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Coordinated regulation of cholinergic motor neuron traits through a conserved terminal selector gene

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Abstract

Cholinergic motor neurons are defined by the co-expression of a battery of genes which encode proteins that act sequentially to synthesize, package and degrade acetylcholine and reuptake its breakdown product, choline. How expression of these critical motor neuron identity determinants is controlled and coordinated is not understood. We show here that in the nematode *Caenorhabditis elegans* all members of the cholinergic gene battery, as well as many other markers of terminal motor neuron fate, are co-regulated by a shared *cis*-regulatory signature and a common *trans*-acting factor, the phylogenetically conserved COE (Collier/Olf/EBF)-type transcription factor UNC-3. UNC-3 initiates and maintains expression of cholinergic fate markers and is sufficient to induce cholinergic fate in other neuron types. UNC-3 furthermore operates in negative feedforward loops to induce the expression of transcription factors that repress individual, UNC-3-induced terminal fate markers resulting in diversification of motor neuron differentiation programs in specific motor neuron subtypes. A chordate ortholog of UNC-3, *Ciona intestinalis* COE, is also both required and sufficient for inducing a cholinergic fate. Thus, UNC-3 is a terminal selector for cholinergic motor neuron differentiation whose function is conserved across phylogeny.

INTRODUCTION

A core identity feature of each specific neuronal type is its neurotransmitter phenotype which is defined by the co-expression of genes encoding proteins that synthesize, package and re-uptake a specific neurotransmitter. In the case of the neurotransmitter acetylcholine (ACh), the enzyme choline acetyltransferase (ChAT) synthesizes ACh from the ubiquitous precursor choline, the vesicular ACh transporter VAChT takes up ACh into synaptic vesicles, acetylcholinesterase (AChE) breaks down ACh into choline after release in the

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synaptic cleft and a choline transporter (ChT) reuptakes choline to shuffle it back in the biosynthetic/release cycle (Fig. 1a)[1]. Moreover, cholinergic synaptic termini often contain autoreceptors to modulate synaptic signaling properties[2]. How the expression of these core features of cholinergic neuron identity is coordinated is not presently known. Elucidating the mechanisms of coordinated expression of neurotransmitter pathway genes also represents one key approach to understand how neuronal identity is controlled. The much anticipated goal of generating specific neuron types *in vitro* for basic and therapeutic studies, is a further motivation to understand how these genes are regulated.

Motor neurons in the spinal and ventral cords of vertebrates and many invertebrates generally use ACh to communicate with their target muscles. The nematode *C. elegans* contains 8 different classes of ventral nerve cord (VNC) motor neurons (MNs) that control locomotion of the animal[3,4]. Two of these 8 classes are GABAergic, the other 6 classes are cholinergic, namely the dorsal A (DA) and ventral A (VA)-class neurons (hereafter referred to as A-type MNs), the dorsal B (DB) and ventral B (VB)-class neurons (hereafter referred to as B-type MNs), the AS neurons and the ventral C (VC)-class neurons (Fig. 1b). Each class contains several members (e.g. DA1, DA2, DA3 etc.) amounting to a total of 56 ventral cord motor neurons [3,4]. The cholinergic nature of these neurons is defined by the expression of *unc-17*/VAChT, *cha-1*/ChaT (both genes are organized into one common genetic locus in many animal species[5]), *ace-2*/AChE (this study), *cho-1*/ChT [6] and the putative ACh autoreceptor subunits *acr-2*, *acr-5* and *acr-14* [7–9].

The phylogenetically conserved transcription factor *unc-3*, the sole *C. elegans* homolog of the COE (Collier/Olf/EBF) family of transcription factors, is expressed in the A-, B- and AS-type MNs, but not the VC MNs [10,11] (this study). *unc-3* mutant animals were previously shown to display defects in the differentiation of the A- and B-type neurons (AS type neurons were not examined), as assessed by aberrant morphology and aberrant expression of three cell surface proteins (the ACh receptor *acr-5*, the DEG-type ion channel *del-1* and the netrin receptor *unc-5*) and one transcription factor (the homeobox gene *unc-4*) [10,11]. If, how and to what extent *unc-3* affects the individual members of the cholinergic gene battery, i.e. the cholinergic phenotype of these neurons is not known. We show here that the cholinergic gene battery, as well as many other terminally differentiated features of the A-, B- and AS-type MNs are co-regulated through a common regulatory strategy, employing a shared *cis*-regulatory signature and *trans*-acting factor, UNC-3. This shared regulatory strategy defines the logic of coordinated regulation of gene expression of terminal cholinergic features. Evidence is presented for an evolutionarily conserved role of UNC-3 in the specification of cholinergic motor neurons in a chordate, the tadpole of *Ciona intestinalis*.

RESULTS

unc-3 is required for coordinated expression of motor neuron genes

We generated a number of fluorescent reporter constructs that monitor expression of the cholinergic gene battery shown in Figure 1a. These reporters either recapitulated known expression patterns in cholinergic ventral cord motor neurons (i.e. *unc-17, cho-1*)[6,12] or demonstrated predicted expression in cholinergic motor neurons (i.e. *ace-2*)[13] (Fig. 1c, Supplementary Table 1). Apart from the cholinergic gene battery, we examined the

expression of 27 additional reporters of putative terminal differentiation genes for which expression in either all or individual types of ventral cord MNs was previously reported, including putative ACh metabotropic and ionotropic autoreceptors (*acr-2, acr-5, acr-14, acr-15, acr-16, gar-2*), other ionotropic and metabotropic neurotransmitter receptors (GABA receptor *gbb-1*, dopamine receptor *dop-1*), ion channels (DEG/ENaC channels *unc-8* and *del-1*, TRP channel *trp-1*, voltage-gated Ca+ channel *nca-1*), gap junction proteins (*inx-12*), signaling proteins (TGFβ-like *tig-2* and *dbl-1*), neuropeptides (*nlp-21*), axon pathfinding factors (*max-1, unc-129, unc-40*) and others (see Methods section for reporter strains used)(Table 1, Fig. 1c, Fig. 2a). We crossed these reporter transgenes into mutants that lack the sole homolog of the COE-type transcription factor *unc-3 (*mutant allele *e151)* and find that 87% (26/30) tested terminal differentiation genes of the A-, B- and/or AS-type motor neurons fail to be properly expressed in *unc-3* mutant animals (Table 1, Fig. 1c, Fig. 2a, Supplementary Table 1, Supplementary Fig. 1). Three of the four genes whose expression is not affected by *unc-3* are broadly expressed throughout the nervous system and some also outside the nervous system. Three known pan-neuronally expressed genes (*rab-3, unc-119, rgef-1*) also show normal expression patterns in the VNC of *unc-3* mutants (Fig. 2b, Supplementary Table 1). We conclude that *unc-3* controls the A-, B-, AS-type MN expression of a large number of genes that display a restricted expression in the nervous system, but does not affect expression of generic components of neuronal fate.

Molecular markers for the other major classes of ventral cord MNs, GABAergic neurons, are not ectopically expressed in the A-, B-, and AS-type MNs in *unc-3* mutants [10]. Three unidentified cells in the ventral cord of *unc-3* mutants ectopically express VC motor neuron fate markers [10], but since *unc-3* affects the fate of a total of 50 cholinergic MNs (A-, B- and AS-type), the *unc-3* phenotype can not be generally explained by a general switch to VC motor neuron fate. Moreover, VC motor neurons are also cholinergic; therefore, if A-, B-, and/or AS-type MNs had simply switched to a VC MN fate, expression of the cholinergic gene battery should be unaffected, which is not the case (Fig. 1c, Supplementary Table 1). We also tested the possibility that cholinergic MNs in *unc-3* mutants convert into a more progenitor-like state characterized by the transient expression of the NeuroD homolog *cnd-1* [8]. Examining *cnd-1* expression in *unc-3* mutants, we find this not to be the case (Supplementary Fig. 2). Therefore, we conclude that in *unc-3* mutants, several major classes of ventral cord cholinergic neurons fail to execute the normal, identity-defining differentiation program, yet retain generic neuronal identity.

unc-3 is sufficient to induce motor neuron markers

To ask whether *unc-3* is not only required but also sufficient to induce cholinergic motor neuron fate, we generated transgenic animals that misexpress *unc-3* in two unrelated, non-cholinergic neuron types – the glutamatergic sensory neurons AWC and ASE, using regulatory sequences from the *ceh-36* homeobox gene (an AWC and ASE fate regulator)[14]. In these transgenic animals, we observe ectopic expression of the cholinergic markers *acr-2* and *unc-17* (Fig. 3a–b).

Misexpression of *unc-3* in the D-type GABAergic motor neurons, using regulatory sequences from the *unc-30* gene (and inducer of GABA fate)[15] also induces ectopic

expression of cholinergic fate (Supplementary Fig. 3). Broader misexpression of *unc-3* using an inducible heat-shock promoter results in very broad expression of cholinergic fate markers throughout the animals if *unc-3* is heat shock-induced at embryonic stages (Fig. 3c–d, Supplementary Fig. 4). Taken together, these findings demonstrate that *unc-3* is not only required but also sufficient to induce cholinergic fate in many spatiotemporal contexts.

Coregulation of motor neuron genes through COE motifs

Mechanistically, the effect of *unc-3* on cholinergic motor neuron differentiation could be explained either by *unc-3* controlling the regulation of a number of other regulatory factors that then control terminal gene expression or by *unc-3* directly controlling expression of the cholinergic terminal gene battery. Such a direct, co-regulatory strategy would represent an ideal and simple way to ensure coordinated expression of the cholinergic synthesis/transport pathway genes. We tested this possibility by systematically examining members of the cholinergic gene battery, as well as all other tested UNC-3-dependent A-, B-, and AS-type motor neuron markers for the presence of putative UNC-3 binding sites. As a guide, we used the binding sites determined for vertebrate orthologs of UNC-3, which in the context of *C. elegans* olfactory neurons, have also been shown to be functional UNC-3 binding sites [16–19] (Fig. 4a, Supplementary Fig. 5). We refer to these *cis*-regulatory binding sites here as “COE motifs”. We found at least one copy of this motif in 20 of the 26 *unc-3*-dependent, VNC-expressed genes (Table 1); most of these motifs are phylogenetically conserved in different nematode species (Fig. 4, Supplementary Table 2, Supplementary Fig. 5). Focusing on 8 of the 26 genes, we first tested whether small regulatory regions that contain these motifs are sufficient to drive expression of a reporter gene in the A-, B-, and AS-type motor neurons and found this to be the case (Fig. 4). We mutated these binding sites in the respective reporter genes and generated transgenic animals expressing these reporters. Compared to the wild-type control, all mutated reporters failed to show correct expression in the A-, B-, and AS-type MNs (Fig. 4, Supplementary Table 2). Expression in other neuron types is unaffected. We note that several of the *unc-3* and COE-motif-controlled genes contain more than one copy of the COE motif. Based on the analysis of other simple *cis*-regulatory motifs that control neuronal gene expression in *C. elegans* [20], we hypothesize that such multiplicity of *cis*-regulatory motifs ensures robustness in gene expression.

We next took a broader genomic view of the occurrence of COE motifs and asked how predictive their occurrence is for expression in cholinergic motor neurons. We first examined a set of genes identified through cell-type specific transcriptome profiling to be expressed in A-type cholinergic ventral cord motor neurons[21]. We found that 27/32 (84.4%) of these genes contain COE motifs (Supplementary Table 3). By comparison, only 5/33 (15.2%) genes whose expression cannot be detected in the A-and B-type motor neurons contain a COE motif (Supplementary Table 3).

Second, we interrogated the entire *C. elegans* genome for the presence of COE motifs using the CisOrtho platform which we previously developed to search genome sequences for matches to a position weight matrix of transcription factor binding sites[22]. We selected 8 genes with unknown expression pattern that contain phylogenetically conserved COE motifs and that encode putative neuronal function genes, focusing on putative ion channels. We

generated *gfp* reporters for these genes and found that seven of eight genes are indeed expressed in cholinergic ventral cord motor neurons (Supplementary Fig. 6). These seven genes encode several potassium channels *(twk-7, twk-13, twk-40, twk-43)*, a ligand-gated ion channel *(lgc-55)*, an acetylcholine receptor subunit *(acr-21)* and the *C. elegans* ortholog of the human transmembrane channel-like gene 1 (TMC1), mutations in which cause deafness in mice and humans[23]. We tested whether the expression of five of these seven genes depend on *unc-3* by crossing reporter genes into an *unc-3* mutant background. We found that four of the five genes indeed require *unc-3* for VNC MN expression (*twk-13, twk-40, twk-43 and* ortholog of TMC1) (Supplementary Fig. 6). With these four *unc*-3-dependent, COE-motif containing genes, we have identified a total of 30 direct target genes of *unc-3* in various classes of VNC MNs, all of which encode for terminal differentiation ("effector") genes (below we describe three more direct targets of *unc-3* that code for transcription factors)(Table 1). We conclude that terminal cholinergic fate is controlled by a co-regulatory strategy through the UNC-3 transcription factor and its cognate *cis*-regulatory target site, the COE motif. The presence of phylogenetically conserved COE motifs appears to be a predictor for expression of genes in cholinergic motor neurons.

Continuous expression and requirement for *unc-3*

Terminal differentiation features of a neuron, such as neurotransmitter gene batteries, need not only be induced but also maintained throughout the life of a neuron. The ability of *unc-3* to directly induce cholinergic fate suggests the possibility that *unc-3* may also maintain cholinergic fate. A prerequisite for such an activity is sustained expression of *unc-3* in cholinergic MNs throughout the life of the animal. While expression of *unc-3* has previously been found to coincide with the generation of A- and B-type motor neurons[11], its expression in these neurons has not been reported during postdevelopmental stages. To monitor *unc-3* expression we generated a fosmid-based reporter that spans about 40 kb of genomic sequences, containing all intergenic regions surrounding the *unc-3* locus plus several genes upstream and downstream of *unc-3*. Consistent with previous studies, we find that the *unc-3* fosmid reporter is expressed in the A- and B-type motor neurons (both the dorsal and ventral A and B-type motor neurons DA, VA and DB, VB), but not in VC (Supplementary Fig. 7) and GABAergic D-type neurons (Fig. 5a). We also detect expression in the postembryonic AS cholinergic ventral cord motor neurons, consistent with the effect of *unc-3* on expression of the cholinergic gene battery in the AS neurons. Importantly, we find that *unc-3* expression persists in all these VNC motor neurons throughout the life of the animal (Fig. 5a).

To assess the physiological relevance of persistent *unc-3* expression, we sought to remove *unc-3* gene activity postembryonically. Since temperature-sensitive alleles for *unc-3* are not available and since *unc-3* gene activity could not be removed by RNAi (data not shown), we generated transgenic animals that lack endogenous *unc-3* gene activity but express heat-shock inducible *unc-3* from an extrachromosomal array under control of the *hsp-16* promoter[24]. We find that the uncoordinated phenotype (data not shown) and loss of *acr-2::gfp or unc-17::gfp* expression of *unc-3(e151); Ex[hs::unc-3]* animals can be rescued through heat-shock induction of *unc-3* expression in mid-larval stages (Fig. 5b, Supplementary Fig. 8). This indicates that embryonically generated MNs remain in a state

that is responsive to *unc-3*, rather than, for example, irreversibly converting into another fate or being irreversibly damaged. If *unc-3* activity is supplied in midlarval stages, but then removed through removal of the inductive heat shock stimulus, a progressive loss of *acr-2::gfp or unc-17::gfp* expression is observed during adulthood (Fig. 5b, Supplementary Fig. 8). This observation indicates that *unc-3* gene activity is continuously required to maintain the gene expression program of cholinergic motor neurons.

Diversification of motor neuron expression programs

Previous work has shown that a set of three phylogenetically conserved homeodomain transcription factors, *unc-4* (Uncx4 in vertebrates), *vab-7* (Evx in vertebrates) and *ceh-12* (HB9 in vertebrates) act as transcriptional repressors to make DA become different from DB and VA different from VB fate [9,25,26]. For example, the B-type specific terminal differentiation marker *acr-5*, coding for an acetylcholine receptor subunit, is repressed by *unc-4* in the DA neurons but not in the DB neurons [9]. The DB-specific *vab-7* homeobox gene in turn represses *unc-4* in DB, thereby allowing the *acr-5* to be expressed in DB-type MNs [25]. These sequential repressive regulatory events beg the previously unexplored question of how *acr-5* expression is activated. We have shown above that positive induction of *acr-5* in DB-type MNs is achieved by direct activation through *unc-3*. Interestingly, we find that the *unc-4* and *vab-7* homeobox genes are also both likely direct targets of *unc-3*. Both genes contain phylogenetically conserved COE motifs in their regulatory regions and both depend on the presence of *unc-3* for their expression in the DA (*unc-4*) and DB (*vab-7*) (Fig. 6a–b). Similarly, the VB-expressed HB9 ortholog *ceh-12*, which is repressed in VA by *unc-4* to allow for the execution of VA-specific fate [26], is also a likely direct target of *unc-3* as it also contains COE motifs in its regulatory region and depends on *unc-3* for correct expression in VB (Fig. 6a–b). Taken together, aside from controlling terminal features of all cholinergic MNs *unc-3* also activates MN subtype-specific transcriptional repressors that act in a negative feedforward loop (a motif commonly found in bacterial gene regulatory networks and also called “incoherent feedforward loop” [27]) to repress subtype-specific terminal differentiation genes, thereby generating diversity in distinct cholinergic MN types (Supplementary Fig. 13c).

Co-regulatory strategies also apply to other cholinergic neurons

unc-3 affects the cholinergic gene battery in the cholinergic A-, B-, and AS-type motor neurons. In addition to those neurons, *C. elegans* contains more than a dozen very distinct types of cholinergic neurons, including sensory and interneurons [1]. For example, the AIY interneuron class, which is located in the ventral head ganglion and processes a variety of distinct sensory modalities, is also cholinergic, as assessed by expression of the *unc-17/cha-1* locus [28]. As expected, we find that AIY also expresses the choline transporter *cho-1* (Supplementary Fig. 9), but it does not express *unc-3* (as assessed with an *unc-3* fosmid-based reporter gene fusion; data not shown). How is the cholinergic battery then induced in AIY? We have previously shown that the *unc-17/cha-1* locus contains a binding site (called “AIY motif”) for the TTX-3/CEH-10 homeodomain heterodimer, which is required for *unc-17/cha-1* expression [29]. We find that *cho-1* expression is also lost in *ttx-3* mutants (Supplementary Fig. 9). Besides containing a COE motif, the *cho-1* regulatory region also contains a phylogenetically conserved AIY motif (Fig. 4c). A reporter gene lacking the COE

motif looses expression in A-, B-, and AS-type cholinergic neurons, but not in AIY, while a reporter that lacks the AIY motif, but contains the COE motif shows expression in A-, B-, and AS-type motor neurons, but not in AIY (Fig. 4c). These findings not only demonstrate co-regulation of cholinergic pathway genes in other cholinergic neuron types, but also suggest that the regulation of cholinergic pathway genes is conferred by a modular array of neuron-type specific *cis*-regulatory elements that are activated by neuron-type specific regulatory factors (Supplementary Fig. 13b).

The function of UNC-3 is conserved across phylogeny

Vertebrate genomes contain multiple orthologs of UNC-3. At least three of the four mouse UNC-3 orthologs are expressed in cholinergic motor neurons of the spinal cord [30,31], yet their role in determining the cholinergic phenotype has not been assessed. The chordate *Ciona intestinalis* contains cholinergic motor neurons with several organizational and developmental similarities to those of vertebrates; their axons extend along the tail of the larvae and, like motor neurons in vertebrates and *C. elegans* they control locomotion[32]. Unlike vertebrates, *Ciona* only contains a single EBF ortholog, called COE[33]. We examined its expression pattern and found it to be also expressed in cholinergic motor neurons, as assessed by a reporter that visualizes expression of the VAChT/ChAT locus (Fig. 7a–b)[34]. The 5′ upstream regulatory region of the VAChT/ChAT locus included in the reporter construct contains a copy of the COE recognition motif, raising the possibility that COE is essential for specifying cholinergic neuronal identity. To investigate this possibility we selectively expressed a dominant-negative form of COE (COE-WRPW) in the visceral ganglion (e.g., the *Ciona* spinal cord) [35]. The resulting transgenic tadpoles fail to induce cholinergic motor neuron differentiation in the spinal cord (Fig. 7c and Supplementary Fig. 10). To ask whether *C. intestinalis* COE is not only required but also sufficient to induce cholinergic fate, we misexpressed the normal versions of the COE protein under the control of regulatory sequences from the *Msx* gene. These regulatory elements drive expression in mainly non-cholinergic territories, including future palp sensory neurons, which are glutamatergic [36]. *Ciona* larvae expressing the COE in these cells show ectopic induction of the VAChT reporter, indicating that palp neurons now have adopted cholinergic features (Fig. 7d).

To further explore the functional conservation of *Ciona* COE and nematode UNC-3, we expressed the *Ciona* COE gene in a *C. elegans unc-3* mutant background. As assessed by analyzing the expression of two *unc-3*-dependent markers (*acr-2* and *unc-17*), we found that *Ciona* COE can functionally compensate for the lack of *unc-3* (Fig. 7e, Supplementary Fig. 12). We conclude that the role of UNC-3 in generating cholinergic motor neurons is conserved from invertebrates to chordates.

DISCUSSION

We have provided insights into the coordinated regulation of the core determinants of the cholinergic phenotype of *C. elegans* motor neurons. While mechanisms of coordinated gene expression are well characterized in, for example, metabolic pathways in bacteria and yeast [37,38], much less is known about how such coordination is achieved within specific cell

types in metazoan organisms. We have shown here that cholinergic pathway genes are co-regulated through a common *cis*-regulatory motif and documented that the cognate *trans*-acting factor for this binding site is both required and sufficient for the expression of the cholinergic phenotype (schematically summarized in Supplementary Fig. 13a). While cholinergic motor neuron fate is affected by various regulatory factors in mice [39], it is not clear whether such effects are a reflection of true co-regulation through the same *trans*-acting factor, or a reflection of an indirect effect in which an upstream regulator controls the expression of several *trans*-acting factors that independently regulate cholinergic pathway genes.

UNC-3 controls the cholinergic phenotype in all but one class of *C. elegans* ventral cord MNs (VC class) and has no apparent impact on the cholinergic phenotype of non-MN cholinergic neurons. Other cholinergic neuron classes may use similar co-regulatory strategies to control their cholinergic phenotype. Indeed, we find that the TTX-3/CEH-10 homeodomain heterodimer coregulates the cholinergic pathway genes in the head interneuron AIY [29]. Together these findings demonstrate that members of the cholinergic gene battery contain a modular array of *cis*-regulatory motifs that respond to distinct cholinergic selector genes in different neuronal cell types (schematically summarized in Supplementary Fig. 13b). This notion is consistent with reporter gene assays that monitor expression of the vertebrate VAChT/ChAT locus in transgenic mice. A longer and a shorter 5′ upstream regulatory region shows that the 5′ regulatory region contains separable regulatory elements [40], whose detailed *cis*-regulatory composition is unknown.

Our results go beyond the demonstration that neurotransmitter-specific pathway genes are co-regulated. Results mainly obtained in *C. elegans* and a few isolated cases in vertebrates have begun to suggest that many features of terminally differentiated neurons, i.e. nuts-and-bolts genes defining the structural and functional properties of a mature neuron (e.g. ion channels, neuropeptides, adhesion molecules), are co-regulated through a common regulatory strategy [41,42]. Unbiased analysis of *cis*-regulatory control regions of terminal gene batteries have revealed shared *cis*-regulatory motifs and *trans*-acting factors that act through these motifs. Such *trans*acting factors have been termed “terminal selectors”, as they control the terminal features of a given neuron [41,42]. Terminal selectors are continually expressed in mature neurons, thereby ensuring the maintenance of key identity genes. Terminal selectors are, at least in specific cellular contexts, also sufficient to induce specific fates. UNC-3 fulfills all the criteria to be classified as a terminal selector, thereby providing critical support for the as yet unproven hypothesis that terminal selector-based gene regulatory strategies are abundantly employed throughout the nervous system to drive terminal neuronal fates. In total, we have identified 33 genes whose expression depends on *unc-3*, most of which are likely direct targets of *unc-3*.

As seen for other terminal selectors, *unc-3* may act differently in distinct neuronal cell types. It functions as a terminal selector that activates gene expression in A-, B-, and AS-type MNs, but represses alternative odorsensory fates in the ASI sensory neuron[16]. This differential activity is presumably conferred by as yet unknown, cell-type specific interaction partners, whose activity may also explain why a subset of terminal markers still show some residual MN expression in *unc-3* null mutants. Cell-type specific cofactors are

also the likely explanation for the diverse function of *unc-3* orthologs in distinct cellular contexts in vertebrates, one being the coregulation of scores of genes involved in B cell terminal differentiation [17].

unc-3 does not represent the regulatory endpoint in cholinergic MN specification. The phylogenetically conserved *unc-4*, *vab-7* and *ceh-12* homeodomain repressor proteins are known to diversify the fate of embryonic and postembryonic A- and B-type MNs through negative regulation of terminal differentiation genes, such as *acr-5* [9,25,26]. Previous work did not address, however, how those repressor targets are activated in those cells in which the repressor is not present. We find that these terminal differentiation genes are direct targets of *unc-3* (e.g. *acr-5*) and that the *unc-4*, *vab-7* and *ceh-12* transcription factors therefore negatively modulate *unc-3*-induced transcriptional programs. Intriguingly, each of these transcription factors are also likely direct targets *unc-3*, as evidenced by their dependence on *unc-3* activity, as well as the presence of conserved COE sites in their regulatory regions. Therefore, *unc-3* acts in a negative feedforward configuration to ensure that some of its terminal targets are only expressed in a subset of *unc-3* expressing neurons (schematically summarized in Supplementary Fig. 13c). Other terminal selectors work through similar negative feedforward loops. For example, the ASE terminal selector *che-1* controls the expression of terminal differentiation genes in ASE neurons, but also directly controls regulatory factors that diversify the ASEL and ASER neuronal subtypes (schematically summarized in Supplementary Fig. 13d) [42]. Apart from conferring specific kinetic properties to a network [27], negative (also called “incoherent”) feedforward loops may be a reflection of a sequential recruitment of target genes for a transcription factor in an evolutionary context, enabling the system to progress from an ancient state of generic activation (*unc-3* activating *acr-5* in all MNs) to finer scaled differential activation in distinct cell types (*unc-3* able to activate *acr-5* only in a subset of MNs).

Our findings in the chordate *Ciona intestinalis* indicate that the role of UNC-3 as a key determinant of cholinergic MN identity is deeply conserved in animal evolution. Mice express several COE family members in postmitotic, cholinergic spinal cord MNs [30,31] and well-conserved COE motifs can be found in the *cis*-regulatory region of the cholinergic genes *VAChT/ChaT*, *CHT1*, and *AChE (*Supplementary Fig. 14). Future loss of function studies in mice may further corroborate the notion of deep conservation of the control mechanisms of terminal MN differentiation. We propose that the simple terminal selector regulatory logic may lie at the evolutionary base of neuronal diversity.

METHODS

C. elegans reporter strains

Details on the reporter strains used in Fig. 1 and 2 are provided in Supplementary Table 1. The following reporter strains were used: *wdEx290* (*acr-15::gfp*), *wdEx419* (*acr-16::gfp*), ctIs43 (*dbl-1::gfp*), *wdEx346* (*tig-2::gfp*), rtEx330 (*nlp-21::gfp), vsIs28 (dop-1::gfp), wdEx351 (tsp-7::gfp), otEx223 (rig-4::gfp), zwEx112 (inx-12::gfp), wdEx345(F55C12.4::gfp), wdEx457 (F39B2.8::gfp), wdEx359 (F29G6.2::gfp), wdIs4 (unc-4::gfp), stIs10140 (vab-7::mCherry), stIs10055 (cnd-1::mCherry), wdIs62*

(ceh-12::gfp), inIs179 (ida-1::gfp), otIs264 (*ceh-36::TagRFP*), *mgIs18 (ttx-3::gfp), ses28 (kvs-1::gfp), nuEx1072 (gar-2::gfp), nuEx1066 (gbb-1::gfp).*

Fosmid recombineering was done as previously described [45], using the following fosmids: *cho-1* - WRM0613dC12, *ace-2* - WRM0641bD11 and *unc-3* - WRM0622bH08. For all fosmid reporters, an SL2 spliced, nuclear localized *gfp (mcherry* for *unc*-3 reporter) coding sequence was engineered at the C-terminus of the respective locus, as previously described [45]. Fosmids were injected as complex arrays [45].

Reporter gene fusions for *cis*-regulatory analysis were done using a PCR fusion approach [46]. Genomic fragments were fused to a nuclear localized DsRed2 coding sequence, which was followed by the *unc-54 3′ UTR.* Mutagenesis was performed using the Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene). PCR fusion DNA fragments were injected into young adult *pha-1(e2123)* hermaphrodites at 50ng/μl using *pha-1* (pBX plasmid) as co-injection marker (50ng/μl).

C. elegans expression constructs and generation of transgenic animals

The *ceh-36::unc-3_cDNA* and *hsp-16.2::unc-3_cDNA* constructs were a generous gift from Piali Sengupta [16]. The *ceh-36::unc-3_cDNA* construct was injected as simple array at 10 ng/μL. The *hsp-16.2::unc-3_cDNA* construct was injected into young adult hermaphrodites as a complex array, using 2 ng/μL linearized plasmid DNA, 150 ng/μL PvuII digested bacterial genomic DNA and 2 ng/μL injection marker. The injection markers were pRF4 (*rol-6d*) for *ceh-36^{prom}::unc-3* and *ttx-3^{prom}::mCherry* for *hsp::unc-3*. The *unc-3* cDNA was fused to 2.8 kb of promoter sequence of the *unc-30* gene and the resulting PCR fragment was injected into young adult hermaphrodites at 30 ng/μL using *myo-2^{prom}::gfp* as co-injection marker (8 ng/μL). The *Ciona intestinalis COE* cDNA was cloned into the pPD49.78 heat-shock vector using EcoRI. The *hsp-16.2::COE* construct was injected into young adult hermaphrodites as a complex array, using 2 ng/μL linearized plasmid DNA, 150 ng/μL PvuII digested bacterial genomic DNA and 2 ng/μL injection marker.

Heat-shock experiments

Two transgenic lines for *hsp-16.2::unc-3 (otEx4536, otEx4441)* were used for the heat-shock experiments. For the ectopic induction experiment shown in Fig. 3 and Supplementary Fig. 4, embryos at 2- and 3-fold stage were heat shocked at 37°C three times for 30 minutes with one hour incubation at 20°C between each heat shock to let worms recover. After heat shock worms were kept at 25°C over-night and scored at the indicated times. The same heat-shock protocol was followed for the rescue experiment shown in Fig. 7e and Supplementary Fig. 12 but the heat shock occurred at L1. For the maintenance experiment shown in Fig. 5, third larval stage (L3) worms were heat shocked at 37°C three times for 30 minutes with one hour incubation at 20°C between each heat shock to let worms recover. After heat shock worms were kept at 25°C over-night and then transferred at 15°C for 5 days.

Microscopy

Worms were mounted on 5% agarose on glass slides and images were taken using an automated fluorescence microscope (Zeiss, AXIO Imager Z1 Stand).). Snapshots were taken for Figures 1 and 2, and Supplementary Figures 1, 2, 6 and 11 using the Micro-Manager software (Version 3.1)[47]. The rest of the images (except Fig. 6) in the remaining figures were created after acquisition of several z-stack images (~1μm thick), and subsequent max-projection using the maximum intensity projection type.

Bioinformatic analysis

The MatInspector program from Genomatix (http://www.genomatix.de) was used to predict the binding sites for the COE transcription factor family in the *cis*-regulatory region of all the tested *C. elegans* genes, as well as the *Ciona intestinalis* and mouse cholinergic pathway genes. The logo for the position weight matrix that describes the COE binding site in 4 nematode species was created using enoLOGOS [48]. Clustalw2 (www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the COE binding sites in different vertebrate species (Supplementary Fig. 14).

Ciona intestinalis expression constructs and animal handling

The *VAChT* -4315/-823 genomic DNA fragment was amplified by PCR using the following primers: VAChT −4315 fwd (CCCTACTGTAACACAGTAAC) and VAChT −823 rev (CTTTCTATTGAATCGTACACCTAAG). This fragment was cloned upstream of unc-76-tagged Venus (YFP). *COE>mCherry* and *FGF8/17/18>H2B::mCherry* have been previously described [44]. HA-tagged *COE::WRPW* [35] and *Ets::WRPW* [43] coding sequences were subcloned downstream of *FGF8/17/18* driver in place of *H2B::mCherry* to make *FGF8/17/18>COE::WRPW* and *FGF8/17/18>Ets::WRPW*, respectively. A fragment spanning from −2304 to +145 bp of the *Msx* gene relative to the start codon was amplified by PCR using the following primers, Msx −2304 fwd (CTACGCATTGATGTCGCAATC) and Msx +145 rev (AGAGGATTGAATGCGATCGG) and cloned upstream of *H2B::mCherry*. A slightly smaller fragment from −2304 to −74 bp was subcloned upstream of the *COE* or *lacZ* coding sequences to produce *Msx>COE and Msx>lacZ*, respectively. All constructs were made using the pCESA plasmid backbone.

Embryo fertilization, dechorionation, electroporation, and fixation were performed according to established methods [49]. Embryos and larvae were mounted in 50% glycerol on glass slides and imaged using an Zeiss LSM 700/Axio Observer. Z1m SP inverted confocal setup (Fig. 7b) or Zeiss AxioImager A.2 compound microscope (Fig. 7c–d). Larvae destined for imaging with the confocal microscope were counterstained with phalloidin-AlexaFluor647 conjugate (Invitrogen).

Statistical analysis

Statistical analysis was performed using the Student's *t*-test (tail 2, type 2). Values are expressed as mean ± standard deviation (s.d). Differences with p value < 0.001 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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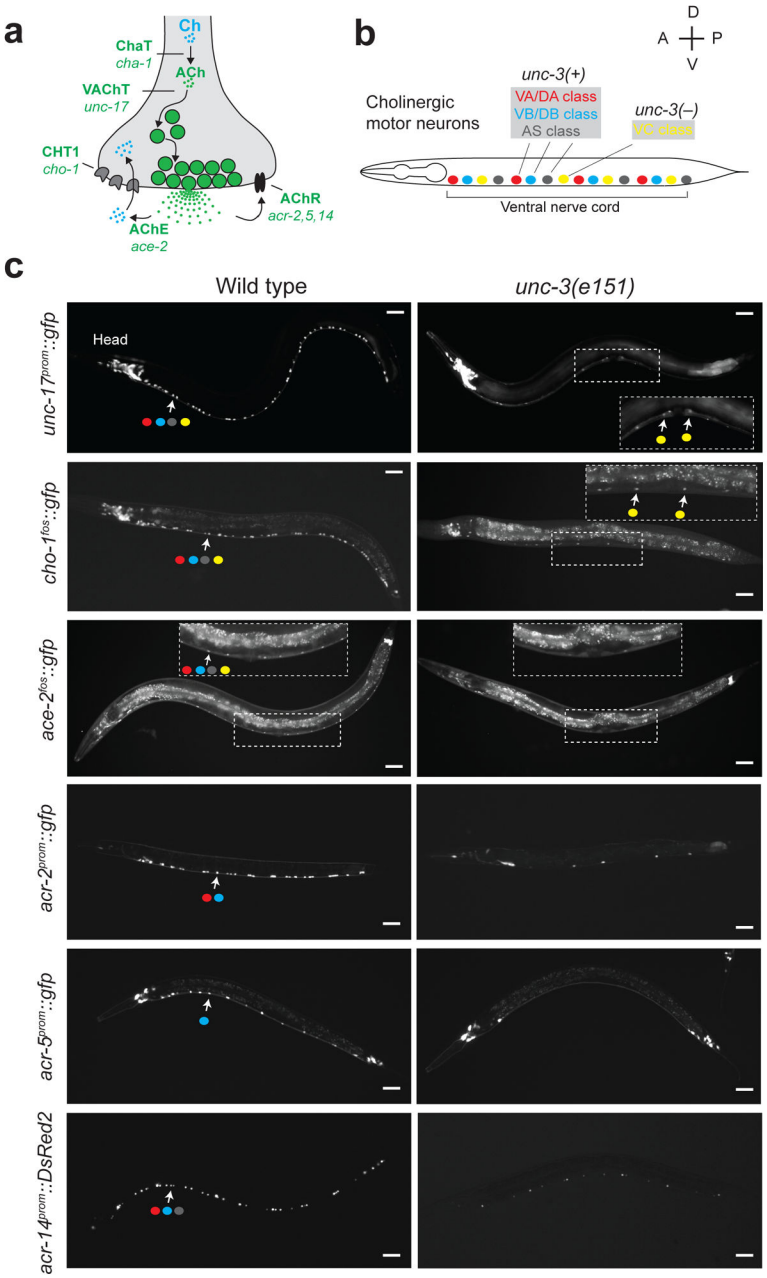

Figure 1. *unc-3* is required for expression of the cholinergic gene battery

a: Cholinergic gene battery. Ch - choline, ACh - acetylcholine. Mouse gene names: ChAT - choline acetyltransferase, VAChT - vesicular ACh transporter, CHT1 - choline transporter, AChE - acetylcholinesterase, AChR - acetylcholine receptors. The *C. elegans* gene names are italicized and shown below the mouse gene names.

b: Schematic of the *C. elegans* ventral nerve cord (VNC) with all cholinergic MN classes indicated. Colored dots indicate each MN class.

c: Expression of cholinergic reporter genes in wild-type and *unc-3(e151)* mutant backgrounds at the larval stage L4. Quantification of data and information on reporter

strains is shown in Supplementary Table 1. *acr-5* has also previously been shown to be affected by *unc-3* [10]. Similar effects were observed when the *acr-2* and *unc-17 gfp* reporters were crossed into three other *unc-3* alleles (*n3435, n3413, n3366*) (Supplementary Fig. 11). The partial penetrance of effects on some reporters is indicative of the existence of factors that collaborate with *unc-3* and are able to partially compensate for the loss of *unc-3*. Arrows indicate MNs in the VNC expressing the cholinergic reporters. Dotted boxes are used in *cho-1^{fos}::gfp* and *ace-2^{fos}::gfp* fosmid reporters in order to highlight VNC expression, which was obscured by autofluorescence of the intestine. The motor neurons that still express the *unc-17* and *cho-1* reporters in the *unc-3(e151)* background belong to the VC class based on their number (six), stereotypical position in the VNC and the fact that *unc-3* is not expressed in the VCs (Supplementary Fig. 7). Scale bar 30μm.

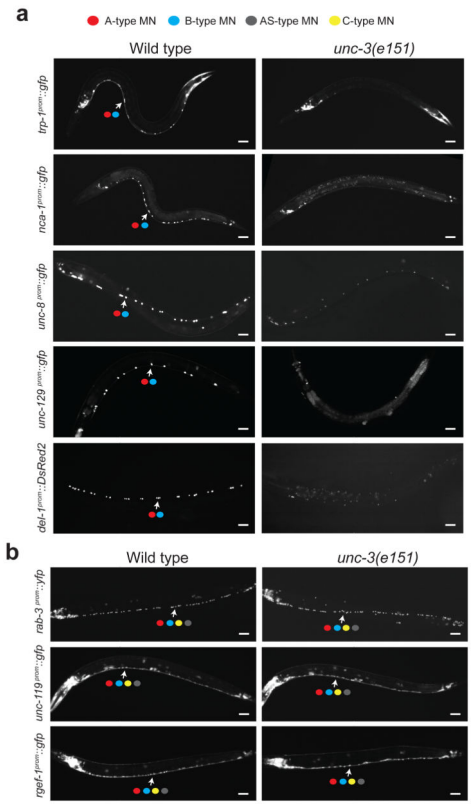

Figure 2. *unc-3* affects A- and B-type motor neuron features but not panneuronal specification

a: Expression of A- and B-type MN markers is severely affected in *unc-3(e151)* mutant backgrounds. Colored dots indicate the types of MNs expressing the reporter, with the color code taken from Fig. 1b. Quantification of data and information on reporter strains is provided in Supplementary Table 1. *del-1* has been previously shown to be affected by *unc-3* [10]. The partial penetrance of effects on some reporters is again indicative of the existence of factors that collaborate with *unc-3* and are able to partially compensate for the loss of *unc-3*. Similar effects were observed in *unc-3(n3435)* mutants (Supplementary Fig. 11). Scale bar 30μm.

b: Expression of panneuronal markers (*rab-3, unc-119, rgef-1*) is unaffected in *unc-3(e151)* mutants. Quantification of data and information on reporter strains is provided in Supplementary Table 1. Scale bar 30μm. Animals were photographed at the larval stage L4.

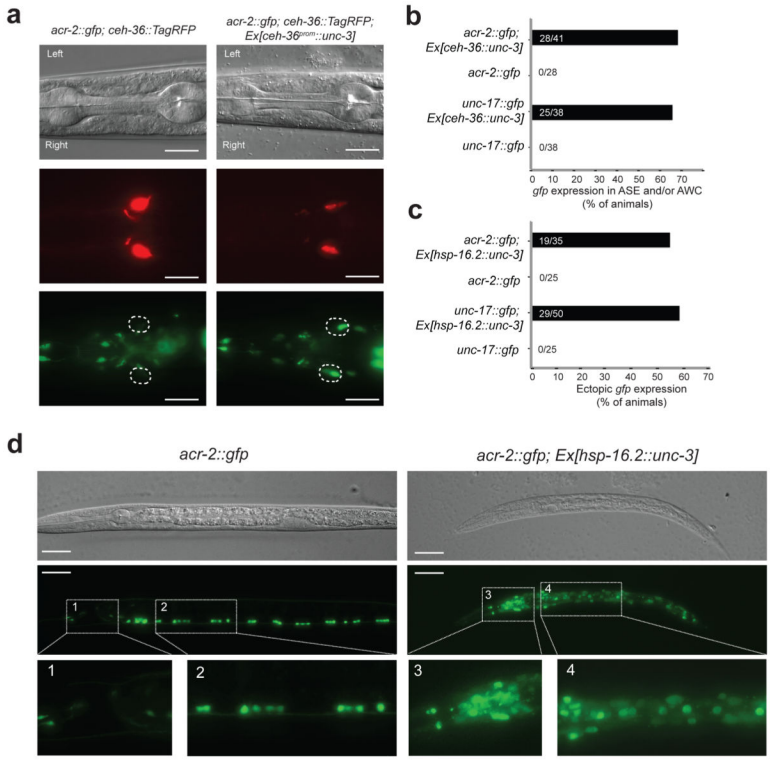

Figure 3. *unc-3* is sufficient to induce cholinergic markers in other neuron types

a: Misexpression of *unc-3* (using the *ceh-36* promoter) in two non-cholinergic neuron types - the glutamatergic sensory neurons AWC and ASE - results in ectopic expression of the cholinergic gene markers *acr-2* and *unc-17*. The *ceh-36*^{prom}::TagRFP strain was used in order to identify the AWC and ASE neurons in the head of the animal. Dashed white circles indicate the position of AWC and ASE neurons at the left and right side of the head based on *ceh-36*^{prom}::TagRFP expression. Scale bar 10μm.

b: Percentage of *ceh-36*^{prom}*::unc-3* animals with ectopic *acr-2::gfp* and *unc-17::gfp* expression in AWC and/or ASE.

c–d: Misexpression of *unc-3* with the inducible heat-shock promoter (*hsp-16.2*) results in broad expression of the cholinergic fate marker *acr-2* in the head and body region of *hsp-16.2::unc-3* animals upon heat shock induction at embryonic stages (2–3 fold embryo). One of the cells we explicitely examined for ectopic induction of *acr-2* expression is the cholinergic AIY interneuron pair, which normally does not express *acr-2*. Ectopic *unc-3* expression induces the *acr-2* reporter in AIY and reduces expression of the AIY marker *ttx-3* expression (Supplementary Fig. 15). Left panels show animals not carrying the *unc-3* array, but also subjected to heat shock. Scale bar 25μm. Ectopic expression of the cholinergic marker *unc-17* was also observed following heat shock induction of UNC-3 (Supplementary Fig. 4).

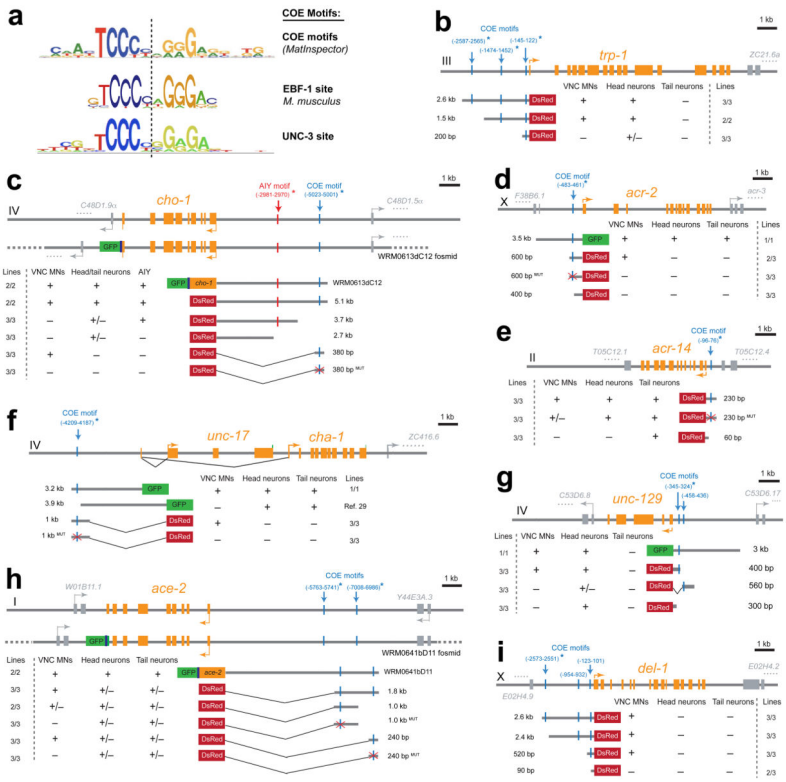

Figure 4. UNC-3 targets are co-regulated through consensus COE motifs

a: Consensus COE motif, as identified by MatInspector (top), by Ebf1 ChIP analysis of mouse B cells [17] (middle) and as present in *unc-3* responsive genes (bottom). See Supplementary Fig. 5 for sequence of the nematode sites.

b–i: *Cis*-regulatory deletion analysis. Multiple transgenic lines were analyzed for each construct. (+) indicates broad and consistent reporter expression in the VNC, or head, or tail (at least 50% of the animals) in at least 2 independent transgenic lines. (+/−) indicates significant reduction in the number of cells expressing the reporter in at least 50% of the animals (in at least 2 independent transgenic lines) compared to longer genomic fragments of the *cis*-regulatory region. (−) indicates complete loss or expression in a very limited number of neurons in the VNC, or head, or tail in at least 50% of the animals (in at least 2 independent transgenic lines) compared to longer genomic fragments of the *cis*-regulatory region. See Supplementary Table 2 for detailed quantification of the promoter analysis data. (*) indicates that the COE motif (vertical light blue line) is conserved in at least 3 other nematode species. Dark blue vertical line shows the introduction of an SL2 intercistronic site between the stop codon and the *gfp* gene. MUT indicates that the COE motif has been mutated by substituting always the same 2 nucleotides in the core sequence(for example, wild-type site: T**CC**CNNGGGA ≫ MUT site: T**GG**CNNGGGA).

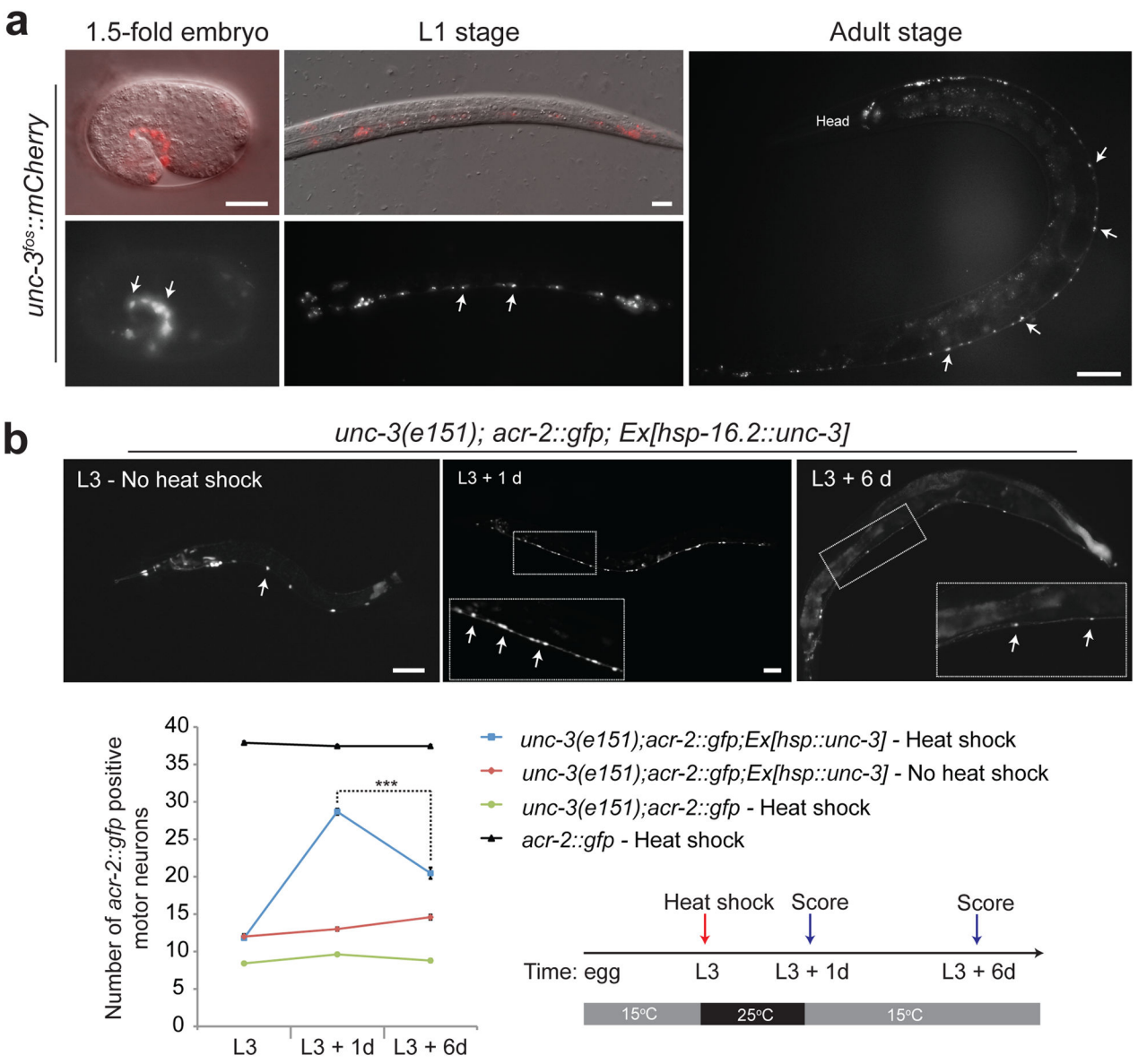

Figure 5. *unc-3* expression is functionally required through the life of the animal

a: The expression pattern of *unc-3* is shown at different stages (1.5-fold embryo, L1 larva, adult) using an *unc-3*^{fos}*::mCherry* fosmid reporter transgene. For embryo and L1 larva, differential interference contrast (DIC) microscopy images are shown at the top, while mCherry fluorescence images are shown at the bottom. Only mCherry fluorescence is shown for the adult stage. Arrows point to cholinergic MNs. Scale bars: 10μm for embryo and L1, 50 μm for adult.

b: *unc-3* is required to maintain cholinergic gene expression. Heat-shock induction of *unc-3* expression at the larval stage L3 restores *acr-2::gfp* expression in the VNC of *unc-3(e151)* mutant animals (note that unlike heat-shock induction in the embryo shown in Fig. 3, this postembryonic induction does not result in the generation of ectopic cholinergic cells). The number of *acr-2::gfp* positive VNC neurons was assessed one (L3 + 1 d) and six (L3 + 6 d) days after heat-shock. Two different *hsp-16.2::unc-3* extrachromosomal lines (*otEx4536*,

otEx4441) were analyzed for this experiment and showed similar effects. 30 – 50 animals were used per time point per genotype. Scale bars 50 μm. See **Methods Section** for details on heat-shock protocol. Quantification of the number of *acr-2::gfp* positive VNC neurons is shown at the indicated time points at the bottom left. Error bars represent standard error of the mean (s.e.m). ***: p value < 0.001.

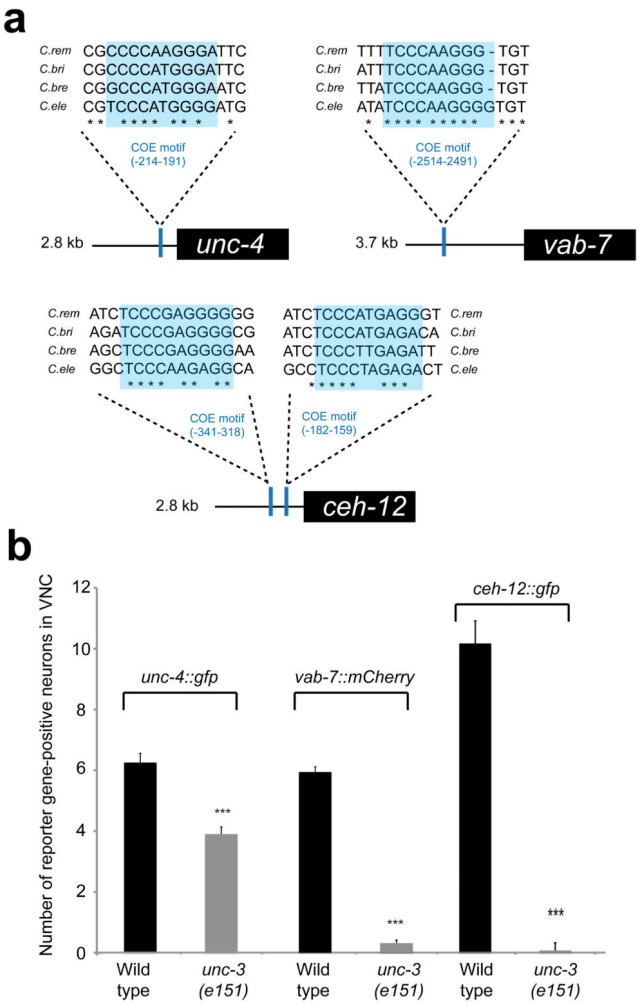

Figure 6. Gene regulatory factors are downstream targets of *unc-3*

a: The homeobox genes *unc-4* and *vab-7*, as well as the HB9 ortholog *ceh-12* contain phylogenetically conserved COE motifs in their respective regulatory regions which drive expression in ventral cord motor neurons. *C.rem; Caenorhabditis remanei, C.bri; Caenorhabditis briggsae, C. bre; Caenorhabditis brenneri, C.ele; Caenorhabditis elegans.*

b: *unc-4*, *vab-7* and *ceh-12* expression require *unc-3* for their expression in the DA (*unc-4*), DB (*vab-7*) and VB (*ceh-12*) class of motor neurons. The number of motor neurons was quantified in wild-type and *unc-3(e151)* mutant animals at 3-fold embryos (*unc-4*), at L1 – L2 (*vab-7*), and at L2 (*ceh-12*). 25 – 30 animals were scored. Error bars represent standard error of the mean (s.e.m). ***: p value < 0.001.

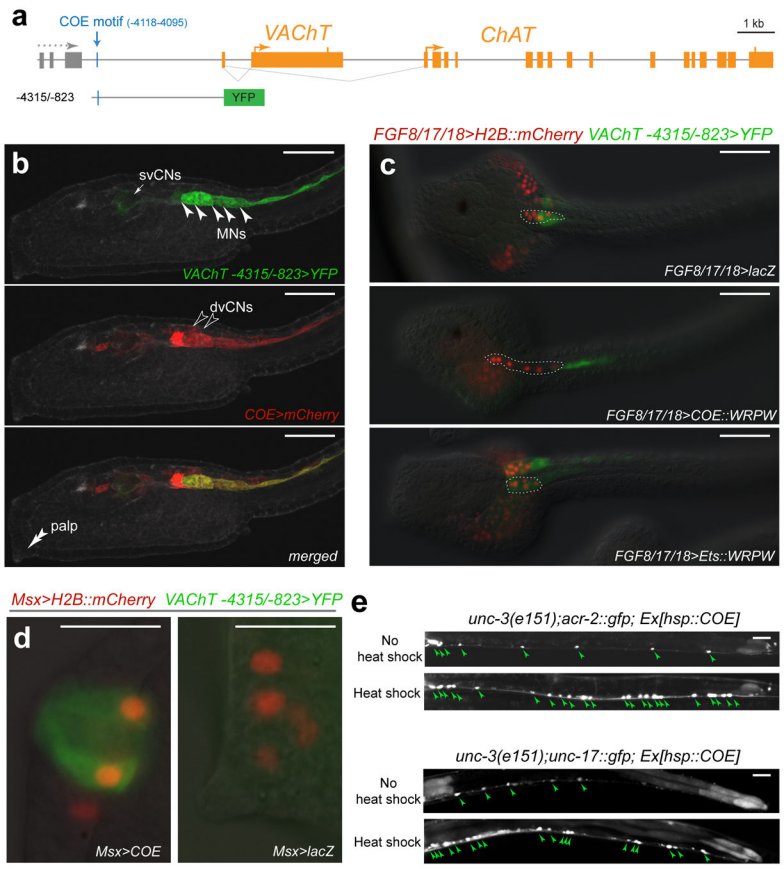

Figure 7. The function of UNC-3 is conserved across phylogeny

a: Schematic of *VAChT/ChAT* locus of *Ciona intestinalis.*

b: Swimming tadpole larva co-electroporated with *VAChT −4315/−823>YFP* (top panel; see Supplementary Table 4 for quantification) and *COE>mCherry* (middle panel) show coexpression in cholinergic MNs (solid arrowheads). Coexpression is also observed in dorsal visceral ganglion cholinergic neurons (dvCNs), but not in *VAChT* positive sensory vesicle cholinergic neurons (svCNs). Scale bar, 50μm.

c: Expression of a dominant negative *COE* gene abolishes *VAChT* expression. *VAChT* reporter expression in the A9.30 lineage (dotted outline) at 15.5 hpf in animals expressing a control construct (*FGF8/17/18>lacZ*, top panel; 89/100 embryos express *VAChT*) and expressing *FGF8/17/18>COE::WRPW* (middle panel), resulting in loss of *VAChT* expression (82/100 animals show such loss) and an apparent conversion to non-neural ependymal cells (Supplementary Fig. 10). The *FGF8/17/18* driver is expressed in the A9.30 lineage, which gives rise to 4 of the 5 cholinergic MNs of the larva, as indicated with a co-electroporated *FGF8/17/18>H2B::mCherry* construct. The *VAChT*-positive MN outside the lineage was not affected by *FGF8/17/18>COE::WRPW* since it does not express *FGF8/17/18*. Electroporation of *FGF8/17/18>Ets::WRPW* [43] converts the entire A9.30 lineage into cholinergic neurons, as predicted [44], demonstrating the specificity of the *COE::WRPW* effect. Scale bar, 50μm.

d: Misexpression of *COE* in palp neurons induces *VAChT* expression (56/100 animals). Spontaneous *VAChT* expression is observed in only 3/100 control embryos co-electroporated with a control construct (right panel). Scale bar = 25μm.
e: *Ciona* COE, expressed using the *hsp-16.2* promoter, rescues the *acr-2* and *unc-17* expression defects of *unc-3* mutant animals (*unc-17* expression is unaffected by *unc-3* in VC-type neurons; Figure 1c). COE expression was induced at the first larval stage and animals were photographed one day later. Quantification of data is shown in Supplementary Fig. 12. Scale bar = 20μm.

Table 1

Motor neuron genes analyzed for regulation by UNC-3

Category	Gene	UNC-3 dependent	COE motif	MN expression in wild type					
				DA	DB	VA	VB	AS	Several MNs [1]
ACh pathway genes and ACh receptors	*unc-17/cha-1*	yes	yes	x	x	x	x	x	
	cho-1	yes	yes	x	x	x	x	x	
	ace-2	yes	yes	x	x	x	x	x	
	acr-2	yes	yes	x	x	x	x		
	acr-5	yes	yes		x		x		
	acr-14	yes	yes	x	x		x	x	
	acr-15	yes	yes	x	x	x	x	x	
	acr-16	yes	no		x				
	unc-63	no	no	x	x		x	x	
	gar-2	yes	no						x
Channel proteins(neurotransmitter receptors, ion channels, gap junction etc.)	*trp-1*	yes	yes	x	x	x	x		
	nca-1	yes	yes	x	x	x	x		
	unc-8	yes	yes	x	x	x	x		
	del-1	yes	yes			x	x		
	inx-12	yes	yes			x	x		
	acc-4	no	yes	x	x	x	x		
	gbb-1	yes	yes						x
	kvs-1	yes	yes						x
Secreted signaling proteins	*dbl-1*	yes	yes	x	x	x	x		
	tig-2	yes	no	x	x	x	x	x	
	nlp-21	yes	yes	x	x	x	x	x	
Axon guidance	*unc-40*	no	no	x	x	x	x	x	
	unc-129	yes	yes	x	x				
	max-1	no	yes	x	x			x	
GPCR	*F39B2.8*	yes	yes	x	x	x	x		

Category	Gene	UNC-3 dependent	COE motif	MN expression in wild type					
				DA	DB	VA	VB	AS	Several MNs [1]
	dop-1	yes	yes						x
Tetraspanin	*tsp-7*	yes	yes	x					x
IgSF	*rig-4*	yes	no	x					
Unknown	*F29G6.2*	yes	no	x	x				
	F55C12.4	yes	no	x	x		x	x	
Transcription factors	*unc-4*	yes	yes	x		x			
	vab-7	yes	yes		x				
	ceh-12	yes	yes				x		
Newly identified MN genes, based on presence of COE motifs	*T13G4.3*	yes	yes						x
	twk-7	no	yes						x
	twk-13	yes	yes						x
	twk-40	yes	yes						x
	twk-43	yes	yes						x
	lgc-55	not determ.	yes						x
	acr-21	not determ.	yes						x

This Table does not summarize the expression of panneuronal markers in *unc*-3 mutants, which are all unaffected (Fig. 2B), as expected from the analysis of other terminal selector transcription factors.

[1] not analyzed which cholinergic MN subtype expresses the reporter.

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