferentiate in males into the PHD neurons (see below), leaving a total of 34 male-specific glia at the end of sexual maturation (Molina-García *et al.* 2020).

Work in the past few years has revealed several important roles for glia, many of which are analogous to their counterparts in other animals, including key roles in sculpting neuronal connectivity (reviewed in Singhvi and Shaham 2019; Singhvi *et al.* 2024). However, our knowledge of the mechanisms that regulate the production and diversity of *C. elegans* glia is rather minimal. All glia that derive from the AB blastomere derive from lineages that exclusively give rise to ectodermal cells, including neurons and hypodermal cells. In many cases, they are sister cells of neurons (Sulston and Horvitz 1977), and, as discussed earlier, in *lin-26* mutants, glial cells undergo cell death or are transformed into neurons (Labouesse *et al.* 1994, 1996). As also touched upon earlier, the postembryonic ray glia derive from the same *lin-32*-dependent neural precursor as the ray neurons themselves and the glial cells are also missing in *lin-32* mutants (Zhao and Emmons 1995). *lin-32* also plays a role, in parallel with 2 other pronerual bHLH TFs, *cnd-1* and *ngn-1*, to negatively regulate the number of embryonically specified AMsh glial cells (Zhang *et al.* 2020). Together, this suggests that proneural proteins play context-dependent roles in neural precursors to regulate the development of glia in *C. elegans*, as they do in other animal systems (reviewed in Guillemot 2007). The Forkhead TF *unc-130* is also known to play roles in the specification of sensory neurons (AWA and ASG) and the ILso glia that arise from the same lineage, likely acting in the neural precursors of that lineage (Sarafi-Reinach and Sengupta 2000; Mizeracka *et al.* 2021). Intriguingly, the 3 pairs of ILso glial cells derive from distinct lineages but converge to the same identity, and UNC-130 is only required in the progenitors of the 2 dorsal ILso cells, where it functions as a transcriptional repressor, and possibly acts to repress alternative cell fates (Mizeracka *et al.* 2021). Recent transcriptome analysis of the mesodermally derived GLR glia, which ensheath the brain neuropil, has revealed they combine astrocytic and endothelial characteristics (Stefanakis *et al.* 2024). This work also defined *let-381*, which encodes the sole *C. elegans* ortholog of the Forkhead TF FOXF as a key regulator of GLR cell fate. LET-381 acts as a terminal selector of GLR fate, where it maintains its own expression and regulates a battery of effector genes, likely directly but also via the HD TF *unc-30* (Stefanakis *et al.* 2024).

At later stages of glial development, it has been demonstrated that *mls-2/Nkx* and *vab-3/Pax6* are required for the differentiation of the CEPsh glia (Yoshimura *et al.* 2008). In addition, they regulate the expression of *hlh-17*, a bHLH TF related to the Olig family, in the CEPsh glia. However, the loss of *hlh-17* does not grossly perturb the CEPsh glia and so its contribution remains unclear (Yoshimura *et al.* 2008). Genetic sex also plays a role in the CEPso glia in allowing them to shape the overlying cuticle in a sex-specific manner during sexual maturation (Fung *et al.* 2023). Postdevelopmentally, it has also been demonstrated that *pros-1* functions to control the expression of a glial secretome (Kage-Nakadai *et al.* 2016; Wallace *et al.* 2016) and that the HD TF *alr-1* functions to maintain the structural integrity of the AMso glial cells (Tucker *et al.* 2005). Glia can also undergo changes in response to the environment (Procko *et al.* 2011). Our understanding of glial development is expected to change rapidly. Many reporters have now been generated that label distinct subsets of glia (Fung *et al.* 2020), and transcriptional profiling (Bacaj *et al.* 2008; Wallace *et al.* 2016) and more recently scRNA-seq for glia have been performed (Purice *et al.* 2023). The next few years are likely to reveal much more about glial development in *C. elegans*.

Postembryonic neural progenitors and the glial origin of neurons

We now know that *C. elegans* glia can act as postembryonic neural progenitors in a similar fashion to vertebrate glia. In males, during sexual maturation, the AMso glial cells divide asymmetrically to self-renew and generate the MCM neurons (Sammut *et al.* 2015). This was the first demonstration that glia act as neural progenitors in an invertebrate, although this role in various vertebrate contexts has been clear for a number of years (reviewed in Doetsch 2003; Kriegstein and Alvarez-Buylla 2009). Also in males, and at a similar time as the division of AMso cells to give rise to the MCMs, the PHso1 glial cells undergo a direct transdifferentiation to generate the PHD neurons (Molina-García *et al.* 2020). These provide paradigms to address interesting questions about cell fate plasticity and the maintenance of neural progenitors using the power of *C. elegans*.

As described earlier, one other direct transdifferentiation has been described in *C. elegans*: the Y cell postembryonically transforms into the PDA motor neuron in hermaphrodites (reviewed in Lambert *et al.* 2021). More recently, the origin of the DVB neuron from the asymmetric division of the rectal epithelial cell K in hermaphrodites has also been characterized as a transdifferentiation event (Riva *et al.* 2022). It is also known that differentiated excretory pore cells G1 and G2 act as neuroblasts during larval development (reviewed in Lambert *et al.* 2021). The fact that in all these cases the progenitors are either epithelial cells or glial cells provides a strong parallel to vertebrate neurogenesis, where it is lineally related neuroepithelia, then radial glia and finally radial astrocytes that act as neural stem cells as development progresses (reviewed in Doetsch 2003; Kriegstein and Alvarez-Buylla 2009). Many of the other postembryonic blast cells that act as neural progenitors in *C. elegans* also display varying degrees of epithelial differentiation (reviewed in Lambert *et al.* 2021). Moreover, as described above and like their vertebrate counterparts, many require the activity of *sox-2* and *ztf-11* to retain their neurogenic potential. This raises the possibility that certain molecular aspects of postembryonic neural progenitor specification in *C. elegans* may be more similar to vertebrate neurogenesis than

that of the embryo, for which *sox-2* and *ztf-11* are largely dispensable. Consistent with this idea, it has recently been shown that a cascade of factors involving *sem-4/Sall* activation of *egl-5/Hox* followed by *egl-5/Hox* activation of *hlh-16/Olig* and *ngn-1/Ngn* is required for Y to PDA transdifferentiation (Rashid *et al.* 2022), analogous to the cascade that drives mammalian spinal cord motor neuron formation.

Mechanisms of neuronal diversification

Principles of neuron class specification by terminal selectors

Out of the almost 900 TFs encoded in the *C. elegans* genome, 73 are known to act as terminal selectors (Reilly *et al.* 2022). This limited number of TFs is used to specify a large neuronal diversity by acting in regulatory logics that are combinatorial, robust, and yet plastic. Below, we summarize several general principles of terminal selector function that have emerged from studying the execution of terminal transcriptional programs across diverse neuron classes, which reveal how neuronal diversity arises.

TFs that act as terminal selectors can also act earlier in development

TFs acting as terminal selectors are not molecularly distinct to other TFs, their expression is not necessarily restricted to postmitotic neurons, and they can display additional functions earlier in development or in nonneuronal tissues, e.g. UNC-86 acts early in several neuroblast lineages to promote their correct specification (Chalfie *et al.* 1981; Finney and Ruvkun 1990) in addition to being a terminal selector (Leyva-Díaz *et al.* 2020). This suggests that some of the inputs for terminal selector activation may originate from earlier stages of embryogenesis. An intriguing case of this is provided by UNC-30/PITX and CEH-36/OTX, 2 different types of HD TFs that act as terminal selectors for the D-type motor neurons and the AWC sensory neurons, respectively (Eastman *et al.* 1999; Lanjuin *et al.* 2003). Both TFs are also expressed in various lineage branches from around mid-embryogenesis with substantially overlapping expression patterns, and they act redundantly in the specification of progenitors of various neurons, glia, and the excretory system as evidenced by cell cycle and mispositioning defects of numerous progenitor cells (Walton *et al.* 2015). One of the shared targets of CEH-36 and UNC-30 is another HD TF *mls-2*, which is necessary for the later expression of *ceh-36* in the AWCs, where CEH-36 acts as a terminal selector (Fig. 5a) (Kim *et al.* 2010). This suggests that CEH-36 (with UNC-30) may set the stage for its own transcription later in development and highlights how intricate regulatory interactions that lead to specific terminal selector expression patterns start forming early in development.

Terminal selectors act in combinations

In most instances of transcriptional activation, multiple TFs are necessary to recruit cofactors and RNA polymerase II to activate transcription (reviewed in Reiter *et al.* 2017). This combinatorial requirement at the enhancer level provides robustness but, most importantly, it enables the generation of exquisite specificity based on intersectional expression patterns of those TFs. For example, the deeply studied HSN motor neuron program relies on at least 6 TFs, both from HD and non-HD families, that act collectively and directly on the regulatory modules of HSN effector genes to ensure specific and robust gene expression (Lloret-Fernández *et al.* 2018). These combinations of TFs have been termed terminal selector collectives in homology to similar TF combinatorial actions described in *Drosophila* (Junion *et al.* 2012). Single and double mutant analysis reveals redundancy and extensive synergistic effects among HSN terminal selectors. In addition, binding site clusters for this terminal selector collective constitute a regulatory signature sufficient for identification of HSN enhancers in the genome (Lloret-Fernández *et al.* 2018). A similar combinatorial logic has also been suggested for dopaminergic neurons (Jimeno-Martín *et al.* 2022).

Modular enhancers direct expression of shared effector genes in different neuron classes

Most effector genes are not expressed in a single neuron class, and their specific patterns of expression are determined by modular *cis*-regulatory landscapes, where different *cis*-regulatory modules (CRMs or enhancers) integrate the input of different sets of terminal selectors. Modularity reveals itself at the level of separate enhancers but also at usage of distinct sets of TFs at the same enhancer (Fig. 5b). For example, different nonoverlapping enhancers drive expression of the effector gene coding for the tryptophan hydroxylase enzyme (*tph-1*) in different serotonergic neuron classes (Fig. 5b, *left*) (Lloret-Fernández *et al.* 2018). In some cases, the same enhancer region drives expression in different neuron classes but is bound and regulated by different terminal selector TFs from the same family. For example, the HD TF binding site present in the CRM controlling *eat-4* VGLUT effector gene expression in several glutamatergic neuron classes is recognized by distinct HD members acting as neuron type-specific terminal selectors in each neuron class (Serrano-Saiz *et al.* 2013) (Fig. 5b, *center*). In other cases, a single enhancer region drives expression in 2 neuron classes because it binds a pleiotropic TF that acts with different additional TFs to activate the enhancer in a class-specific manner. This is illustrated by the *bas-1*, an amino acid decarboxylase effector gene enhancer that is active in NSM and HSN serotonergic neurons. UNC-86 binds this enhancer in both neurons, but acts together with distinct neuron class-specific TFs that bind different motifs in the same CRM (Zhang *et al.* 2014; Lloret-Fernández *et al.* 2018) (Fig. 5b, *right*).

Expression and function of terminal selectors are maintained throughout the life of the animal

Terminal selector activity is required for sustained expression of the effector genes and thus for maintenance of neuron class identity throughout life. This was first uncovered with the use of temperature-sensitive alleles and RNAi experiments and, more recently, through inducible depletion of TFs with the auxin-inducible degron system and CRISPR/Cas9-mediated analysis of *cis*-regulatory regions (Etchberger *et al.* 2009; Lloret-Fernández *et al.* 2018; Feng *et al.* 2020; Li, Osuma, *et al.* 2020; Maicas *et al.* 2021; Traets *et al.* 2021). Sustained expression of terminal selectors is usually achieved by a feedback loop of autoactivation (Etchberger *et al.* 2007; Leyva-Díaz and Hobert 2019). In addition to promoting stable effector gene expression throughout the life of the neuron, terminal selectors present in adult neurons can work in concert with other temporally restricted TFs that provide additional information for modifying gene expression in a neuron class-specific manner. This intersectional activity of terminal selectors with other dynamically expressed TFs is discussed in extensive detail in the sections on time, genetic sex, and environment.

A handful of terminal selectors is recurrently used to define multiple neuron identities

Among all the terminal selectors described in *C. elegans*, 5 TFs are recurrently used to specify 70 distinct neuron classes (Table 2), in each case acting in combination with other TFs: UNC-86, CEH-14, UNC-42, CEH-34 (4 HD TFs), and UNC-3 (Colier/Olf/EBF COE TF family). CEH-34 acts as master terminal selector of all 14 pharyngeal neuron classes, which share some transcriptional commonalities

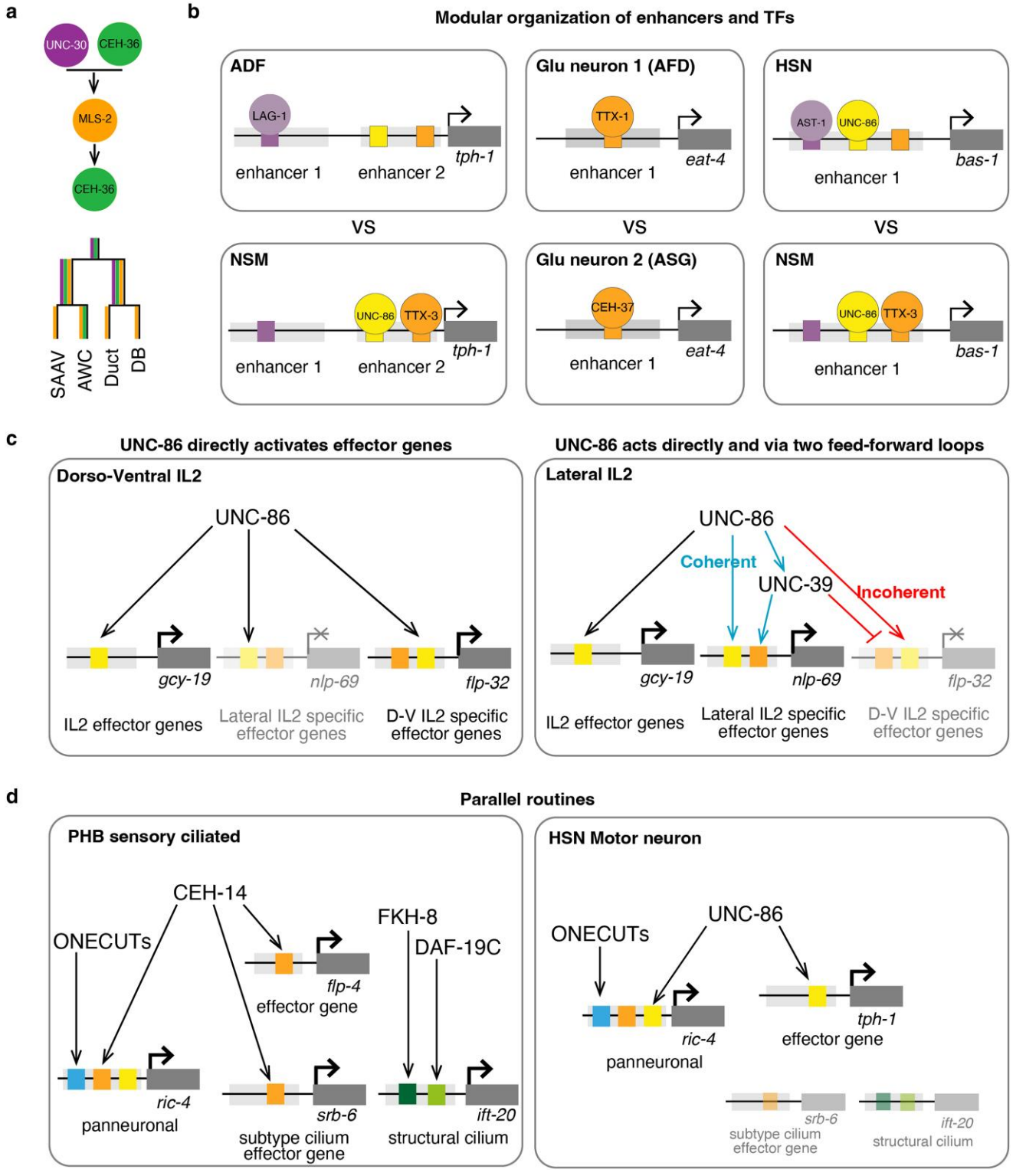

Fig. 5. Terminal selector functions in neuronal diversification. a) Example of the accumulation of regulatory factors along a lineage to result in specific (and robust) patterns of gene expression (from Walton *et al.* 2015). b) Terminal selector TFs act combinatorially on modular *cis*-regulatory sequences, enabling cell-specific control of various terminal effector genes. The modular architecture of *cis*-regulatory elements enables integration of distinct combinations of terminal selectors, as well as integration with other transcriptional inputs as discussed in the rest of the chapter. The 3 illustrated examples correspond to those described in the text. c) The output of a terminal selector (e.g. UNC-86) can be diversified by integration into gene regulatory networks with other transcriptional regulators. These can be organized in regulatory motifs such as coherent feedforward loops (in which a terminal selector activates an effector gene as well as an additional transcriptional activator, blue arrows) and incoherent feedforward loops (in which a terminal selector activates an effector gene as well as a repressor of the same target gene, red arrows). Such motifs are recurrently used to modify the terminal battery activated by a terminal selector. d) Regulation of the different components of the neuronal transcriptome is achieved thanks to combinatorial TF action on modular *cis*-regulatory sequences that result in parallel modules that are interconnected. For example, panneuronal gene expression relies on CUT TFs but also receives terminal selector input. Gene modules such as those coding for the structural cilium components are under control of a parallel program driven by FKH-8 and DAF-19C, but other neuron class-specific components of the cilia, just like other neuron-type effector genes, are under terminal selector control.

(Vidal et al. 2022). In support of the HD code model, CEH-34 cooperates with other specific HD TFs to individuate distinct pharyngeal neuron classes. In contrast to CEH-34, the other 4 prevalent TFs act as terminal selectors in sensory neurons, interneurons, and motor neurons that share no obvious similarities. Of note, CEH-34, UNC-3, and UNC-42 have been described as "circuit" TFs, because neurons sharing each of these terminal selectors tend to be more interconnected and mutants for these TFs show defects in axon pathfinding and synapse formation between neurons that share the same terminal selector (Pereira et al. 2015; Berghoff et al. 2021; Vidal et al. 2022). The reiterative use of specific TFs in neuron-sharing connectivity has been postulated to be linked to a common evolutionary origin for those neurons (Arendt 2008). Alternatively, specific TFs may be reused as terminal selectors because they have particular functional properties, e.g. extensive protein–protein interactions that may allow them to effectively act combinatorially, the ability to induce accessibility of chromatinized DNA, or other unknown features. A better description of the complete array of TFs that act in the activation of neuron class regulatory modules and the biochemical properties of those TFs will be important for advancing our understanding of neuron-type regulatory programs and of the noncoding regulatory genome.

Terminal selectors act with other TFs in regulatory motifs that enable developmental and evolutionary flexibility

In addition to targeting effector genes, terminal selectors also activate other TFs that act in various regulatory motifs to ensure robustness of gene expression and provide flexibility to the transcriptional program and a structure on which evolution can act to diversify neuron subclasses. For example, the TFs UNC-86 and UNC-39 form a regulatory motif known as a coherent feedforward loop in which UNC-86 activates transcription of *unc-39*, and the 2 TFs together activate their target genes (Fig. 5b) (Cros and Hobert 2022). As will be explained later, this motif is used to diversify the 6 IL2 sensory neurons along the D–V axis. Similarly, incoherent feedforward loops, where a terminal selector activates expression of a repressor of a subset of its gene targets, are also present in *C. elegans* as another strategy for increasing neuron subclass diversity. For example, UNC-39 not only induces IL2 subclass-specific effector genes but also represses UNC-86-driven effector genes from a different IL2 subclass (Fig. 5c) (Cros and Hobert 2022). A similar incoherent feedforward loop is also found for RMD D–V vs L–R diversification (Cros and Hobert 2022).

Parallel yet interconnected regulation of the panneuronal and neuron-specific transcriptional programs

Interestingly, CUT TF sextuple mutants in which panneuronal gene expression is severely affected do not show defects in expression of neuron class effector genes, neuronal migration, or morphology, indicating that panneuronal and neuron class regulatory programs run "in parallel" (Fig. 1). Nevertheless, these 2 parallel regulatory routines, which are triggered directly or indirectly by proneural TFs, are interconnected: panneuronal gene expression is controlled by redundant CRMs and some of these modules are under direct control of terminal selectors (Stefanakis et al. 2015). Thus, as expected, combinations of sextuple CUT mutants with specific terminal selectors further affect panneuronal gene expression in a neuron class-specific manner (Fig. 5d) (Leyva-Díaz and Hobert 2022). As a corollary of this regulatory interaction, it has been shown that ectopic expression of neuronal terminal selectors in a heterologous cell type (e.g. the germline) can induce expression of panneuronal features when specific chromatin factors, e.g. the histone chaperone LIN-53, are removed (Tursun et al. 2011). Similarly, terminal selector mutants do not affect structural cilia effector gene expression, which is dependent on the parallel action of DAF-19 and FKH-8 TFs (Fig. 5d). However, as described earlier, the neuron class-specific ciliome components are under terminal selector regulation (Fig. 5d).

Lineage-based mechanisms of neuronal diversification

The precise regulatory landscape that determines the specific identity of a neuron includes not only the expressed terminal selector TFs but also each cell's specific chromatin state as well as numerous other posttranscriptional and posttranslational regulators. This regulatory landscape is the result of a combination of cell autonomous and nonautonomous regulatory events along the developmental history of a neuron. During every cell division, cell autonomous, lineage-specific information (including regulatory factors present and the existing chromatin landscape) is integrated with external signaling inputs from other cells to eventually give rise to the different neuron classes. This sequence of lineage-specific regulatory events results in every neuron class expressing a unique combination of regulatory factors that act on a unique nuclear and cellular setting.

Intriguingly, in *C. elegans*, bilateral members of the same neuron class are not necessarily related by lineage. This means that the same cell type, as defined by shared effector gene batteries, can be produced from different lineage branches following distinct developmental regulatory trajectories, a processed termed developmental convergence. At the same time, cells that are produced from the same lineage branch typically adopt different identities upon terminal division, indicating that a single regulatory input/event at the end of embryonic development is sufficient to create 2 different cell types. These features of neuronal development are not exclusive to *C. elegans*, but the worm once again provides a model for addressing these with unparalleled precision. For example, the worm is perfectly suited to address the 2 main open questions that arise with respect to convergence: (1) how do distinct transcriptional histories result in the same terminal selector output? and (2) how do the differences in the history of 2 convergent cells impact their terminal identity?

Below, we discuss some of the regulatory mechanisms that lead to the expression of specific combinations of terminal selectors and other regulators in specific branches of the lineage.

The Wnt asymmetry pathway is a general mechanism for diversifying cells along the lineage

The Wnt/β-catenin asymmetry pathway is a central mechanism for defining changes in gene expression associated with most asymmetric cell divisions during *C. elegans* development (Fig. 6a). This pathway has been extensively reviewed (Sawa and Korswagen 2013; Bertrand 2016) and discussed in the context of the *C. elegans* lineage (Bertrand and Hobert 2010; Barrière and Bertrand 2020; Liu and Murray 2023), so we only provide a brief description here. However, we underscore the critical role it plays in defining progressive/sequential, binary cell fate decisions along the lineage. Wnt ligands secreted by a posterior source polarize cells or the products of their division, leading to asymmetric localization of Wnt pathway components across the anterior and posterior daughter cells. This leads to the asymmetric nuclear localization of the transcriptional effector of the pathway, the TF POP-1/TCF, and the asymmetric degradation of its cofactor SYS-1/β-catenin. The resulting asymmetry of POP-1 and SYS-1 results in distinct gene regulation in the anterior and posterior cells, which is integrated with TFs already present in the 2 daughter cells to generate 2 new asymmetric regulatory states. Below, we

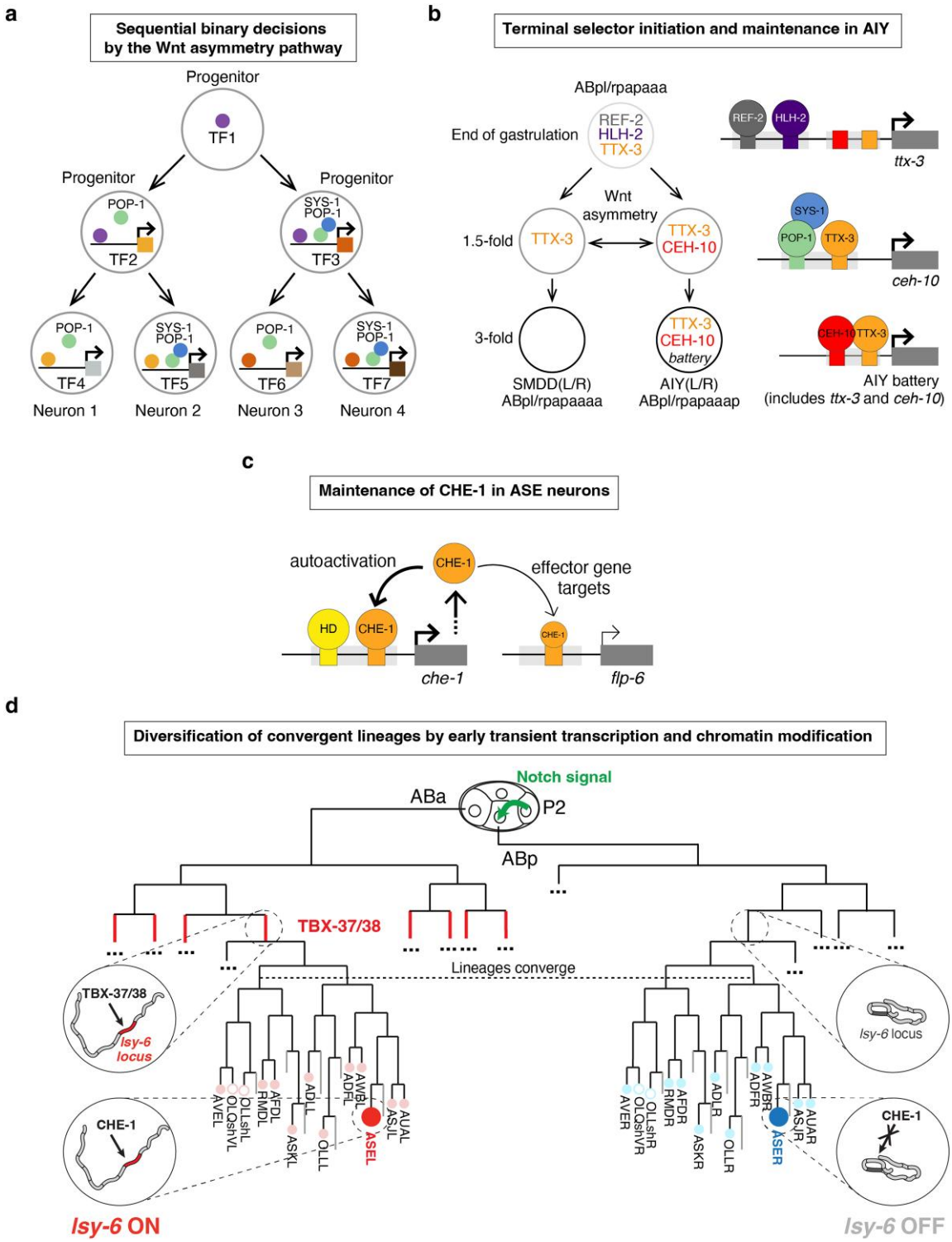

Fig. 6. Generation of terminal selector combinations in space and time. a) Schematic of how the Wnt asymmetry pathway can be integrated with existing TFs to generate a novel regulatory state during every cell division (adapted from Bertrand 2016). The asymmetry in POP-1 and SYS-1 results in distinct transcriptional outputs in the anterior vs the posterior daughter cell. Note that this schematic is highly simplified and POP-1 in the anterior cell can also activate or repress targets (Murgan and Bertrand 2015). b) Specification of the AIY neurons illustrates a number of points described in the text. The vertical axis denotes time. Transient expression of a trigger, REF-2, is integrated with the Wnt asymmetry pathway and autoactivation of the terminal selectors TTX-3 and CEH-10 to result in specific and robust activation in the posterior cell. This distinguishes the SMDD (anterior) from the AIY (posterior) neurons. c) The autoactivation mechanism of *che-1* has been proposed to be sensitive to fluctuations in the CHE-1 protein by preferential activation of its own locus (Traets *et al.* 2021). d) The differentiation of the left and right ASE neurons also illustrates a number of mechanisms described in the text. These 2 neurons derive from lineages that diverge at the 4-cell stage owing to the first Notch induction and represent one of the best described examples of convergence. The point where the lineages converge is marked with a dashed line. The converging branches generate 11 pairs of bilaterally symmetric neurons and 2 pairs of bilaterally symmetric glia. The early lineage asymmetry is integrated with the terminal selector CHE-1 in the form of differential chromatin states of the *lsy-6* locus. This determines whether *lsy-6* is expressed (ASEL) or not (ASER) and the downstream acquisition of the respective terminal gene batteries (Hobert 2014) (Fig. 7a).

illustrate the key role of the Wnt/β-catenin asymmetry pathway with the case of AIY interneuron specification.

Specific initiation plus maintenance by autoactivation of terminal selectors

The expression of a terminal selector at sufficient levels to drive acquisition of neuronal identity has been shown in several cases to require a specific initiation trigger and a maintenance mechanism that relies on the produced TF itself. This enables a regulatory logic in which transient transcriptional inputs early in development can be converted into stable, robust, and specific expression patterns. The positive autoregulation of most terminal selectors also results in their expression throughout the life of a neuron, consistent with their requirement to establish and maintain neuronal identity. In all but a few cases, the specific triggers that determine the time and onset of expression of terminal selectors are unknown. We discuss the best understood cases, but this remains an area that requires further exploration.

One of the best-defined examples that showcases this regulatory logic is the activation of the 2 terminal selectors that cooperatively define the AIY interneuron identity, CEH-10 and TTX-3 (Fig. 6b) (Wenick and Hobert 2004). Transcription of *ttx-3* is specifically activated in the mothers of the AIY pair of neurons by the intersection of lineage-specific TFs and signaling through the Wnt/ß-catenin asymmetry pathway. Specifically, the Zic TF REF-2 is expressed early in a number of ABa and ABp branches, including the mothers and grandmothers of the AIY neurons. In the mother of AIY and its sister SMDD (ABplpapaaa), *ttx-3* transcription is activated by the transiently expressed REF-2 together with multiple proneural bHLHs (HLH-2, HLH-3, HLH-16, and NGN-1) and activity of SYS-1/β-catenin and POP-1/TCF, which is high in the posterior daughter as a result of asymmetric Wnt signaling. This neuroblast divides asymmetrically following another round of polarization by Wnt signaling, resulting in 2 neurons that contain TTX-3 but no REF-2 and differ in their POP-1 and SYS-1 distribution. In the posterior cell (AIY), but not in the anterior cell (SMDD), the existing TTX-3 acts together with POP-1 and the proneural bHLHs to activate *ceh-10* transcription. CEH-10 and TTX-3 then form a heterodimer that locks the AIY identity by promoting their own transcription as well as the AIY gene battery (Bertrand and Hobert 2009; Murgan *et al.* 2015; Filippopoulou *et al.* 2021). This tiered activation of 2 TFs that ultimately act together to sustain their own expression and drive expression of the terminal battery is analogous to the action of UNC-86 and MEC-3 in specification of the mechanosensory neurons (Way and Chalfie 1989).

The case of the terminal selector CHE-1 in the ASE sensory neuron pair showcases important mechanistic aspects of the autoregulatory activity of terminal selectors. CHE-1 is a zinc-finger TF that is exclusively expressed in the ASE left (ASEL) and right (ASE) neurons. Based on dissection of the *che-1 cis*-regulatory sequences, we know that at least 3 initiator motifs are necessary for ASE-specific transcription (Etchberger 2008). The complement of TFs that initiate *che-1* transcription has not been fully defined, but it seems likely that the Tailless/TLX homolog, nuclear hormone receptor (NHR)-67, is among them (though a direct binding site has yet to be defined) (Sarin *et al.* 2009). The CHE-1 binding site, however, has been well defined, and a single site in the *che-1* promoter has been shown to be necessary for positive autoregulation (Etchberger *et al.* 2007; Leyva-Díaz and Hobert 2019; Traets *et al.* 2021). Importantly, this positive feedback is not only required for maintenance of CHE-1 throughout the life of the neuron but also for achieving the suprathreshold level of this TF required for initial activation of the effector battery (Leyva-Díaz and Hobert 2019). The maintenance of CHE-1 has also been studied in the context of the fluctuations in levels that are expected from the stochastic nature of the gene expression process. Below, a certain CHE-1 threshold autoregulation fails, and loss of CHE-1 results in loss of ASE function (Traets *et al.* 2021). This study revealed a mechanism that ensures continued *che-1* expression under small fluctuations of CHE-1 levels: the CHE-1 binding site in the *che-1* promoter is stronger than CHE-1 binding sites in most other targets, allowing prioritization of its own locus among different targets. This is likely due to cooperative binding with CEH-36 on the *che-1* promoter, as removal of this additional binding site resulted in spontaneous loss of CHE-1 and downstream targets in the ASEs (Fig. 6c) (Traets *et al.* 2021).

Both these cases highlight an important requirement for this regulatory logic to be effective: activation of a terminal selector must be strong enough for the levels of the TF to cross a threshold where autoregulation can be sustained—the TTX-3 case highlights the role of the many bHLH TFs in driving robust initiation, and the CHE-1 case highlights the importance of the strength of the autoregulatory feedback. Another consequence of such robust activation mechanisms that require multiple combinatorial inputs to reach and maintain terminal selector levels is that they help determine specificity, since they make it unlikely that the positive feedback loop would be spuriously triggered in the wrong place or at the wrong time.

The effect of chromatin on TF activity and the generation of temporal TF combinations

As discussed above, the generation of neuronal diversity relies on specific combinations of TFs that result in unique patterns of cooperative transcriptional activity. In other words, the generation of cellular diversity relies on the fact that the activity of most TFs is context dependent, with the same TF driving activation (or repression) of different sets of target genes in different cell types or even at different stages in the same cell type. In addition to coexpression with other TFs, context dependence is also determined by another important parameter, the chromatin landscape acquired by each cell during its developmental trajectory. Different chromatin states can determine accessibility of TFs to certain binding sites, and therefore, the same TF could act on different target gene sets in cells with different chromatin landscapes. The defining effect that chromatin state can have on the activity of a TF and ultimately on neuronal identity is best illustrated by the modification of activity of the ASE terminal selector, CHE-1, in 2 different contexts.

1) The ectopic expression of many terminal selectors, including CHE-1, at embryonic stages before cells are fully differentiated readily activates transcription of target genes in those ectopic cellular contexts. However, the same expression in differentiated cells will only rarely cause target transcription largely because of the acquisition of heterochromatin during differentiation (Meister *et al.* 2011). Loss of function of chromatin factors associated with repressive function enables CHE-1, but also UNC-3, UNC-30, and other TFs, to activate their targets in cells where they would normally be inactive (Tursun *et al.* 2011; Patel *et al.* 2012; Kolundzic *et al.* 2018; Hajduskova *et al.* 2019).
2) In its endogenous context, activity of CHE-1 is also affected by chromatin status. Both left and right ASE neurons are specified by CHE-1, but the 2 cells execute different terminal differentiation programs that ultimately result in asymmetric gene expression and neuronal functions (Fig. 6d). This asymmetry has been traced back to the expression of a microRNA (miRNA) called *lsy-6* in ASEL but not ASER. We

explain the role of *lsy-6* below and rather focus here on the fact that transcription of *lsy-6* requires CHE-1, but the locus is only accessible for transcriptional activation in ASEL and not in ASER. The difference in accessibility has been linked to the distinct lineage origins of the 2 ASE neurons, which diverge at the 4-cell stage: ASEL derives from the ABa blastomere and ASER from the ABp blastomere. ABa and ABp are originally equivalent in fate, but an A–P asymmetry is introduced by the first Notch signaling event in the embryo (Priess *et al.* 1987; Priess 2005). The lineage that gives rise to ASEL transiently expresses TBX-37 and TBX-38, 2 redundant T-box TFs, 4–5 cell divisions before ASEL is born. TBX-37/38 acts directly on the *lsy-6* locus, priming it for later activation by CHE-1. In the ASER-producing lineage, absence of TBX-37/38-mediated priming results in a "closed" *lsy-6* locus that becomes refractory to CHE-1 activation (Poole and Hobert 2006; Cochella and Hobert 2012; Charest *et al.* 2020). Thus, early L/R lineage differences in TF expression result in chromatin differences that are maintained throughout several divisions and result in *lsy-6* being an accessible target for CHE-1 only in the left side. This enables the combinatorial action of TFs that are never coexpressed in the same cell—a mechanism that has been termed "temporal intersection" (Cochella and Hobert 2012; Charest *et al.* 2020).

It seems possible that similar temporal integration mechanisms will be at play in other cells, with the most likely candidates being the 10 additional bilateral neuron pairs and 2 bilateral glial pairs that descend from the same asymmetric lineages as the ASEs and converge to the same terminal identities (Fig. 6d). This mechanism may more generally be used to diversify other convergent cell types that develop through different lineage trajectories but ultimately express the same terminal selector TFs. The *C. elegans* lineage is greatly suited to exploration of how temporal integration through the establishment of permissive chromatin states may more broadly contribute to neuron diversification, but this will require cellular or at least lineage-resolved analyses of chromatin states over time.

Differences in TF accessibility to chromatin-embedded binding sites correlate with histone modifications, particularly methylation and acetylation of lysine residues. In the case of CHE-1, accessibility to the *lsy-6* locus requires the activation-related methylation of histone 3 on lysine 4 (H3K4me) (Poole *et al.* 2011; Charest *et al.* 2020). Silencing modifications such as H3K9 methylation have been implicated in the diversification of the 6 VC (VC1–VC6) motor neurons. The vulval VC neurons, VC4 and VC5, express the HD TF UNC-4, but expression is not observed in the other VCs. However, loss of the H3K9 methyltransferase MET-2 or of other proteins that are necessary for formation of H3K9me2/3 heterochromatin results in ectopic expression of UNC-4 in the other VCs (Zheng *et al.* 2013). Based on this and on work in multiple other systems, it is expected that lineage or cell-specific chromatin modification patterns play critical roles in defining the output of most TFs. Obtaining lineage-specific, genome-wide patterns of histone modifications will be crucial for understanding how cell-specific transcriptomes arise.

The role of repressors in shaping TF activity and terminal gene batteries

Repression is an essential mechanism to establish the precise combinations of terminal selectors and their downstream gene batteries. During early embryonic events, the Notch and Wnt pathways deploy numerous repressive inputs to establish binary splits along the lineage. Critical targets of the Notch pathway include members of the REF-1 family of bHLH TFs, which act together with the corepressor UNC-37/Groucho to repress specific targets (Priess 2005). The Wnt/β-catenin asymmetry pathway defines whether POP-1/TCF acts as a repressor or an activator of its targets, depending on its association with SYS-1/β-catenin and depending on additional cofactor contexts (Murgan and Bertrand 2015). We also discussed above the effect of repressive chromatin environments on TF activity. In this section, we focus on the roles of repressors at more terminal stages of diversification. These examples reveal how specific cell identities are built by superimposing layers of repression (and repression of repression) over broader activating inputs. This is particularly evident in examples of subclass specification, in which a neuron class is specified by a terminal selector but then diversified by the superimposition of repressive modules. We highlight the roles of both transcriptional and posttranscriptional repressors in diversifying neuron types and subtypes at 4 levels.

Double-negative motifs that result in distinct expression of terminal selectors

As mentioned above, the 2 ASE neurons are diversified into distinct subclasses by the expression of the miRNA *lsy-6* in ASEL but not in ASER (reviewed in Hobert 2014) (Fig. 6d). miRNAs are 21–22-nt long RNAs that form part of an RNA-induced silencing complex and guide that complex to target mRNAs via sequence-specific interactions with the 3′-UTR (reviewed in Ketting and Cochella 2021). The effect of miRNA action is to repress translation and promote decay of its targets. In the case of *lsy-6*, the target is *cog-1*, which codes for a HD TF (Fig. 7a). *cog-1* is transcribed in both ASEs, but COG-1 protein is restricted to ASER given the *lsy-6*-mediated repression in ASEL. COG-1 is itself a transcriptional repressor that acts with the UNC-37/Groucho corepressor on various targets (Flowers *et al.* 2010). One of those targets is *die-1*, a zinc-finger TF, which as a result is repressed in ASER but expressed in ASEL. This double repressive interaction results in the mutually exclusive expression of DIE-1 and COG-1. Mutations in *lsy-6*, *die-1*, or *cog-1* result in complete subclass switches from ASEL to ASER for the first 2, and vice versa for the latter. As a result of this double-negative module, animals have 2 ASE neurons with numerous symmetric morphological and molecular properties (defined by CHE-1, see e.g. Etchberger *et al.* 2007) but also substantial molecular and thus functional asymmetries defined by the expression of DIE-1 or COG-1 (Hobert 2014) (Fig. 7a).

Repression of the terminal selector's activity resulting in distinct target repertoires

The COE/EBF-type terminal selector UNC-3 specifies the identity of 5 classes of cholinergic motor neurons in the ventral nerve cord. In each of these, UNC-3 activates transcription of shared genes (e.g. the complete ACh biosynthesis and packaging machinery) but also of neuron class-specific effector genes (Kerk *et al.* 2017). The transcriptional specificity of these effector genes is achieved by a network of repressor TFs that largely act through binding sites in the vicinity of the UNC-3 binding sites to counteract its activity (Kerk *et al.* 2017). Mutations in any of these repressors result in a "mixed" molecular identity, with motor neurons expressing effector genes from different classes. For example, UNC-4, together with the corepressor UNC-37/Groucho, acts in the VA and DA classes to repress features of VBs and DBs (Pflugrad *et al.* 1997; Kerk *et al.* 2017) (Fig. 7b). Analogously, BNC-1, with the corepressor CtBP, acts in the VA and VB classes to repress genes expressed in the DAs and DBs (Kerk *et al.* 2017). Interestingly, the specificity of expression of UNC-4 and BNC-1 is also determined by repressors: VAB-7 is expressed in the DBs where it represses UNC-4 (Esmaeili *et al.* 2002),

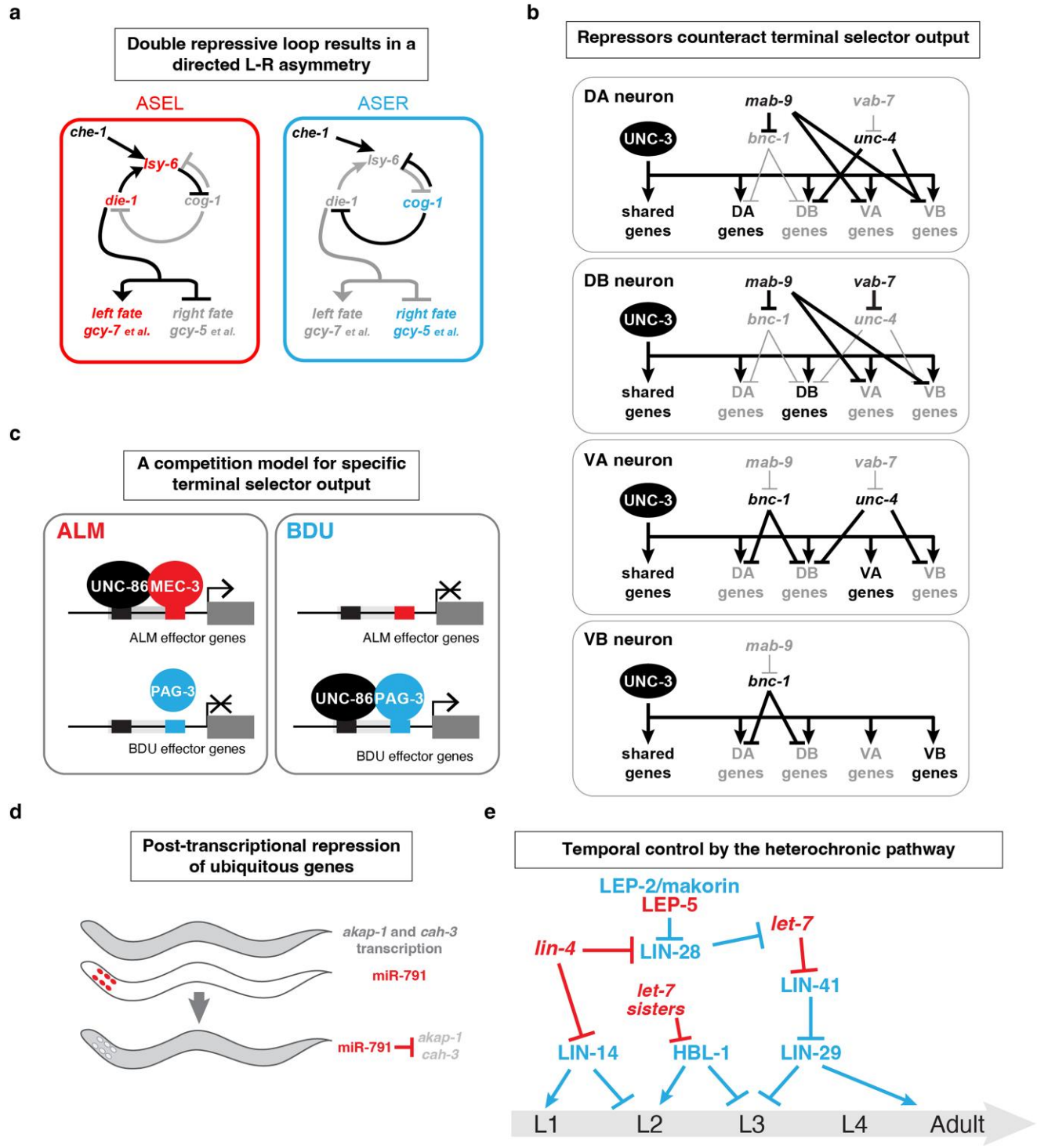

Fig. 7. Repressor-based mechanisms for neuronal diversification. a) Schematic of the double-repressor interactions that result in ASE asymmetry. The miRNA lsy-6 in ASEL represses the production of the TF COG-1. *cog-1* is transcribed in both ASEs but only produces COG-1 protein in ASER where lsy-6 is absent. COG-1 acts with the corepressor Groucho/UNC-37 to repress transcription of *die-1*, which encodes another TF. The mutually exclusive expression of COG-1 and DIE-1 define downstream molecular asymmetries that determine the asymmetric sensory function of the 2 ASEs. b) Scheme of the repressor-based diversification of distinct motor neuron classes. All classes rely on the terminal selector UNC-3 for their differentiation, but each class expresses a distinct combination of repressors that counteract the action of UNC-3 on specific gene batteries (Kerk *et al.* 2017). c) The action of the terminal selector UNC-86 in the ALM and BDU sister neurons is diversified by the asymmetric expression of MEC-3 in ALM. In the absence of MEC-3, UNC-86 acts cooperatively with PAG-3 to activate BDU-specific gene expression. In ALM, MEC-3 binds UNC-86, which now activates the ALM-specific battery and is titrated away from PAG-3-dependent genes (Gordon and Hobert 2015). d) The miRNA miR-791 is specifically expressed in the BAG, AFD, and ASE neurons, where it posttranscriptionally represses 2 otherwise ubiquitously transcribed genes, *cah-3* and *akap-1*. This repression is necessary in the BAG neurons to elicit the correct avoidance response to high CO_2 levels (Drexel *et al.* 2016). e) Key components of the heterochronic pathway and their regulatory interactions (Ambros 2000; Ketting and Cochella 2021). Proteins are in blue; regulatory RNAs are in red and include the miRNAs *lin-4* and *let-7* and the *lep-5* lncRNA that associates with Makorin/LEP-2 (Herrera *et al.* 2016; Kiontke *et al.* 2019). LIN-14, HBL-1, and LIN-29 are TF outputs of the pathway that are integrated with other TFs (e.g. terminal selectors) on the *cis*-regulatory regions of neuronal genes discussed in the text.

and MAB-9 is expressed in both the DAs and DBs where it represses BNC-1 (Kerk *et al.* 2017) (Fig. 7b). Upstream regulators of this intricate network remain to be uncovered.

In contrast to the mechanism above, where specific repressors counteract UNC-3 function at the level of individual promoter/enhancers, a different mechanism has been proposed for modifying the target repertoire of UNC-86 across the sister neurons ALM (TRN, glutamatergic) and BDU (interneuron, neuropeptidergic). In the BDU neuron, UNC-86 acts with the Senseless/Gfi ortholog PAG-3 as terminal selectors, while in its sister neuron, ALM, UNC-86 acts as a heterodimer with MEC-3 (Fig. 7c). The ALM neuron also expresses PAG-3, but the presence of MEC-3 in this neuron serves not only to activate ALM-specific genes but also to outcompete PAG-3 for its interaction with UNC-86. MEC-3 is restricted to ALM by the Wnt asymmetry pathway. Thus, MEC-3 is a transcriptional activator that effectively acts as a repressor of the BDU gene battery in ALM (Gordon and Hobert 2015). UNC-86 is 1 of the 5 recurrently used terminal selectors, and the UNC-86/MEC-3 heterodimer not only specifies the ALMs and other gentle TRNs but also the FLP nociceptor neurons. However, the FLPs express a different gene battery than the TRNs owing to the presence of 2 TFs, EGL-44 and EGL-46, that act together to repress TRN genes (such as the mechanosensory channel *mec-4* and the specific tubulin *mec-7*) and to activate FLP genes (Zheng *et al.* 2018). Showcasing once again how distinct cell identities are built by superimposing layers of repression, the TRNs express a zinc-finger TF, ZAG-1, that represses *egl-44* and *egl-46* expression. It seems that EGL-44/46 and ZAG-1 form a bistable repressor loop that acts as a switch on the output of UNC-86/MEC-3 (Zheng *et al.* 2018). It is worth noting that repression of alternative gene batteries may not need to be complete. For example, the FLPs contain low levels of mRNAs that code for TRN genes, but these do not seem to produce detectable protein in wild-type animals (Topalidou and Chalfie 2011).

Repression of ubiquitous genes to tune the functional properties of a neuron

The function of specialized cells requires the expression of the specific effector gene battery and, at least in some cases, may also require specific regulation of ubiquitous genes that would be considered to have general housekeeping functions (Thorrez *et al.* 2011). Specifically, the CO_2-sensing neurons of *C. elegans*, the BAGs, require the function of miR-791 to repress 2 genes that are otherwise ubiquitously transcribed, *akap-1* and *cah-3* (Drexel *et al.* 2016) (Fig. 7d). In the absence of miR-791, or the binding sites for miR-791 in these 2 targets, these 2 proteins are derepressed and animals show decreased responsiveness to CO_2. The expression of such ubiquitously transcribed genes may be best tuned by posttranscriptional repression as their compact enhancer/promoters may be less amenable to cell-specific transcriptional regulation (Drexel *et al.* 2016).

Neuronal diversification across body axes

A–P axis

As described above, the 6 HOX cluster TFs of *C. elegans* (*ceh-13*, *lin-39*, *mab-5*, *egl-5*, *php-3*, and *nob-1*) provide positional information along the A–P axis during early stages of neurogenesis. In addition to these roles in early A–P patterning, they are integrated into terminal differentiation programs to introduce diversity. Our knowledge of this comes mainly from studies of the motor neurons along the ventral nerve cord and of the TRNs that occupy distinct positions along the A–P axis. We briefly discuss these roles here and direct the reader to recent reviews (Feng *et al.* 2021; Smith and Kratsios 2024).

The 6 gentle TRNs are specified by the terminal selectors UNC-86 + MEC-3. Among these, the 2 pairs of embryonically born TRNs, the ALMs (anterior) and PLMs (posterior), are distinguished by the expression and function of specific HOX cluster genes (Zheng, Diaz-Cuadros, *et al.* 2015). While both neurons need to converge to a very similar mechanosensory neuron fate, they differ substantially in position, morphology, and connectivity (Chalfie and Sulston 1981; Zheng, Diaz-Cuadros, *et al.* 2015). The anterior HOX cluster gene CEH-13 is necessary for acquisition of ALM features, together with the cofactors UNC-62/Meis and CEH-20/Pbx. In contrast, posterior HOX cluster TFs like EGL-5 and PHP-3 are necessary for PLM features. In agreement with the "posterior dominance" rule deduced from HOX cluster gene function in *Drosophila* and mouse (Duboule and Morata 1994), EGL-5 acts by (directly or indirectly) repressing other anterior HOX cluster genes, *lin-39* and *mab-5*. EGL-5 also prevents the establishment of anterior ALM-like features in the posterior PLM by repressing the cofactors UNC-62 and CEH-20. In addition to distinguishing the ALM and PLM identities, CEH-13 and EGL-5 act together with UNC-86 (expressed in both neuron classes) to ensure activation of the terminal selector MEC-3 and thus provide robustness to the execution of these terminal identities by UNC-86 + MEC-3 (Zheng, Jin, et al. 2015). Importantly, the loss of HOX cluster genes does not result in complete, but rather partially penetrant loss of TRN fate. It has thus been proposed that these factors do not determine, but act as transcriptional "guarantors" of the terminal fate (Zheng, Jin, et al. 2015). Such combinatorial TF use to ensure reliable differentiation seems to be a widespread need and may involve other non-HOX cluster TFs (e.g. Topalidou *et al.* 2011, or the use of bHLH TFs described above).

The ventral cord motor neurons comprise 2 classes of GABAergic (DD and VD) and 7 classes of cholinergic motor neurons (SAB, DA, DB, VA, VB, AS, and VC). Each of these classes contains anywhere from 3 to 12 members that intermingle along the A–P axis (e.g. DA1-9 from anterior to posterior). Neurons from most (all) of these classes can be further subclassified based on connectivity (www.wormwiring.org) and effector gene expression, initially with selected reporter genes and more recently with scRNA-seq (Smith *et al.* 2024, http://celegans.spinalcordatlas.org). The regulatory mechanism that defines subclass diversity has been best studied for cholinergic motor neurons, which are specified by the terminal selector UNC-3. While the output of UNC-3 is modified by various repressor TFs to generate diversity among motor neuron classes along the A–P axis, as discussed above, the mechanism for subclass diversification is different and relies on integration of UNC-3 with region-specific HOX cluster TFs (Kratsios *et al.* 2017). Specifically, LIN-39 (a mid-body HOX cluster gene) and MAB-5 and EGL-5 (posterior HOX cluster TFs) are necessary for the expression of subclass-specific effectors in the corresponding motor neurons. UNC-3 cooperates with these HOX cluster TFs to directly activate both intermediate regulators and specific effector genes. In the GABAergic motor neurons, UNC-30 similarly acts together with LIN-39 and MAB-5 to define different subclasses. The role for HOX cluster genes in motor neuron subclassification is as terminal selectors: they directly activate (Fig. 1) batteries of specific effector genes and they are maintained throughout the life of the neurons (Feng *et al.* 2020, 2022). Importantly, HOX cluster TFs are expressed in many other neuron classes beyond the TRNs and motor neurons and play roles in the differentiation of other neurons (Reilly *et al.* 2020; Taylor *et al.* 2021; Zheng *et al.* 2022).

D–V axis

A number of D–V asymmetries are observed in the worm nervous system. The one whose function is best understood is the

asymmetric innervation of ventral and dorsal muscles by V-type and D-type motor neurons, as this is essential for the undulatory locomotion pattern of *C. elegans* (Lu *et al.* 2022). Diversification of the ventral VA and VB classes from the dorsal DAs and DBs through gene repression has been explained above (Kerk *et al.* 2017). For the 2 classes of GABAergic motor neurons, the dorsal DDs and ventral VDs, what we know thus far points to a somewhat different mechanism. Both classes are specified by UNC-30/Pitx with additional cofactors (McIntire *et al.* 1993; Jin *et al.* 1994; Gendrel *et al.* 2016). The DDs are born in the embryo and undergo a programmed rewiring in the L1 that we will discuss below. The VDs are born late in L1 and, in addition to UNC-30, express UNC-55, a TF of the NHR family that is necessary for the VD identity, including wiring pattern, and seems to act as a transcriptional activator. UNC-55 is also sufficient to induce ventral VD-like features in the dorsal DDs (Walthall and Plunkett 1995; Shan *et al.* 2005). Analysis of binding of UNC-30 and UNC-55 by chromatin immunoprecipitation revealed largely overlapping binding sites of both TFs and uncovered a prominent role for cAMP levels in the acquisition of the specific properties of both neuron classes (Yu *et al.* 2017).

Asymmetries along the D–V axis are also observed in various neuron classes composed of 3, 4, or 6 members that distribute in a radial manner. Several morphological and some molecular asymmetries have been described (Ward *et al.* 1975; Hobert *et al.* 2016); however, in most cases, we do not know how these arise. For 2 of these classes, we have some mechanistic understanding of how they are diversified, at least at the level of differentially expressed TFs that contribute to the observed differences. As already noted above, the 6 IL2 sensory neurons have been subclassified in 2 based on anatomy and connectivity (Ward *et al.* 1975; Schroeder *et al.* 2013), the dorsal and ventral pairs (DL, DR, VL, and VR) are most similar to one another and have been named IL2Q (for quadrant), while the 2 lateral neurons (L, R) are similar to one another and distinguishable from IL2Q (Fig. 5b). Both are specified by the recurrently used UNC-86 together with 2 cofactors, CFI-1 and SOX-2. Single-cell profiling has revealed molecular distinctions between the lateral pair and the IL2Q pairs, with various differentially expressed channels and neuropeptides (Taylor *et al.* 2021). The extensive profiling of HD TF expression (Reilly *et al.* 2020) uncovered that UNC-39/SIX4/5 is expressed in the lateral IL2 pair but not in the IL2Q pairs. UNC-39 is necessary for lateral IL2 identity and sufficient to induce lateral features in the IL2Qs; thus, UNC-39 acts as a "subclass terminal selector" (Fig. 5b) (Cros and Hobert 2022). Similarly, the 6 RME motor neurons, which are defined by the recurrently used terminal selector UNC-42, are further subclassified by the expression and function of CEH-32/SIX3/6 in the D–V RMEs but not in the lateral pair (Cros and Hobert 2022). In both cases, these subtype selectors and their downstream subbatteries are also under the control of the class-specific terminal selectors, generating feedforward motifs as described above. While other molecular asymmetries are known in other radial neuron classes (Hobert *et al.* 2016), the scRNA-seq analysis of L4 neurons did not reveal other molecular D–V asymmetries and a deeper or more focused analysis may help refine these subclassifications.

L–R axis

Caenorhabditis elegans has a largely bilaterally symmetric nervous system; however, a number of lateral asymmetries can be observed in the position of neurons, their projections and connections, and also in the gene expression and function of symmetrically positioned neurons. Two types of L–R asymmetries exist, and both are used to diversify the *C. elegans* nervous system: directed and stochastic.

1) Directed asymmetries are stereotypic, with the particular feature of being found always on one side of the animal in >95% of individuals (Palmer 2004). Morphologically, the most obvious asymmetry is found in the ventral nerve cord, where 50 axons follow the right tract while only 4 are present in the left tract. This asymmetry originates in the embryo, when interneuron axons that approach the ventral cord from the left side cross the midline and join the right axon tract, the 2 tracts being separated by a hypodermal ridge. Multiple pathways affecting cell adhesion, signaling pathways, and the extracellular matrix have been shown to be important for the establishment (Taylor and Hutter 2019) and maintenance of the ventral cord asymmetry (Hobert and Bülow 2003). Another asymmetry related to position and morphology is the asymmetric migration of the Q neuroblasts: the left neuroblast and its descendants migrate posteriorly and give rise to the PVM, SDQL, and PQR neurons, and the right neuroblast and its descendants migrate anteriorly to give rise to the AVM, SDQR, and AQR neurons (Sulston and Horvitz 1977).

In terms of directed asymmetry of otherwise symmetrically positioned neurons, the only known example with clear functional consequences is that of the ASE neurons, which were initially realized to be asymmetric because of their asymmetric expression of guanylyl cyclase receptors (Yu *et al.* 1997). This asymmetry extends beyond this expression and has well-described functional consequences (Pierce-Shimomura *et al.* 2001; Suzuki *et al.* 2008; Ortiz *et al.* 2009). The mechanism of ASE lateralization has been described in detail above. Another directed asymmetry is the biased expression of HLH-16 across 2 pairs of neuroblasts that generate 4 pairs of bilateral neurons (sisters SMDD and AIY and sisters SIAD and SIBV). The neuroblasts that give rise to the left members of these neuron pairs express higher levels of HLH-16 than their right counterparts. This difference is induced by a Delta/Notch signal derived from cells from mesodermal lineage located on the left side (Bertrand *et al.* 2011). It is unclear whether this has consequences in terms of lateralized function, and it remains possible that it is in fact a mechanism to ensure symmetry in the context of an asymmetrically developing embryo (Bertrand *et al.* 2011). Another example is the asymmetric use of different proneural genes in the lineages giving rise to left and right AVH and CAN neurons explained in the section on proneural bHLH TFs.

1) Antisymmetry or stochastic asymmetry is an L–R asymmetry in which a particular feature can be found on one or the other side of the animal with equal probability (Palmer 2004). In the worm, this is observed in the AWC pair of olfactory neurons. These neurons are specified by at least 2 terminal selectors, CEH-36 and SOX-2 (Lanjuin *et al.* 2003; Koga and Ohshima 2004; Alqadah *et al.* 2015), and a screen for expression of G protein-coupled receptors revealed that *str-2* is asymmetrically expressed (Troemel *et al.* 1999). The AWC neuron that expresses STR-2 was called the AWC^{ON} neuron, the one that does not is the AWC^{OFF} neuron. AWC^{OFF} expresses different odorant receptors and responds to different cues. Whether the left or the right AWC neuron adopts the ON or OFF state is stochastic, but there is always one of each, indicating that the decisions of the 2 neurons must be coordinated. Initially, both AWC neurons adopt the default OFF state through the activation of a calcium-dependent protein kinase pathway that activates 2 P38 MAPKs, encoded by *pmk-1* and *pmk-2* (recently reviewed in

Alqadah *et al.* 2016, 2018). The developing AWC neurons then establish contact through their axons and communicate through transient gap junctions made of the innexin NSY-5. This enables a "comparison" of calcium levels, and the cell with the lower level induces the AWC^{ON} fate. Expression of AWC^{OFF}-specific markers depends on the TF DIE-1, which intriguingly is also asymmetrically expressed in the ASE neurons through a very different mechanism (Cochella *et al.* 2014). In AWC^{ON}, the transient signaling during embryogenesis results in lasting expression of at least 2 TFs, NSY-7 and HMBX-1, which are required for expression of the correct effector gene battery (reviewed in Alqadah *et al.* 2016, 2018). The function of NSY-7 is dependent on its nuclear import by a specific importin, IMB-2, suggesting that control of nuclear import/export could be used for regulation (Alqadah *et al.* 2019).

Neuronal diversification over developmental time and across sexes

In addition to the molecular mechanisms described above, which provide extensive layers of diversification among neuron classes and subclasses, a given neuron class can display different functions depending on its postembryonic larval stage or the sex of the animal. Here, we summarize our understanding of how temporal and sexual information is translated into neuron diversification mechanisms.

Time

At the end of embryogenesis, the L1 larva is born with 222 neurons in the hermaphrodite (202 somatic and 20 pharyngeal) and 224 in the male (WormAtlas). The remaining neurons are generated during late L1 to L2, and in the male, additional neurons required for sex-specific reproductive behaviors are born in L3 and L4. As new neurons are added and the animal reaches sexual maturity, existing sex-shared neurons change over time, and they do so in a sex-specific manner. Most processes that occur with temporal dynamics during larval development in *C. elegans* are controlled by the heterochronic pathway: a regulatory cascade composed of transcriptional and posttranscriptional regulators that coordinate temporal progression across different tissues. Prominent players in this pathway are the miRNAs, *lin-4*, *let-7*, and the *let-7* sisters, a number of RNA-binding proteins (e.g. LIN-28), and TFs (most prominently LIN-14 and LIN-29), which together define the transitions between different larval stages (Ambros 2000). We highlight 3 contexts that illustrate the extent of cellular and molecular changes of the worm nervous system over time.

1) *Remodeling of the VNC wiring between L1 and L2*: the newly hatched L1 larva has only 3 classes of motor neurons, DA, DB, and DD, while the remaining VA, VB, VC, VD, and AS classes are born in a second wave late in L1. In adults, all D-types innervate dorsal muscles and receive input from the V-types on the ventral side. However, in early L1, the DDs display a reversed synaptic connectivity: they innervate ventral muscles and receive input from the DAs and DBs through projections to the dorsal side, providing the sole synaptic input for the ventral side until the V-types are integrated into the network. Based on EM reconstructions of L1 and L2 larvae, it became evident that synaptic remodeling of the DDs happened between these 2 stages (White *et al.* 1978). New serial EM reconstructions of the remodeling process have further uncovered that it follows a gradual transition where structural changes necessary for the adult wiring occur before the juvenile synapses are dismantled (Mulcahy *et al.* 2022).

The timing of this remodeling is under control of the heterochronic pathway; specifically, it is repressed by the TF LIN-14 (Hallam and Jin 1998). LIN-14 levels are high at the beginning of L1 but it is then repressed by the miRNA *lin-4* (Lee *et al.* 1993; Wightman *et al.* 1993), enabling the remodeling to take place. As discussed above, the VDs are distinguished from the DDs by the expression of the TF UNC-55. In *unc-55* mutants, the VDs first establish synapses with the ventral muscles but then incorrectly remodel these toward the dorsal muscles in a process very similar to the DDs (Petersen *et al.* 2011; Thompson-Peer *et al.* 2012), indicating that UNC-55 represses the transcriptional program required for remodeling in the VDs. Remarkably, the temporal and cellular specificity for DD remodeling converge on the regulation of the immunoglobulin domain gene *oig-1*, with UNC-55 and LIN-14 acting together with UNC-30, the terminal selector for both DDs and VCs (Howell *et al.* 2015). The cellular mechanisms behind the actual synaptic remodeling have been extensively studied, and we will not cover them here as they were recently reviewed (Cuentas-Condori and Miller Rd 2020).

1) *Molecular and functional changes in postmitotic neurons in larvae and adults*: the nervous system continues to develop during juvenile stages, and this is reflected in molecular changes in postmitotic neurons as they mature during larval stages. Some of these changes are related to sexual maturation. For example, both in males and hermaphrodites, sex-specific neurons mature at the L4 stage regardless of their time of birth, and this is largely under control of the heterochronic pathway (Olsson-Carter and Slack 2010; Lawson *et al.* 2019; Tekieli *et al.* 2021). Maturation events that are not directly linked to sexual maturation have also been observed. For example, the cholinergic motor neuron terminal selector, UNC-3, acquires new targets after the L2 stage (Li, Osuma, *et al.* 2020). The mechanism that determines the timing with which targets become dependent on UNC-3 is unknown but may reflect the regulatory architecture of these loci, although a connection with the heterochronic pathway cannot be excluded. Recently, a transcriptome-wide view of the changes in gene expression over larval development was obtained from neuronal nuclei and complemented with a deep characterization of locomotor behavior at matched stages (Sun and Hobert 2021). This analysis revealed extensive maturation of the transcriptomes of postmitotic neurons under control of the heterochronic pathway, specifically *lin-4/lin-14*, and identified the change in expression of a neuropeptide, *nlp-45*, as the cause for changes in exploratory behavior as the animal matures. Intriguingly, the same study identified a number of genes that changed over time in a manner that was independent of *lin-4/lin-14* (Sun and Hobert 2021). What promotes these temporal changes remains to be explored.
2) *Loss of regeneration capacity over time*: *C. elegans* neurons lose the ability to regenerate their axons as they mature. In the case of AVM, regeneration after laser axotomy declines by ~70% between the L2 and young adult stages, and this is dependent on the heterochronic pathway (Zou *et al.* 2013). This decline is practically abolished in *let-7* mutants.

Genetic sex

The nervous systems of hermaphrodites and males differ in the number of neurons, 302 vs 387, respectively. Of these, 294 neurons (belonging to 116 classes) are shared between both sexes. The

remaining 8 hermaphrodite-specific neurons belong to the HSN and VC classes and are both involved in egg laying. Males have an additional 93 specific neurons, 2 classes in the head (CEM sensory neurons and MCM interneurons), 2 in the VNC (CA and CP motor neurons), and 23 additional classes in the tail. In addition to the evident differences in sex-specific neurons, there are several molecular, connectivity, and functional dimorphisms across the shared neuron classes (recently reviewed in Portman 2017; Barr et al. 2018; Goodwin and Hobert 2021). All these differences are defined by the sex determination pathway (Oren-Suissa et al. 2016), which results in the hermaphrodite-specific expression of a zinc-finger TF called TRA-1. TRA-1 promotes female identity and prevents adoption of male identity in all somatic cells in a cell autonomous manner. It can act directly on its targets, but it also controls the expression of other TFs, most prominently those belonging to the DMRT family. There are 10 DMRT TFs in *C. elegans*, 4 of which are male specific (MAB-3, MAB-23, DMD-3, and DMD-10) and 1 (DMD-4) hermaphrodite specific.

The sex determination pathway acts during embryogenesis and affects the development of sex-specific neurons. For example, TRA-1 directly represses *egl-1* and results in survival of HSN in hermaphrodites but cell death in males (Conradt and Horvitz 1999). TRA-1 also regulates specific CEM death in hermaphrodites by directly repressing the transcription of the *ceh-30* HD TF that represses *egl-1* and *ced-3* (Nehme et al. 2010).

Time and genetic sex

Many of the dimorphisms in the nervous system arise as animals approach sexual maturity. At the regulatory level, this requires integration of time (via the heterochronic pathway), sex (via the sex determination pathway), and specific neuronal identity (via terminal selectors). *Caenorhabditis elegans* has been instrumental for understanding mechanisms of sexual maturation. Importantly, conserved members of the heterochronic pathway, such as *lin-28* and *let-7* (Ambros 2000) and the more recently characterized regulator of LIN-28, makorin/*lep-2* (Herrera et al. 2016; Kiontke et al. 2019; Lawson et al. 2019), have been found to be involved in timing of sexual maturation in mammals (Cao et al. 2020; Liu et al. 2023).

Some examples of sexual maturation in the *C. elegans* nervous system are as follows: the maturation of the hermaphrodite-specific HSNs (Desai et al. 1988; Lloret-Fernández et al. 2018) and the male-specific CEM neurons at the L4 stage (Pereira et al. 2015); the downregulation of the olfactory receptor *odr-10* in adult male AWA neurons to promote exploration over feeding (Ryan et al. 2014); and the induction of the TGF-β ligand *daf-7* in adult male ASJ neurons that promotes mate-searching behavior (Hilbert and Kim 2017). We only briefly highlight key features of how sexual and temporal integration takes place, as this was recently reviewed (Sun and Hobert 2023).

1) TRA-1 is upregulated in hermaphrodites in the L3 to L4 transition by a member of the heterochronic pathway, *daf-12* (Bayer et al. 2020). At the same time, TRA-1 regulates other members of the heterochronic pathway: TRA-1 and the heterochronic miRNA *lin-4* regulate the TF gene *lin-14* resulting in dimorphic expression of the neuropeptide gene *nlp-45* (Sun and Hobert 2021), and TRA-1 directly represses *lin-29a* in hermaphrodites. LIN-29A is a zinc-finger TF that is also a downstream target of the heterochronic pathway and is required for male-specific AIM, AVG, and ASJ gene expression, PDB synaptic remodeling, and male-specific locomotor patterns at sexual maturity (Pereira et al. 2019).
2) The heterochronic miRNA *let-7* represses the translation regulator LIN-41 at the L4 to adult transition. LIN-41 represses 3 TFs that are also targets of TRA-1 repression: DMD-3, MAB-3, and LIN-29. These TFs are expressed in specific neurons with sexual and temporal specificity (Pereira et al. 2019). DMD-3 controls the sexually dimorphic remodeling of PHC (Serrano-Saiz et al. 2017). MAB-3 and LIN-29A promote AIM maturation in males, as evidenced by *srj-54* expression (Lawson et al. 2019; Pereira et al. 2019).
3) Terminal selectors also provide inputs to generate the neuron class-specific expression patterns of *lin-29a*, *dmd-3*, and *mab-3*. For example, CEH-14, a terminal selector of PHC neuron identity, is required for *dmd-3* expression in male PHC neurons (Pereira et al. 2019). Moreover, the action of terminal selectors can intersect with genetic sex and time determinants directly on the CRMs controlling effector gene expression. *unc-6/netrin* expression in the AVG neuron is directly activated by LIN-11, the AVG terminal selector, both in males and hermaphrodites, until the L3 stage. At later stages, TRA-1 acts upon the same CRM to directly repress *unc-6* expression in hermaphrodites, which leads to pruning of synaptic contacts from the PHB neuron (Weinberg et al. 2018). Similarly, in addition to repressing *dmd-3* expression in PHC neurons, TRA-1 also represses the expression of the *eat-4/vglut* effector gene in hermaphrodites, acting on the CRM activated by UNC-86 and CEH-14, while PHC terminal selectors lead to effector gene activation only in males (Serrano-Saiz et al. 2017).

Environmental effects on neuron gene expression

Neuron class identities and synaptic connections are largely stable throughout the life of an organism. Nevertheless, as already described, intrinsically programmed mechanisms such as genetic sex or postembryonic larval development can partially modify these identities. In addition, the robust scaffolding of an animal's behaviors displays experience-dependent plasticity, providing the animal with flexible and adaptative responses to a changing environment, an illustrative example being the remodeling of dendritic arbors upon dauer entry (Schroeder et al. 2013). In order to generate flexible behaviors, nervous systems integrate past experiences and physiological and metabolic status into particular internal states that determine the specific responses elicited upon newly encountered environmental stimuli. Internal states can be seen as persistent changes in the function of the nervous system whose effects span multiple sensory modalities and/or motor systems. Well-studied internal states in animals are fear, arousal, hunger, motivation, and aggression. In *C. elegans*, sleep, hunger, pathogen exposure, and exploration represent internal states that influence functional circuit connectivity and sensorimotor transformations (Flavell and Gordus 2022). For example, the nutritional status of the animal modifies its chemosensory behaviors, and exposure to pathogenic bacteria changes the initially elicited attractive response to avoidance. Understanding the cellular and molecular mechanisms responsible for the establishment, persistence, or transition between internal states, and how these affect behavior, has been the subject of intense investigation in recent decades (reviewed in Flavell and Gordus 2022; Flavell et al. 2022).

Environmentally induced changes in neuronal gene expression

Plastic behavioral outputs depend on specific gene expression changes in particular neuron classes triggered by environmental stimuli. These expression changes are largely of 2 types: (1)

changes in sensory receptors or downstream effectors expressed by sensory neurons, which result in a state that produces different primary sensory responses to particular environmental stimuli, or (2) changes in neuromodulators (such as monoamines or neuropeptides) and endocrine signaling (such as insulin peptides and TGF-β ligands) that produce long-range changes in the coupling between neurons and thus create persistent behavioral internal states. More recently, transcriptome-wide analyses have revealed more comprehensive responses to environmental stimuli. Temperature, food deprivation, oxygen levels, and pathogen exposure produce changes in expression of hundreds of genes in a single neuron type (Li, Marcu, *et al.* 2020; Kyani-Rogers *et al.* 2022; McLachlan *et al.* 2022; Wu *et al.* 2023; Harris *et al.* 2023). In agreement with previous work on specific genes, G protein-coupled receptors and neuropeptides are enriched among differentially expressed genes. Thus, modulation of neuronal gene expression seems to be a key player in the regulation of behavioral plasticity and in shaping behavioral internal states. Examples of known gene expression changes of sensory neurons responsible for plastic behaviors include the following:

1) Cultivation temperature determines the preferred location of *C. elegans* in a temperature gradient (Hedgecock and Russell 1975). This plastic behavior is mediated by the AFD thermosensory neuron, whose activity increases at temperatures above the cultivation temperature. The remarkably precise resetting in the threshold of activity is achieved by changes in expression of guanylyl cyclase genes *gcy-8*, *gcy-18*, and *gcy-23* that correlate with cultivation temperature (Yu *et al.* 2014; Harris *et al.* 2023).
2) Exposure to *Pseudomonas aeruginosa* (PA) induces *str-44* receptor expression in AWA sensory neurons allowing for its activation upon pheromone exposure, which in turn suppresses the avoidance behavior to pheromones observed in naive animals (Wu *et al.* 2023). *str-44* receptor expression in AWA neurons is also induced upon starvation, conferring responsiveness to putative food odors and promoting starved-like foraging behaviors (McLachlan *et al.* 2022). Similarly, AWA neurons in adult males increase *odr-10* receptor expression upon food deprivation, promoting food attraction (Ryan *et al.* 2014).

As mentioned, internal states can also be modified by neuromodulators induced in response to environmental conditions, and a few illustrative examples follow.

1) Activity, temperature, nutritional state, and pathogens modulate serotonin synthesis in ADF polymodal neurons by controlling expression of their rate-limiting enzyme tryptophan hydroxylase (*tph-1*) (Zhang *et al.* 2005; Xie *et al.* 2013; Qin *et al.* 2013; Bayer and Hobert 2018). In addition, increased serotonin signaling via *tph-1* expression modulation has also been reported in ASG neurons upon hypoxic conditions that lead to changes in chemosensory perception (Pocock and Hobert 2010).
2) Modulation of TGF-β signaling by external stimuli also modifies internal behavioral states. Pathogenic bacteria, nutritional status, and also genetic sex control *daf-7*/TGF-β expression in ASJ polymodal neurons, which in turn leads to changes in exploratory behaviors (Meisel *et al.* 2014; Hilbert and Kim 2017).
3) Expression of insulin-related peptides and neuronal insulin signaling, which produce a broad range of effects in the organism, are not only modulated by pheromones and nutritional state but also by pathogenic bacteria (Li *et al.* 2003; Chen *et al.* 2013) or salt concentration (Tang *et al.* 2023). A recent report described plastic asymmetric connectivity between ASE salt-sensing and the AWC olfactory neurons in response to environmental salt concentration. Newly hatched larvae show bilaterally symmetric connections between ASE–AWC that in naive larvae develop into a left-biased connectivity in the adult animals. However, exposure of adults to high salt conditions inverts this bias. Mechanistically, insulin signaling released from ASI and ASJ neurons is sensed by ASE leading to rewire connectivity and plastic locomotory behaviors (Tang *et al.* 2023).

Integration of "environmental sensor" TFs with terminal selectors

In order to generate plastic behavioral responses, neurons need to alter gene expression in response to environmental stimuli. One important question is how this is achieved, given the robustness of neuronal identity specification during development. The activity of some broadly expressed TFs depends on external signals or neuronal activity. For example, DAF-16/FOXO is inactivated upon insulin signaling, and CRH-1/CREB and MEF-2/MEF2 are activated downstream of calcium increase upon neural activity (van der Linden *et al.* 2007; Gruner *et al.* 2014, 2016; Chen *et al.* 2016; Rojo Romanos, Petersen, *et al.* 2017; Wexler *et al.* 2020; Kyani-Rogers *et al.* 2022; McLachlan *et al.* 2022; Harris *et al.* 2023; Tang *et al.* 2023). These TFs have conserved roles as "environmental sensors" both in invertebrates and vertebrates (McLaughlin and Broihier 2018; Yap and Greenberg 2018; Assali *et al.* 2019). A single neuron can respond to a variety of external stimuli thanks to the modular regulatory ability of these TFs (Xie *et al.* 2013; Gruner *et al.* 2014; Maicas *et al.* 2021; McLachlan *et al.* 2022) (Fig. 8a).

These stimulus-activated TFs are broadly expressed in the nervous system. However, their targets are neuron-type specific (Fig. 8b). This can be explained by the integration of stimulus-activated TFs with neuron class-specific terminal selectors (Fig. 1), similar to the intersectional mechanisms for the integration of space, time, or genetic sex signals in a neuron type-specific manner. In other systems, enhancers that become activated in response to stimulation have been termed latent enhancers (Ostuni *et al.* 2013; Stroud *et al.* 2020). Importantly, even transient activation of these enhancers results in a persistent activated state and in faster and stronger transcriptional responses upon restimulation. In *C. elegans*, the integration of activity-dependent and neuron-specific TFs at specific enhancers has also been observed in several cases (Fig. 8c):

1) The GPCR serpentine receptor *shr-234* is expressed in ADL under direct control of its terminal selector HLH-4. However, upon starvation, *shr-234* transcription is repressed by the direct action of MEF-2 acting on the same CRM as HLH-4. Interestingly, insulin signaling in well-fed animals seems to repress MEF-2 levels in head neurons, avoiding *shr-234* repression (Gruner *et al.* 2014, 2016).
2) The induction of *tph-1* in ADF upon several different environmental stimuli is achieved thanks to a modular *cis*-regulatory architecture on which different TFs cooperate. For example, the induction upon exposure to pathogenic bacteria or to cilia morphology defects requires the direct activity of LAG-1, an ADF terminal selector, together with stimulus-dependent TFs, on those activity-dependent CRMs (Moussaif and Sze 2009; Xie *et al.* 2013; Maicas *et al.* 2021).
3) Induction of the gap junction gene *inx-6* in the AIB interneuron upon dauer entry requires direct activation by

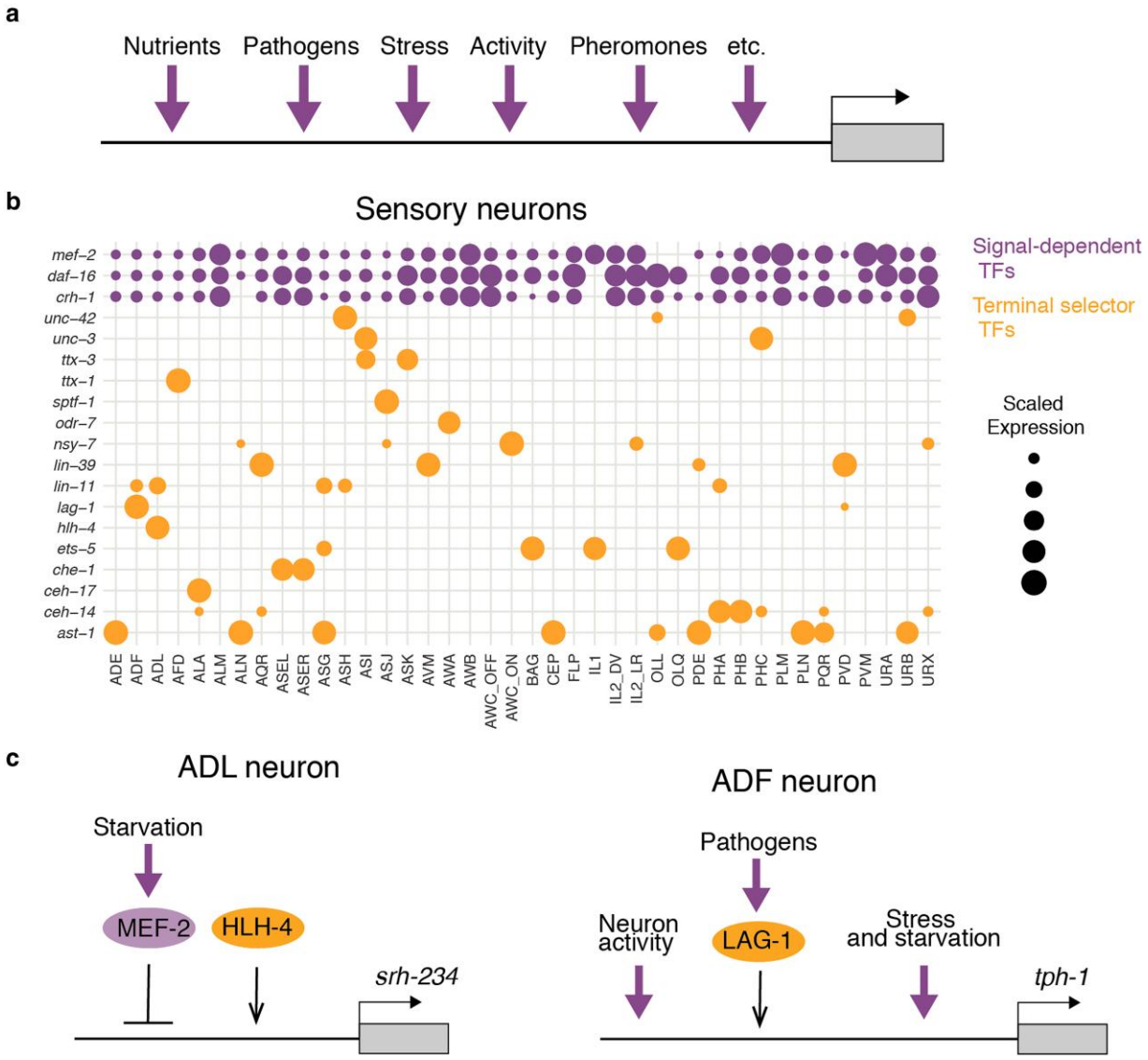

Fig. 8. Environmental effects on neuron diversity. a) Different environmental triggers are integrated into gene expression changes through parallel modules acting in the neuron. b) L4 expression data of signal activated TFs and some examples of terminal selectors in sensory neurons. Signal-activated TFs, such as MEF-2, CRH-1, and DAF-16, are broadly expressed in the nervous system; however, they produce neuron class-specific responses acting together with terminal selectors that show a more restricted pattern of expression. Data source CENGEN. c) The 2 illustrated examples correspond to those described in the text. CRMs of plastic effector genes can integrate input from signal-regulated TFs as well as terminal selectors. ADL expression of *srh-234* is directly regulated by the HLH-4 terminal selector but upon starvation is repressed by the MEF-2 TF. *tph-1* expression in the ADF neuron is plastic to several environmental conditions, and pathogen exposure induces *tph-1* expression through the activity of the LAG-1 ADF terminal selector.

UNC-42, an AIB terminal selector, and by the DAF-16/FOXO (Bhattacharya *et al.* 2019).

As indicated in the previous section, terminal selectors often intersect with additional regulatory inputs such as positional cues, time, or genetic sex. It is therefore not surprising that time and genetic sex inputs also intersect with environmental stimuli to increase the array of plastic responses observed in animals. These examples show how some of the targets in an intrinsically determined program, such as genetic sex, can be suppressed if the animal encounters particular environments.

1) Serotonin signaling promotes male-specific pruning of sexually dimorphic synaptic connectivity under well-fed conditions. However, if juvenile animals are exposed to starvation, pruning is suppressed, resulting in increased chemosensory responsiveness in adult males (Bayer and Hobert 2018).
2) The stochastic addition of multiple neurites in the DVB neuron of adult males is modulated by exposure to hermaphrodites (Hart and Hobert 2018).
3) ASJ neurons express *daf-7* in adult males (not in hermaphrodites), where it is involved in mate-searching behavior. However, males subjected to starvation at the L4 stage fail to express *daf-7* and prioritize feeding over mate searching (Hilbert and Kim 2017).

The examples above show the importance of integrating internal and external factors before deciding to reproduce. The fact that metabolic signals can modify or even override sexual behaviors indicates a hierarchical organization of internal needs. Nevertheless, it is still unclear how these specific examples impact reproductive fitness in the wild.

In addition, NHRs, which are extensively expressed in sensory neurons, have also been proposed as good candidates for the perception of external cues (that could act as activating ligands of the NHRs) for directly modulating expression of chemosensory receptors or neuromodulators (Sural and Hobert 2021). However, the identification of relevant ligands and environmentally triggered targets for such factors is still missing.

In summary, transcriptional integration of activity-related TFs and terminal selectors is the best characterized molecular mechanism underlying the generation of specific behaviorally plastic responses. Whereas posttranscriptional and posttranslational mechanisms undoubtedly will also play important roles in the modulation of plastic responses, these remain to be explored.

Evolution of neuronal diversity

Developmental programs are the result of complex evolutionary paths, and, at the same time, they constitute the raw material for the emergence of novel cell types and functionalities. Thus, understanding how nervous systems evolved is important for a full understanding of their development.

Neurons are proposed to have originated from 2 possible ancestral cell types, contractile or secretory cells, both with experimental support from comparative analyses and single-cell transcriptomics (Arendt 2021; Wang *et al.* 2021; Najle *et al.* 2023). Intriguingly, the extreme molecular diversity of neuronal types found in early branching metazoans suggests that neuronal cell types may have emerged more than once from distinct tissues and in different metazoan lineages (reviewed in Arendt 2021; Moroz 2021). Regardless of their cell type of origin, the defining features of neurons, e.g. their secretion, presynaptic and postsynaptic machineries, and excitability and sensory capacities, are encoded by gene modules that are also found in nonneuronal contexts and were already present in early branching metazoans without nervous systems. Thus, the "neuron identity" most likely arose by integration and divergence of these preexisting modules (Arendt 2020). Those protoneurons then diversified to generate the neuronal complexity we see in nervous systems today. Comparative studies can shed light on different aspects of how this diversification occurs.

Evolutionary lessons from *C. elegans* gene regulatory network architecture

We have described in this chapter cases of neuronal diversification by which the output of a terminal selector is modified such that the presence or absence of an additional regulatory factor produces 2 different neuron classes or subclasses (such as ALM vs BDU, ASEL vs ASER, and IL2D/V vs IL2L/R). It has been proposed that such examples, in *C. elegans* and other animals, reveal possible evolutionary paths for cell diversification through which an ancestral cell became duplicated and acquired the expression of a novel regulator (e.g. MEC-3 TF or *lsy-6* miRNA) or the *cis*-regulatory elements recognized by such a regulator (Arlotta and Hobert 2015).

The deep mechanistic understanding we can gain using *C. elegans* will likely yield further models and hypotheses for possible evolutionary trajectories. Ideally, however, these should be complemented with comparative analyses across divergent species to probe these questions more directly. For example, the *Caenorhabditis* species that have been analyzed all have *lsy-6*, but this miRNA is absent from the satellite nematode species *Pristionchus pacificus*, providing an exciting paradigm for understanding how a L–R functional asymmetry may evolve (Hong *et al.* 2019).

Comparative analyses among *C. elegans* isolates

A large amount of genotypic and phenotypic variation occurs across *C. elegans* isolates. The *Caenorhabditis* Natural Diversity Resource has sequenced and classified hundreds of different *C. elegans* wild isolates from around the world that exhibit substantial genetic variation (Lee *et al.* 2021). These also display a high degree of natural variation in behaviors such as starvation resistance (Webster *et al.* 2022), dauer induction (Lee, Zdraljevic, *et al.* 2019; Billard *et al.* 2020), egg laying and egg-laying response to natural products (Chen *et al.* 2020; Vigne *et al.* 2021; Mignerot *et al.* 2024), response to O_2 or CO_2 (Persson *et al.* 2009; Beets *et al.* 2020), behavioral responses to drugs (Dennis *et al.* 2018), foraging (Bendesky *et al.* 2011; Zhao *et al.* 2020), pheromone detection (Greene, Brown, *et al.* 2016; Greene, Dobosiewicz, *et al.* 2016), phoretic behavior (Lee *et al.* 2017), decision-making (Bendesky *et al.* 2011), and locomotion (Schwarz *et al.* 2015). Laboratory adaptation has also generated novel insights into *C. elegans* nervous system evolution (de Bono and Bargmann 1998; McGrath *et al.* 2011; Sterken *et al.* 2015).

The molecular basis underlying these diverse phenotypes has already been identified in some of these cases, revealing natural variation with functional consequences on nervous system (reviewed in Evans *et al.* 2021). The full characterization of gene regulatory network changes responsible for behavioral and phenotypic variability among *C. elegans* wild isolates could uncover principles of neuron effector gene expression evolution. The extensive toolsets available give us an advantage in trying to understand evolution by comparing isolates of *C. elegans*, although of course the extent of regulatory differences expected across isolates of the same species is limited.

Comparative analyses among other nematode species

The development of new technologies in the last decades, in particular CRISPR, deep sequencing, and single-cell sequencing, has revolutionized the field of evolutionary biology. For a vast array of model and nonmodel organisms, genomes and transcriptional profiles at single-cell resolution of different organs and tissues are now available. These advances enable comparative studies with other nematodes that may offer larger differences with *C. elegans*. A prerequisite for such comparisons is the unequivocal identification of orthologous and/or homologous neuron types among species, which is a challenge in organisms with more complex nervous systems. Nematodes are an excellent model for this. Despite often exploiting different habitats, displaying different behaviors, and having considerably distant genomes at the molecular level, morphological diversity is restricted among many nematode species. Comparison of embryonic lineages suggests a similar number of neurons placed in similar locations are present in 4 studied *Caenorhabditis* species (*C. elegans*, *Caenorhabditis briggsae*, *Caenorhabditis remanei*, and *Caenorhabditis brenneri*) (Zhao *et al.* 2008; Memar *et al.* 2019), although postembryonic lineage differences among these species could still be present. Indeed, different numbers of ventral nerve cord neurons have been reported in more distant species such as the free-living organism *P. pacificus*, where 46 neuronal nuclei are identified by DAPI staining, in contrast to the 57 neurons present in *C. elegans* (Han *et al.* 2015) and the parasitic nematode *Ascaris suum*, with 72 ventral nerve motor neurons identified by light microscopy of serial sections (Stretton *et al.* 1978). Thus, it is tempting to propose that based on embryonic lineage conservation, many neuronal homologies among nematode species could be established. Nevertheless, some differences

in morphologies or gene expression patterns in mature neurons might pose a challenge when assigning homologies by comparing postembryonic stages (Hong *et al.* 2019). Well-resolved phylogenies, availability of genomes, and amenability to genetic manipulation of several species make nematodes, in particular the *Caenorhabditis* genus, a uniquely suited experimental framework for studying neuron-type evolution (Stevens *et al.* 2019). This must be complemented with morphological, functional, and molecular comparisons to uncover diversity among these nervous systems and gain a mechanistic understanding of how that may arise. At the morphological level, recent EM reconstructions of the *P. pacificus* nervous system have unraveled important connectivity differences with *C. elegans*, at least for pharyngeal and sensory neurons, as well as large differences in the morphology of sensory cilia (Bumbarger *et al.* 2013; Hong *et al.* 2019). At the functional level, a number of *Caenorhabditis* species have been analyzed by staining for dopamine and serotonin. While there is a high degree of conservation in staining patterns, some differences have been found both for hermaphrodites and males, such as VC4 and VC5 hermaphrodite-specific and CA male-specific neurons stained for serotonin in *Caenorhabditis angaria* (Loer and Rivard 2007; Rivard *et al.* 2010). A recent study has compared gonochoristic (male/female) *Caenorhabditis nigoni* with hermaphroditic *C. elegans* and *C. briggsae* to characterize the shift from female to hermaphrodite behavior (Ebert and Bargmann 2024). In *C. elegans*, NRH TF *odr-7* is the terminal selector for AWA and is necessary for male chemotaxis to females. The same TF acts in AWA neurons of *C. nigoni* females to induce chemotaxis to males (which is not present in *C. elegans* hermaphrodites). Thus, these results suggest that female and male *C. nigoni* use the sex-shared AWA olfactory neurons to detect sex-specific attractants from potential mates. Future experiments should address whether similar sex-dependent gene regulatory networks displayed in *C. elegans* for neuronal dimorphisms act in *C. nigoni* to select differential AWA effector genes in males and females that in turn determine sex-specific attraction to mates.

These types of comparative studies are particularly relevant to the characterization of parasite-specific behaviors. Parasite-specific neural adaptations are necessary to target humans or livestock. Different parasitic nematodes respond differently to different cues, such as chemicals or body temperature (Gang *et al.* 2020). Interestingly, homolog neurons mediate the same type of sensory cues (e.g. AFD sensing temperature or BAG sensing CO_2), but in each species, sensory neurons respond with different activation patterns and produce different behavioral outputs (Bryant *et al.* 2022; Banerjee *et al.* 2024). Once more, the wealth of knowledge from *C. elegans* nervous system development and functionality together with new available tools opens the possibility of identifying gene regulatory network changes important for the evolution of parasitic behaviors.

Generation of scRNA-seq data sets from these and other nematode species will be crucial in assessing the degree of divergence in orthologous gene expression for all neuron types. The first scRNA-seq report comparing *C. elegans* and *C. briggsae* gene expression profiles along embryonic development shows a high level of gene expression similarity, which is consistent with their conserved developmental lineages (Large *et al.* 2024). Despite this conservation, thousands of genes show divergence in their cell type-specific expression patterns. This is particularly evident in neurons, compared to other tissues such as the intestine and body wall muscle, and for gene categories involved in environmental response and behavior (Large *et al.* 2024). This and similar data sets will lead to testable hypotheses on the molecular mechanisms driving expression changes in neuronal effector gene batteries that could underlie the emergence of novel neuron functionalities.

In summary, although past studies have provided examples of diversity in behaviors and gene function or expression changes among different species or isolates, much work remains to be done to start identifying patterns for the evolution of nervous system gene regulatory networks. We expect that the wealth of knowledge already available on *C. elegans* nervous system diversification combined with the use of single-cell technologies, the generation of better annotated genomes (Athanasouli *et al.* 2020; Moya *et al.* 2023), and the amenability of different nematodes for genetic modification will provide fruitful insights on nervous system evolution in the coming years. It is worth noting that the advantage of strong conservation in the complement of neurons in different nematode species also has the drawback that it might limit their use in studying the cellular mechanisms underlying neuron gains or losses along evolution and emergence of new neuronal identities. Although current evidence points toward broad changes in neuronal effector gene expression in homologous neurons in different species, these studies might be more suited in characterizing how evolution shapes gene expression to drive adaptation to new environments. Importantly, similarities in gene expression patterns and TF regulators among homologous neurons have also been described for different neuronal populations in vertebrates (Shi *et al.* 2021; Hain *et al.* 2022). Nevertheless, greater lineage divergence across other groups of nematodes may allow for addressing these additional questions (reviewed in Barrière and Bertrand 2020).

Concluding remarks

Overall, the last 2 decades of studies on neuron specification and differentiation of the complete nervous system of *C. elegans* have allowed us to start building a holistic model of the regulatory networks involved in neurogenesis, with a degree of molecular and cellular resolution that is difficult to attain in other animal models. We have distilled the vast genetic and molecular analyses generated by the *C. elegans* community, described here, into the regulatory framework summarized in Fig. 1. This view also highlights open questions.

For example, given the apparent lack of neural inductive signals in *C. elegans*, we do not currently know what induces expression of proneural factors and how these, together with other inputs from the lineage, ultimately result in the expression of the combinations of terminal selectors. We have described glimpses into this obtained from studying specific cases but with further single-cell sequencing and, eventually, chromatin accessibility profiles for every cell and stage in the embryo, we can aim to understand the regulatory integration of dynamic signals that results in the production of the complete array of neuron classes. This information will allow us to identify new principles of neuronal diversification that could open new, unforeseen paths of research. Given the dynamic nature of these events along the lineage progression, the use of in vivo temporally resolved imaging of proteins and RNA will likely be necessary to allow us to achieve this goal.

Moreover, despite the knowledge acquired over the last 2 decades on neuron terminal differentiation mechanisms, important technical limitations preclude a better understanding of this process: (1) characterization of terminal selector mutants has typically been based on analysis of a handful of reporter genes, and future experiments with cell type-specific RNA-seq profiles in mutants for specific TFs will be necessary to assess the breadth of action

of these TFs; (2) genomic profiling of TF binding is only available for whole worm samples so, considering the extent of neuron class-specific combinatorial action of TFs, it will be necessary to obtain binding profiles in a cell type-specific manner to better understand the function of terminal selector collectives; (3) cell type-specific chromatin accessibility profiles would complement TF binding information to improve the understanding of neuron class regulatory logics; and (4) while the use of classical *cis*-regulatory reporter analysis by conventional transgenesis has been extremely informative, its low throughput limits the number of different constructs that can be tested in vivo. Implementing massively parallel reporter assays or MPRAs (Inoue and Ahituv 2015) in *C. elegans* would provide a fantastic tool for advancing toward building a complete model for coregulation and cross-talk among regulatory routines. Present technologies should allow us to start exploring these questions in greater depth.

In addition, our knowledge of gene regulation at the transcriptional level far exceeds that of our understanding of mechanisms that operate at later steps of gene expression. Despite some known cases of regulation of alternative splicing in *C. elegans* (reviewed in Zahler 2012; Sharifnia and Jin 2014), this likely plays more extensive roles than are currently appreciated (Raj and Blencowe 2015). The use of long-read sequencing approaches like Oxford Nanopore will allow more systematic analyses of these events (e.g. Bernard *et al.* 2023). Also, beyond a limited number of reports (e.g. Sharifnia *et al.* 2017), translation regulation is currently underexplored. Work in mice suggests that ~30% of the neuronal transcriptome is translationally regulated (Rodrigues *et al.* 2020), and that neurons use an intriguing mechanism of translation regulation in order to reshape their proteome and sustain plasticity (Popper *et al.* 2024). Moreover, regulation of translation can contribute to diversification of neuronal function, e.g. during the selection of a specific olfactory receptor in mouse sensory neurons (Dalton *et al.* 2013). Following along these lines, posttranslational modification of proteins also deserves greater attention in this context, particularly since bHLH TFs are known to be strongly regulated by phosphorylation (Guillemot and Hassan 2017).

The unparalleled resources available to the *C. elegans* and broader nematode communities, together with developing technologies for measuring different outputs of gene expression with increasing depth and manipulating RNA and protein production and stability with increasing precision, promise an exciting time in continuing to explore how these fascinating cells develop and function.

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Conflicts of interest

The authors declare no conflict of interest.

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