ZAG-1/ZEB prevents the expression of repressors and protects neuronal cell fate in *Caenorhabditis elegans*

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Summary statement: Transcriptional repressors regulate binary fate choices through reciprocal inhibition during terminal neuronal differentiation. Specifically, ZEB family transcription factor indirectly promotes fate specification of sensory neuron by inhibiting TEA domain-containing repressor.

Abstract

Terminal differentiation generates the specialized structures and functions that allow postmitotic cells to acquire their distinguishing characteristics. This process is thought to be controlled by transcription factors called "terminal selectors" that directly activate a set of effector genes. Using the mechanosensory touch receptor neurons (TRNs) in *Caenorhabditis elegans*, we extend this concept by identifying the ZEB family transcriptional repressor ZAG-1 as a non-selector regulator of cell fate. ZAG-1 does not activate TRN genes directly but promotes TRN differentiation by preventing the expression of the TEA domain transcription factor EGL-44 and its binding partner EGL-46, which are potent repressors of TRN fate. The EGL-44/EGL-46 complex normally blocks the activation of TRN genes by the terminal selectors, UNC-86 and MEC-3, in the multidendritic nociceptor FLP neurons, whose fate also requires UNC-86 and MEC-3. Thus, TRN and FLP neurons share the same terminal selectors and a common ground state. Diversification beyond this ground state is controlled by ZAG-1 and EGL-44, which form a negative feedback loop through the reciprocal inhibition to regulate the choice between the two neuronal fates.

Introduction

The terminally differentiated state or cell fate of neurons distinguishes them from other neurons through specialized features and functions and the expression of effector genes, termed as "terminal differentiation genes" (Hobert, 2011). Although individual effector gene may not be expressed in a cell type-specific manner, the collective expression of a battery of terminal differentiation genes serve as a signature for the cell fate. For example, the cell fate of mammalian photoreceptor is defined by the expression of over 600 genes, including photoreceptor-specific retinol-binding proteins, rhodopsin, G protein Gnat1, phosphodiesterase, and others that are directly involved in light detection (Blackshaw et al., 2001; Hsiau et al., 2007). The expression of the terminal differentiation genes is often activated by transcription factors called "terminal selectors" (Garcia-Bellido, 1975; Hobert, 2011) that act alone or in combination and bind to common *cis*-regulatory elements in the terminal differentiation gene.

Terminal selectors or transcriptional activators are not, however, sufficient to specify neuronal fates. Neuron type-specific transcriptional repressors are an important type of non-selector regulators that sculp the terminal differentiation gene expression profiles by repressing effector genes that do not contribute to a given fate (Kerk et al., 2017; Mitani et al., 1993; Pflugrad et al., 1997; Vallstedt et al., 2001; William et al., 2003). Such repressors appear to be particularly important since the same terminal selectors are often expressed in several distinct types of neurons and promote their differentiation into distinct fates (Hobert, 2016). Shared selectors can presumably work in coordination with other neuron type-specific regulators to generate a unique combinatorial code for each cell fate. A recent study suggests that combinations of repressors can be employed to antagonize the activity of terminal selectors on selective effector genes (Kerk et al., 2017), thus diversifying the selector function. The use of

such repressors presents a problem: how can cells prevent the inappropriate expression and action of repressors, so that the correct cell fate results?

To address this problem, we have studied the choice of cell fate that generates either the touch receptor neurons (TRN) or the FLP neurons in *C. elegans*. The six TRNs are mechanosensory neurons that detect gentle mechanical stimuli along the body (Chalfie and Sulston, 1981), and the two FLP neurons are multidendritic nociceptors that sense harsh touch, noxious temperature, and humidity (Chatzigeorgiou and Schafer, 2011; Chatzigeorgiou et al., 2010; Kaplan and Horvitz, 1993; Russell et al., 2014). The heterodimer of the POU homeodomain transcription factor UNC-86 and the LIM homeodomain transcription factor MEC-3 acts as a terminal selector that promotes TRN cell fate (Xue et al., 1993). Specifically, the heterodimer activates a set of TRN terminal differentiation genes (the mechanosensory channel genes *mec-4* and *mec-10*, the tubulin genes *mec-7* and *mec-12*, the tubulin acetyltransferase gene *mec-17*, and others) by binding to conserved regulatory elements in their proximal promoters (Duggan et al., 1998; Zhang et al., 2002).

In addition to directing the differentiation of the TRNs, MEC-3 and UNC-86 are expressed in and needed for the differentiation of two pairs of multidendritic neurons, the embryonic and anterior FLP neurons and the postembryonic and posterior PVD neurons. The expression of *unc-86* and *mec*-3 in these cells does not lead to the normal expression of many TRN terminal differentiation genes, resulting in cells whose fate is very different from the TRNs (Finney and Ruvkun, 1990; Way and Chalfie, 1988). Our previous work suggested that the TEA domain transcription factor EGL-44 and the zinc-finger protein EGL-46 in FLP neurons prevented these cells from acquiring the TRN fate (Wu et al., 2001). Thus, the active inhibition of TRN fate by EGL-44 and EGL-46 in FLP neurons distinguished these two types of neurons

that express common selector genes. However, whether EGL-44 and EGL-46 also activate a FLP-specific genetic program is unclear.

In this paper, we extend this model of TRN and FLP neuronal determination, by asking whether other transcription factors help promote the TRN fate and cause the cells to differentiate away from the FLP fate. We screened for the effects on TRN development of the loss of 392 transcription factors using RNA interference and identified ZAG-1 as a positive TRN fate regulator. The Zn-finger homeodomain protein ZAG-1 is expressed in TRNs but not FLP neurons. ZAG-1 promotes the TRN fate not by directly activating the TRN terminal differentiation genes but by preventing *egl-44* and *egl-46* expression, which blocks the activation of TRN genes by UNC-86/MEC-3. In FLP neurons, EGL-44/EGL-46 complex simultaneously inhibits the TRN genes and activates FLP genes. EGL-44/EGL-46 also represses the expression of *zag-1* in FLP neurons, and this negative feedback loop establishes a bistable switch between TRN and FLP fates. Our work suggests that UNC-86/MEC-3 serves as a ground-state selector resulting in a common state in both TRNs and FLP neurons, and that individual fates are subsequently controlled by ZAG-1 and EGL-44/EGL-46 acting in a bistable switch. In particular, ZAG-1 regulates the choice of cell fate by inhibiting fate inhibitors and safeguarding the activation of terminal differentiation genes.

Results

An RNAi screen for transcription factors affecting TRN cell fate

The anterior ALML and ALMR neurons and the posterior PLML and PLMR neurons are two pairs of bilaterally symmetric TRNs generated in the three-fold embryo, i.e., late in embryonic development. Two other TRNs, the AVM and PVM neurons, arise post-embryonically (Chalfie and Sulston, 1981; Sulston and Horvitz, 1977). To search systematically

for transcription factors specifying TRN fate, we knocked down the expression of transcription factor genes using RNAi and looked for animals that no longer expressed *mec-17p::RFP*, a TRN marker (see Materials and Methods for details). Using RNAi against *unc-86* as a positive control, we tested various genetic backgrounds that were previously found to enhance the effects of RNA interference and found that *eri-1; lin-15B* mutants had the highest penetrance for the loss of RFP expression. About 80% of these animals did not express RFP in either of the two ALM neurons when treated with *unc-86* RNAi (Figure 1A and B). The posterior TRNs (PLM neurons) were less affected by the RNAi treatment (Figure 1B), as seen previously (Calixto et al., 2010). Therefore, we focused on the disappearance of *mec-17p::RFP* expression in the ALM neurons in the screen.

Among the 443 bacterial clones expressing dsRNA against 392 transcription factors and associated proteins (Table S1), we identified 14 genes that were required for the expression of TRN markers (Figure 1). Four genes (*unc-86*, *mec-3*, *ldb-1*, and *ceh-20*) were previously known to affect the expression of TRN terminal differentiation genes (Cassata et al., 2000; Way and Chalfie, 1988; Zheng et al., 2015). We examined null mutants for all the remaining ten genes but could only confirm the loss of *mec-17p::RFP* expression in *zag-1* mutants.

We were surprised that RNAi against nine genes affected TRN expression through six rounds of testing but loss-of-function mutations in these genes did not. These false positive results are unlikely to result from the specific genetic background of the RNAi strain, since mutation of several of the genes (*zip-4*, *hmbx-1*, and *nhr-119*) in *eri-1; lin-15B* animals did not affect TRN fate. Activating the RNAi pathway non-specifically by RNAi against GFP in those triple mutants (e.g. *zip-1; eri-1; lin-15B* mutants) did not cause the loss of *mec-17p::RFP* expression either. Therefore, we reason that the discrepancy between the RNAi and mutant

phenotypes was likely due to mistargeting of the dsRNAs (this problem was also encountered by Poole et al. (2011) in an RNAi screen for genes specifying the ASE neurons).

ZAG-1 is required for the expression of TRN fate markers independently of MEC-3

zag-1 encodes the sole *C. elegans* ZEB transcription factor. Human ZEB transcription factors, which like ZAG-1 have a homeodomain flanked by clusters of C2H2-type zinc fingers, induce the epithelial to mesenchymal transition (EMT) and are essential for normal embryonic development (Vandewalle et al., 2009). Mutations in human ZEB genes cause defects in neural crest development and are linked to malignant tumor progression (Vandewalle et al., 2009). *C. elegans zag-1* prevents the posterior post-embryonic TRN neuron PVM from adopting the morphology of the multidendritic nociceptor neuron PVD, since a hypomorphic *zag-1* allele (*zd86*) led to the development of dendritic arbors in PVM (Smith et al., 2013). Here, we found that a *zag-1* null mutation (*hd16*) led to developmental arrest at the L1 stage (AVM and PVM do not arise until late in the L1 stage) and the loss of expression of all the tested TRN fate markers in ALM and PLM neurons (Figure 2A-C). Thus, the role of ZAG-1 is important for general TRN fate specification.

We next tested the genetic interaction between *zag-1* and *mec-3* and found that the *zag-1 (hd16)* null allele did not affect the expression of the *mec-3* using a transcriptional reporter (Figure 2A), quantitative mRNA measurements with single molecule fluorescent *in situ* hybridization (smFISH; Figure 2B), or MEC-3::GFP translational fusions (Figure 2C). In addition, expression of *zag-1(+)* from the *mec-3* promoter restored the expression of the TRN fate marker *mec-17p::RFP* in *zag-1* mutants, suggesting that ZAG-1 induces the expression of TRN terminal differentiation genes cell-autonomously. Since *mec-3* expression is completely

dependent on *unc-86*, we expected and found that the expression of *unc-86* was not changed in *zag-1* mutants.

Using a fosmid-based GFP translational fusion, we found that *zag-1* was expressed in the six TRNs but not FLP and PVD neurons (Figure 2D), and the expression of *zag-1* in TRNs was not affected by mutations in *mec-3* (Figure 2E). Therefore, *zag-1* and *mec-3* are transcriptionally independent of each other. We also failed to find any physical interaction between ZAG-1 and MEC-3 in yeast two-hybrid assays (Figure S1). Together, our results suggest that ZAG-1 promotes TRN fate independently of the previously identified TRN fate determinants UNC-86 and MEC-3; the expression of the three transcription factors only overlap in the TRNs and thus can form a unique combinatorial code for TRN fate.

Smith *et al.* (2013) found that another conserved transcription factor AHR-1 (aryl hydrocarbon receptor) controls the differentiation of the anterior post-embryonic TRN neuron AVM; in *ahr-1* null mutants, AVM cells adopted a PVD-like multidendritic shape. We found that although expressed in all six TRNs, *ahr-1* is only required for the expression of TRN markers in AVM neurons but not in the other TRNs (ALMs, PLMs, and PVM), suggesting that AHR-1 plays a subtype-specific role in TRN fate specification (Figure S2A-C). Thus, our findings disagree with the hypothesis presented by Smith *et al.* (2013) that AHR-1 and ZAG-1 function in parallel to specify TRN morphology in the AVM and PVM, respectively. Instead, ZAG-1 is required for TRN fate adoption in general, whereas AHR-1 functions only in AVM. Moreover, Smith *et al*. (2013) found that AHR-1 specified TRN fate in AVM by regulating *mec-3*, whereas we found that ZAG-1 promoted TRN fate independently of *mec-3*.

ZAG-1 promotes TRN fate by suppressing TRN fate inhibitors EGL-44/EGL-46

The TEA domain transcription factor EGL-44 and the Zn-finger protein EGL-46 inhibited the TRN fate in the FLP neurons (Wu et al., 2001). These proteins appeared to work together to regulate gene expression and physically interacted in yeast two-hybrid assays (Figure S3; the EGL-44 and EGL-46 complex is hereafter abbreviated as EGL-44/EGL-46). Both genes were normally expressed in the FLP neurons, and the expression of *egl-46* was dependent on *egl-44* (Figure 3A and B). Our lab previously found and we confirmed that mutations in *egl-44* and *egl-46* caused the ectopic expression of *mec-17p::GFP* and other TRN reporters in FLP neurons (Figure 3A and C; Table S2). *egl-44* and *egl-46* were not expressed in differentiated TRNs (Figure 3A and B).

Since *zag-1* was selectively expressed in TRNs but not FLP neurons, we tested if ZAG-1 promoted the TRN fate by preventing the activation of EGL-44/EGL-46 in these cells. Consistent with this hypothesis, GFP reporters for both *egl-44* and *egl-46* were ectopically expressed in the TRNs in *zag-1* mutants (Figure 3D). In addition, *egl-44* mRNA, as measured by smFISH, was increased in *zag-1* TRNs, an indication that ZAG-1 transcriptionally repressed *egl-44* (Figure 3E). Importantly, *egl-44* and *egl-46* are epistatic to *zag-1*, since mutations in them restored the expression of TRN fate markers in *zag-1*-deficient animals (Figure 3C and Table S2). Thus, the TRN cell fate did not require ZAG-1 in the absence of EGL-44/EGL-46. Instead, ZAG-1 promoted the TRN fate through a double inhibition mechanism by preventing the expression of the TRN fate inhibitors *egl-44* and *egl-46*.

We also found that ZAG-1 loss affected general neurite growth and guidance in TRNs, since *egl-44; zag-1* and *zag-1; egl-46* mutants showed shortened and misguided TRN neurites. These results extend those of Smith *et al.* (2013), who found that ZAG-1 inhibited dendritic

growth in PVM neurons, and are consistent with the function of ZAG-1 in regulating axon guidance in other neurons (Clark and Chiu, 2003; Wacker et al., 2003).

Misexpression of ZAG-1 converts FLP neurons into TRN-like cells

To address whether *zag-1* could affect FLP differentiation, we misexpressed *zag-1* in FLP neurons using the *mec-3* promoter. This misexpression led to the expression of *mec-17p::GFP* and other TRN markers (Figure 4A and Table S2) and diminished expression of *egl-44* and *egl-46* (Figure 4A and B) in FLP neurons. These data suggest that misexpressed ZAG-1 converts FLP neurons to a TRN-like fate by inhibiting *egl-44* and *egl-46*. The expression of *mec-3p::zag-1* transgene also activated TRN markers in PVD neurons (Figure 4A), which is consistent with previous findings that ZAG-1 prevents PVM neurons from adopting a PVD-like morphology (Smith et al., 2013). Thus, ZAG-1 not only prevents PVM neurons from taking on the PVD fate but can also turn PVD neurons into TRN-like cells. Since *egl-44* was not expressed in PVD neurons, the misexpressed ZAG-1 presumably directs this conversion of cell fate by inhibiting some unidentified factor(s) that normally suppresses the TRN fate in PVD neurons.

We also expressed *ahr-1* from the *mec-3* promoter and found that misexpressed AHR-1 could activate TRN fate markers in PVD but not FLP neurons (Figure S2D-E). Overexpression of AHR-1 in TRNs also caused morphological defects, such as the growth of an ectopic ALM posterior neurite (Figure S2D), which does not occur when ZAG-1 was overexpressed. These results further support the hypothesis that ZAG-1 and AHR-1 have different functions.

EGL-44/EGL-46 prevents *zag-1* expression in FLP neurons

Given the mutually exclusive patterns of *zag-1* and *egl-44/egl-46* expression in FLP neurons and TRNs, we next tested whether EGL-44/EGL-46 regulated *zag-1* expression.

Mutations in *egl-44* and *egl-46* resulted in ectopic expression of a *zag-1::EGFP* reporter in FLP neurons. Misexpression of *egl-44* from the *mec-3* promoter is sufficient to suppress the TRN fate, because it activates the endogenous *egl-46*, a cofactor that is required for EGL-44 functions (Figure 5B and D; Wu et al., 2001). We found that EGL-44 misexpression reduced, but did not completely eliminate, the expression of *zag-1* in TRNs (Figure 4B and C). Therefore, the positive TRN fate regulator ZAG-1 and the negative regulator EGL-44/EGL-46 reciprocally inhibit each other's expression, and their expression must be regulated, so one or the other predominates.

Simultaneous misexpression of both EGL-44 and ZAG-1 from the *mec-3* promoter, however, led to the activation of TRN marker *mec-17p::GFP* in all *mec-3*-expressing neurons (TRNs, FLP, and PVD; Figure 4E), because ZAG-1-mediated repression of endogenous *egl-46* blocked the effects of EGL-44. In fact, supplying EGL-46 using the *mec-3* promoter removed the dominance of ZAG-1 and turned off the expression of TRN markers again (Figure 4F). Moreover, expression of an *egl-46*::GFP reporter was reduced by ZAG-1 despite the presence of misexpressed EGL-44 (Figure 4B), suggesting that ZAG-1 can repress *egl-46* transcription both through *egl-44* and independently of *egl-44*. Functionally, animals carrying the *uIs211[mec-3p::egl-44]* transgene were completely insensitive to gentle touch; co-expression of *zag-1* from *mec-3* promoter could restore the sensitivity in animals where the TRN markers were reactivated but failed to do so when *mec-3p::egl-46* was expressed (Figure S3). Thus, the activity of EGL-44/EGL-46 complex predominates over ZAG-1 when both expressed from the same heterologous promoter in the same cell, leading to the suppression of TRN fate. The action of these competing inhibitors is not likely through direct interaction, since we did not observe any

physical interaction of ZAG-1 with either EGL-44 or EGL-46 or of MEC-3 with EGL-44, EGL-46, and ZAG-1 in yeast two-hybrid assays (Figure S4).

EGL-44/EGL-46 and ZAG-1 regulate a switch between FLP and TRN fates

We next investigated how EGL-44/EGL-46 and ZAG-1 regulated FLP fate using *sto-5* (Topalidou and Chalfie, 2011) and several other genes (*dma-1*, *bicd-1*, and *flp-4*) that were expressed in FLP neurons (Aguirre-Chen et al., 2011; Kim and Li, 2004; Liu and Shen, 2011) but not the TRNs (Figure 5A). Expression of all four genes in the FLP neurons depended on EGL-44 and EGL-46 (Figure 5B and C). Moreover, the expression of the FLP and TRN markers were mutually exclusive; FLP neurons in *egl-44* and *egl-46* mutants or animals carrying *mec-3p::zag-1* transgene never showed mixed expression of the FLP- and TRN-specific genes (Figure 5E). Mutations in *zag-1* or the misexpression of *egl-44* in TRNs activates the FLP markers in addition to turning off the TRN markers (Figure 5B and S5), and we did not observe any mixed expression of both types of markers under those conditions (Figure 5E). Since FLP markers were not expressed in *egl-44; zag-1* double mutants, our results suggest that EGL-44/EGL-46 not only repressed the TRN fate but also promoted the FLP fate. Thus, the action of EGL-44/EGL-46 and ZAG-1 results in two mutually exclusive fates.

This bistable switch affected neuronal morphology as well as transcriptional reporters. For example, ALM neurons that assumed a FLP-like fate because of *egl-44* misexpression grew an ectopic posterior neurite (Figure S6), and *zag-1*-expressing and thus TRN-like FLP and PVD neurons had far fewer dendritic branches than the wild-type cells (Figure S6). This latter result is consistent with ZAG-1's role in preventing PVM from adopting a PVD-like morphology (Smith et al., 2013).

dma-1, *bicd-1*, and *flp-4* were also expressed in PVD neurons, suggesting that FLP and PVD share a common genetic program. The expression of these PVD markers was blocked by the misexpression of ZAG-1 in PVD neurons, suggesting that ZAG-1 represses the unknown PVD fate selector. On the other hand, the *sto-5* reporter was expressed in FLP but not PVD neurons, indicating difference in the transcriptome of the two cell types (Figure S7).

The activation of the FLP and PVD markers, like the TRN markers, required UNC-86 and MEC-3, which are the common terminal selectors for the three fates (Figure 5E and S5B). We identified one common terminal differentiation gene, *mec-19*, which was expressed in TRN, FLP, and PVD neurons at similar levels (and no other cells); its expression depended on UNC-86 and MEC-3 but was not affected by the loss of EGL-44/EGL-46 and ZAG-1 (Figure 5D-E). Thus, *unc-86* and *mec-3* serve as the "ground-state selectors" that promote a common ground states shared by the three types of neurons, and *mec-19* is a marker for this ground state. Subsequently, *zag-1* and *egl-44* act as "modulators" that shift the ground state towards specific fates. Therefore, the development of a particular fate requires the activities of both the ground-state selectors and the fate-restricting modulators.

EGL-44/EGL-46 inhibits the expression of TRN genes by competing with UNC-86/MEC-3 for DNA binding and by suppressing ALR-1

We next investigated the mechanism, by which EGL-44/EGL-46 prevented the expression of TRN terminal differentiation genes. Although we found two UNC-86/MEC-3 binding sites required for the expression of a minimal TRN-specific promoter (a 184-bp *mec-18* promoter) in TRNs, we failed to identify any additional, discrete *cis*-regulatory element that mediated its repression in FLP neurons (Figure S8). Thus, EGL-44/EGL-46 seems unlikely to suppress TRN markers *via* distinct, repressive elements. We then tested the possibility that EGL-

44/EGL-46 acts through the UNC-86/MEC-3 binding site, since EGL-44 belongs to the TEA domain class transcription factors, which recognize DNA sequences similar to the UNC-86/MEC-3 binding site (Figure 6A) (Jiang et al., 2000; Zhang et al., 2002). EGL-44 and the EGL-44/EGL-46 complex bound to previously identified UNC-86/MEC-3 motifs in the *mec-4*, *mec-7*, *mec-17*, and *mec-18* promoters in electrophoretic mobility shift assays (Figure 6B and C). These results suggest that EGL-44 mediates the direct contact with the TRN promoters and EGL-46 acts as a corepressor. More importantly, EGL-44/EGL-46 outcompeted UNC-86/MEC-3 for the binding to these same sequences (Figure 6C), suggesting that the *cis*-regulatory sites normally bound by UNC-86/MEC-3 biochemically prefer EGL-44/EGL-46. Therefore, EGL-44/EGL-46 in the FLP neurons may prevent the activation of TRN genes by occluding the UNC-86/MEC-3 binding sites essential for the expression of TRN fate.

However, how EGL-44/EGL-46 avoids inhibiting UNC-86/MEC-3 targets that are commonly expressed in FLP and TRNs is unclear. One such target is *mec-3* itself, whose activation and maintenance depend on two UNC-86/MEC-3 binding sites (Xue et al., 1992). We found that the two sites were not bound by EGL-44 (Figure 6B), which explains why *mec-3* expression is not affected by the presence of EGL-44/EGL-46 in FLP neurons. Comparing the *cis*-regulatory motif sequences on *mec-3* promoter with those on TRN-specific promoters, we found that the four nucleotides (positions 1 to 4 in Figure 6A) following the UNC-86/MEC-3 binding sequence showed significant divergence between EGL-44-binding and nonbinding sites. In particular, the EGL-44-binding sites all contain adenine at the fourth position and the nonbinding sites do not (Figure 6A). Changing this adenine to thymine eliminated the binding of EGL-44 to the site on *mec-4* promoter (Figure 6B). However, converting other nucleotides to adenine at the fourth position was not sufficient to enable the binding of EGL-44 to *mec-3*

promoter motifs and coordinated change of the nucleotides on the first and second positions are needed to evoke EGL-44 binding (Figure 6B). Similar results were also obtained for the UNC-86/MEC-3 target gene *mec-10*, which is expressed in both TRN and FLP cells (Huang and Chalfie, 1994). The UNC-86/MEC-3 site on *mec-10* promoter was not bound by EGL-44 and changing the nucleotides on the first and fourth position enabled EGL-44 binding. Thus, our results suggest that EGL-44/EGL-46 can differentiate TRN/FLP common genes from TRN-specific genes *via* the *cis*-regulatory sequences in their promoters.

We next tested whether converting the EGL-44 binding site to a nonbinding site in a TRN promoter could prevent the suppression by EGL-44/EGL-46 in FLP neurons *in vivo*. Contrary to our expectation, a *mec-4* promoter reporter harboring the adenine-to-thymine change at the fourth position was not activated in FLP neurons, although its TRN expression was preserved. In the 184-bp *mec-18* promoter, changing the four nucleotides following the UNC-86/MEC-3 binding sequence from ACCA to CTAT completely abolished EGL-44 binding *in vitro*; but the mutant reporter was only weakly expressed in ~30% of FLP neurons and remained silenced in the rest ~70% (Figure S9). In addition, creating an EGL-44-binding site in the *mec-3* promoter by mutating the regulatory motif did not suppress *mec-3* expression in FLP neurons (Figure S9A). The discrepancy between the *in vitro* and *in vivo* results suggests that the lack of EGL-44 binding to TRN differentiation genes *per se* is not sufficient to distinguish TRN fate from the TRN/FLP ground state. In addition to directly binding to the *cis*-regulatory elements of TRN promoters, EGL-44/EGL-46 may also inhibit TRN genes by activating or repressing the expression of other *trans*-acting factors.

One such factor may be ALR-1, which is an ortholog of human Arx and Drosophila *aristaless* and is needed for the robust differentiation of TRN fate (Topalidou et al., 2011). Loss

of *alr-1* variably reduced but did not eliminate the expression of TRN markers in the TRNs in wild type and strongly eliminated the ectopic expression of TRN markers in FLP neurons in *egl-44* and *egl-46* mutants (Figure 6D and S10A). This difference between reduction and elimination may result from the fact that FLP neurons have lower *mec-3* expression than the TRNs (Topalidou et al., 2011) and thus a stronger need for ALR-1 to activate TRN markers. The observation that *alr-1* is epistatic to *egl-44* and *egl-46* suggested that ALR-1 is a downstream effector suppressed by EGL-44/EGL-46. Indeed, a fosmid-based *alr-1* translational reporter, which was normally expressed in TRNs but not FLP neurons, became de-repressed in FLP cells in *egl-44* and *egl-46* mutants (Figure 6D and S10A).

The lack of ALR-1 and the lower level of *mec-3* in wild-type FLP neurons may explain the inactivation of the mutated TRN fate reporters that cannot be bound by EGL-44/EGL-46. Supporting this hypothesis, forced expression of ALR-1 in FLP neurons ectopically activated even the wild-type TRN fate reporters (Figure 6D) (Topalidou et al., 2011), suggesting that ALR-1 not only promotes TRN fate but can also overcome the direct suppression from EGL-44/EGL-46 on the TRN genes. This ability of ALR-1 may result from ALR-1 upregulating *mec-3* (Topalidou et al., 2011) and directly interacting with TRN promoters (ChIP-seq data; Figure S10B). Therefore, EGL-44/EGL-46 inhibits TRN-specific genes by both occupying the UNC-86/MEC-3 site in the TRN promoters and suppressing TRN fate-promoting transcription factor ALR-1. Consistent with this model, overexpression of MEC-3 in FLP neurons could overcome EGL-44/EGL-46-mediated inhibition and activate the TRN program (Topalidou and Chalfie, 2011) presumably by both retaking the UNC-86/MEC-3 sites and by activating *alr-1*, which is a *mec-3*-dependent gene (Topalidou et al., 2011).

Spatial and temporal expression of *zag-1* and *egl-44* are mutually exclusive

Given the mutual inhibition of *zag-1* and *egl-44* in the FLP neurons and the TRNs, we asked whether the two genes were generally expressed in different cells in the nervous system. Using the neurotransmitter maps for glutamatergic, cholinergic, and GABAergic neurons (Gendrel et al., 2016; Pereira et al., 2015; Serrano-Saiz et al., 2013), we found that, in addition to TRNs, *zag-1* was expressed in the AIB, AIM, AIN, AIZ, AVA, AVB, AVD, AVE, AVG, AVK, AVL, M4, M5, RIA, RIB, RIF, RIG, RIM, RIV, RMD, RME, RMF, RMH, SIA, and SMD neurons in the head, all the DD, VD, and VC neurons in the ventral cord, and the DVA, DVB, LUA, PDA, PVC, PVP, PVQ, PVR, and PVT neurons in the tail. *zag-1* is also expressed in the serotonergic HSN neurons. In comparison, *egl-44* expression was much more restricted, being found only in the FLP, ADL, and SAB neurons, as well as a few VA and VB motor neurons. Moreover, *egl-44* was widely expressed in hypodermis, pharynx, and intestine, whereas *zag-1* expression was absent in these tissues. We also constructed an *egl-44::RFP* reporter and cross it with *zag-1:EGFP* and did not observe overlapping expression in any cell (Figure S11A). The above data support the hypothesis that *zag-1* and *egl-44* expression are mutually exclusive in the nervous system and throughout all tissues of the animal. Given the mutual inhibition of EGL-44 and ZAG-1 in the TRNs/FLPs, we expected that the loss of *zag-1* might affect *egl*-44 expression more broadly. We did not, however, observe a systematic upregulation of EGL-44::GFP expression in the nervous system in either the *zag-1(zd86)* hypomorphic mutants or the arrested L1 animals of *zag-1(hd16)* null mutants (Figure S11B and C). Similarly, tissues like hypodermis, pharynx and intestine did not gain *zag-1* expression upon the loss of *egl-44* either, suggesting that, in addition to the reciprocal inhibition, other activating signals are needed to create the expression pattern of the two transcription factors.

A few neurons expressed both *zag-1* and *egl-44* but did so at different times. *egl-44* and *egl-46* were expressed in the precursors of the postembryonic TRNs (the AVM and PVM neurons) and persisted in these cells for a few hours after their generation to promote cell cycle exit (Feng et al., 2013; Wu et al., 2001), but they were not expressed in terminally differentiated AVM and PVM. These differentiated cells expressed *zag-1*, which promoted the TRN fate. Similarly, *egl-44* and *egl-46* were transiently expressed in the early embryos in HSN neurons before they migrated and differentiated (Wu et al., 2001) (Figure S11D), whereas *zag-1* was expressed in the terminally differentiated HSN neurons in adults and was required for the activation of the HSN fate marker *tph-1p::GFP* (Figure S11E and F).

Discussion

RNAi screen is a systematic method to identify genes involved in cell fate determination

We demonstrate here that a systematic RNAi screen of a library of transcription factors can be used to identify neuronal cell fate regulators, particularly genes whose mutations lead to lethality or sterility. Our previous forward genetic screens, which searched for viable mutants with touch-sensing defects, despite reaching saturation, only identified *unc-86* and *mec-3* as the TRN fate determinants (Chalfie and Au, 1989; Chalfie and Sulston, 1981). Using the RNAi screen, we not only recovered *unc-86* and *mec-3* blindly, but also identified *ceh-20*, *ldb-1*, and *zag-1* as genes required for the expression of TRN fate. These latter genes would not have been identified in our previous screens, because null mutations in them lead to early larval arrest. Moreover, our previous neuronal RNAi screen of nonviable genes would not have identified these genes either, because it only looked for postembryonic effects on touch sensitivity after TRN differentiation was completed (Chen et al., 2015). In contrast, the current RNAi screen

knocked down gene expression in the embryos and could identify genes whose silencing perturbed TRN fate specification.

Although previous studies of individual genes identified *ceh-20* (Zheng et al., 2015) and *ldb-1* (Cassata et al., 2000) as regulators of TRN fate, RNAi screens, such as we have done, provide a systematic, complementary approach to identify genes controlling neuronal differentiation. Importantly, through the screen, we identified ZAG-1 as a new TRN fate determinant and the third piece in a combinatorial code (UNC-86, MEC-3, and ZAG-1) that defines TRN fate. Although previous studies suggested that ZAG-1 regulates PVM morphological differentiation using a viable hypomorphic *zag-1* allele (Smith et al., 2013), our RNAi screen and the subsequent studies using a null allele found that ZAG-1 is essential for the acquisition of TRN fate more generally. Nevertheless, our screen failed to recover *ceh-13*, which is known to affect the TRN fate in ALM neurons (Zheng et al., 2015), suggesting the existence of false negatives. In addition, the bias of feeding RNAi towards the anterior TRNs led to the failure of recovering *egl-5*, whose loss only affects PLM differentiation (Zheng et al., 2015).

ZAG-1 prevents the inappropriate expression of repressors and safeguards cell fate determination

Terminal selectors (mostly transcriptional activators) play pivotal roles in inducing neuronal cell fate, but they are often shared by many different types of neurons. The employment of non-selector regulators (often transcriptional repressors) can help diversify the function of selectors by forming unique combinations for different cell fates. One well-characterized example is the diversification of motor neurons in *C. elegans*, during which neuron type-specific transcriptional repressors selectively antagonize selector-induced activation of effector genes to generate diverse gene expression profiles (Jung et al., 2010; Kerk et al., 2017; Pflugrad et al.,

1997; Winnier et al., 1999). Because the repressors potently block selector function, their expression must be tightly regulated to prevent inappropriate activation.

In this study, we found that neurons develop protective mechanisms to prevent the improper activation of repressors and to ensure the differentiation of the correct cell fate. Specifically, ZAG-1 prevents the expression of repressors EGL-44 and EGL-46 in the TRNs. Because the EGL-44/EGL-46 complex is a powerful inhibitor of TRN fate (its derepression in *zag-1* mutants or misexpression in TRNs can completely shut off the expression of TRN genes), the function of ZAG-1 is essential to ensuring the specification of TRN fate.

As a differentiation regulator, ZAG-1 is required for the adoption of TRN fate but does not directly bind to the *cis*-regulatory elements within TRN promoters and so does not act as a selector. Moreover, unlike the highly confined expression of terminal selectors and repressors (e.g. both *mec-3* and *egl-44* are expressed in few neurons), *zag-1* is expressed in many neurons in the head and tail ganglia and ventral cord motor neurons, as well as various muscles (this study and Wacker et al., 2003). Thus, ZAG-1, as a transcriptional repressor, may serve as a cell fate protector for many different neurons. Already ZAG-1 is found to be required for the fate specification of at least TRN, HSN, and PVQ neurons (this study and Clark and Chiu, 2003), although whether ZAG-1 also regulates HSN and PVQ fates by preventing repressor expression is unclear.

Diverse mechanisms of repressor function

Repressors affect effector gene expression in several ways. Repressors can act through discrete *cis*-regulatory elements that are close to but separate from the selector binding site on the promoter of effector genes (Kerk et al., 2017), and the mechanism of repression may involve histone modification and the recruitment of histone deacetylase (Winnier et al., 1999). Our study

suggests that the repressors may directly occupy the selector binding site, thus blocking the activation of effector genes. EGL-44/EGL-46 binds to the same *cis*-regulatory elements as UNC-86/MEC-3 but can outcompete UNC-86/MEC-3 for the binding to the same DNA sequence, suggesting that repressors can directly antagonize the physical association of selectors at effector genes.

Repressors that inhibit effector genes directly can also activate effector genes indirectly by inhibiting other repressors (Kerk et al., 2017). We found that in the decision between the FLP and TRN fates, EGL-44/EGL-46 simultaneously inhibits TRN genes and activate the FLP genes. But whether EGL-44/EGL-46 promotes the expression of FLP genes by repressing yet another repressor is unclear; this other repressor presumably prevents the activation of FLP genes by UNC-86/MEC-3.

All of these methods shape the final collection of effector genes that define a particular cell fate. ZAG-1, however, appears to act differently because it does not interact with effector genes directly (we did not find any UNC-86/MEC-3-induced effector gene that is directly repressed by ZAG-1). Instead, the only function of ZAG-1 in TRNs is to prevent the expression of repressors, including EGL-44/EGL-46 and possibly the unknown repressor that inhibits TRN fate in PVD neurons. Thus, ZAG-1 may be devoted to inhibiting the alternative FLP and PVD fates, while UNC-86/MEC-3 induces TRN fate. The uncoupling between the two modules suggests that they may have evolved independently.

Negative feedback loop controls binary fate choice and neuronal diversification

The repressors reported by Kerk et al. (2017) formed a hierarchy that regulated effector gene expression in motor neurons. In this study, however, we showed that repressors can also form, through mutual repression, bistable switches that can ensure proper cell differentiation.

The diversification of three types of sensory neurons (TRNs, FLP, and PVD neurons) that share the same selectors (UNC-86 and MEC-3) use a set of binary fate choices. The TRN versus FLP fate choice is controlled by the negative feedback loop between ZAG-1 and EGL-44, while the TRN versus PVD choice might be similarly controlled by mutual inhibition between ZAG-1 and an unknown repressor that helps specify PVD fate and represses TRN fate.

In the absence of both components of the switch (*egl-44; zag-1* mutants), both TRNs and FLP neurons expressed the TRN genetic program, suggesting that the ground state is a TRN-like fate and the selectors UNC-86/MEC-3 activate TRN genes by default. EGL-44/EGL-46-induced modification of the default genetic program gives rise to the FLP fate. Our results are different from the observation in motor neurons, where the loss of repressors leads to a "mixed ground state" that is not similar to any of the five motor neuron types (Kerk et al., 2017). This distinction may result because ZAG-1 only inhibits EGL-44 and does not directly regulate effector genes, whereas in motor neurons all repressors interact with effector genes.

The choosing of one of two alternative fates appears to be a common way to diversify neuronal types and subtypes during development (e.g., Mikeladze-Dvali et al., 2005; Sarin et al., 2007). In vertebrates, neuronal fate, especially neurotransmitter identity, is also subjected to such binary regulation (Lodato et al., 2014; Nakatani et al., 2007). The function of ZEB family transcription factors in regulating such binary cell fate choices appears to be evolutionary conserved. The Drosophila homolog of ZAG-1, Zfh1, promotes a GW motor neuron fate over an EW interneuron fate in the 7-3 neuroblast lineage, and its expression is suppressed by the steroid receptor family transcription factor Eagle in the EW interneuron (Lee and Lundell, 2007); whether Zfh1 also suppressed Eagle in GW motor neuron is unclear. In another example, the mutual antagonism between Zfh1 and another zinc-finger transcription factor, Lame duck,

regulates the decision between pericardial cell and fusion competent myoblast fates in the mesoderm (Sellin et al., 2009). Although a genetic interaction between Zfh1 and the Drosophila EGL-44, Scalloped, has not been reported, these results suggest that Zfh1 can form regulatory switches with other transcription factors. Mouse homologs of ZAG-1, ZEB1 and ZEB2, repress tissue differentiation during early embryogenesis, induce epithelial mesenchymal transition (EMT), and are essential for neural tube development (Vandewalle et al., 2009). Their roles in the specifying terminal cell fates, however, are unclear, because their knockout leads to embryonic lethality (Miyoshi et al., 2006).

Binary fate switches may enable the generation of neuronal diversity

Because the introduction of self-reinforcing, bistable switches can generate diversity within pre-existing neuronal fates, we envision that the extraordinary variety of neuronal types in the nervous system evolved from a few primitive neuronal fates through stepwise addition of binary switches. For example, since TRN, FLP, and PVD fates all require the same selectors, UNC-86 and MEC-3, they may be derived from a common ancestral fate. In fact, the existence of UNC-86/MEC-3 target genes like *mec-19*, which is expressed in all the FLP, PVD, and TRNs but not any other neuron, suggests such an ancestral fate.

One possible evolutionary derivation of these cells is that some of the ancestral TRN-like cells acquired the ability to express *egl-44* and *egl-46*. This expression led to the suppression of TRN genes, the activation of FLP genes, and the emergence of this new cell type. The fact that the ground state is a TRN-like state seems to support this hypothesis. Moreover, since *egl-44* is primarily expressed in non-neuronal tissues, mutations in the regulatory elements of *egl-44* might have allowed expression in some neurons. Thus, EGL-44 may have been co-opted to induce divergence among neurons that share a common fate, and this cell fate divergence is

subsequently stabilized by the establishment of a negative feedback loop between EGL-44 and ZAG-1.

Alternatively, a regulatory element in the *zag-1* gene, which was already present in the ancestral TRNs to repress *egl-44* and *egl-46*, may have been mutated to prevent its expression in some ancestral TRN cells. This loss led to the de-repression of *egl-44* and *egl-46* and the subsequent acquisition of the FLP fate. The widespread expression of *zag-1* in neurons suggests that it may be in general required for the differentiation of many neuronal fates, and selective loss of *zag-1* expression in some cells through changes in *cis*-regulatory sequences may prevent these cells from committing to particular fates and allowing them to adopt alternative ones.

Overall, we imagine that a limited number of ground-state selectors may first define a handful of shared, and subsequently a series of binary fate switches carry out further differentiation that modifies the ground state to generate a diverse array of terminal neuronal fates. The broad expression of ZAG-1 in the nervous system and the lack of increased EGL-44 expression in *zag-1* mutants suggests that ZAG-1 may form bistable regulatory loops with many different inhibitors. Moreover, the fact that ZAG-1 expression is only found in a subset of neurons that express the same selector supports the hypothesis that ZAG-1 may ensure diversification from a shared ground state through down-regulation of inhibitors; e.g. *zag-1* is expressed in 7/17, 6/15, and 3/11 classes of neurons that use UNC-86, UNC-3, and CEH-14 as a terminal selector, respectively (classification according to Hobert, 2016). Although several UNC-3-expressing neurons, including some motor neurons, express ZAG-1, none of the ventral cord motor neurons described by Kerk et al. (2017) do. We speculate that ZAG-1 may prevent expression of repressors, such as those found in the ventral cord neurons, in these other UNC-3-expressing cells. Finally, the broad expression of ZEB family transcription factors in the nervous

system of both Drosophila (Lai et al., 1991) and mice (Vandewalle et al., 2009) suggests possibly a conserved role for them in regulating binary fate choices and the generation of neuronal diversity.

Author Contributions

C.Z., F.Q.J., and B.L.T. performed experiments and analyzed the data. C.Z. and M.C. conceived the study and wrote the manuscript. J.W. performed the initial study about EGL-44 binding to TRN promoters. M.C. supervised the work and provided funding. F.Q.J. and B.L.T. contributed equally to the work.

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Materials and Methods

Strains, Constructs, and transgenes

C. elegans wild type (N2) and mutant strains were maintained as previously described (Brenner, 1974). Most strains were provided by the *Caenorhabditis* Genetics Center, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440), or the National BioResource Project of Japan. VH514, *zag-1(hd16)/unc-17(e113) dpy-13(e184)* was used as the balanced null allele for *zag-1* and *zag-1(zd86)* was used as a hypomorphic allele. For other genes identified from the RNAi screen (see below), we tested *zip-4(tm1359)*, *nhr-119(gk136908)*, *nhr-166(gk613)*, *nhr-159(tm2323)*, *egl-38(ok3510)/nT1[qIs51]*, *hmbx-1(ok3467)*, *fkh-2(ok683)*, *lin-*

40(ku285), *lin-40(s1506) unc-46(e177)/eT1*, *elt-6(gk723)*, and *elt-6(gk754)*. Other mutant alleles used in this study include *ahr-1(ju145)*, *egl-44(n1080)*, *egl-46(n1127)*, *mec-3(u184)*, and *unc-86(u5)*.

A 2.4 kb *mec-3* promoter, a 2.2 kb *zag-1* promoter, a 2.2 kb *bicd-1* promoter, 4.9 kb *dma-1* promoter, 3.1 kb *flp-4* promoter, 2.3 kb *sto-5* promoter, and 1.3 kb *mec-19* promoter were cloned from wild type (N2) genomic DNA into the Gateway pDONR221 P4-P1r vector. The genomic coding region of GFP, *zag-1*, *egl-44*, *egl-46*, *alr-1*, and *ahr-1* were cloned into Gateway pDONR221. The resulted entry vectors, together with pENTR-*unc-54*-3' UTR and the destination vector pDEST-R4-R3 were used in the LR reaction to create the final expression vectors. Gateway cloning was performed according to the manual provided by Life Technologies (Grand Island, NY).

DNA constructs TU#625 and TU#626 (Wu et al., 2001) contain translational GFP fusion of *egl-44* and *egl-46*, respectively, and were injected into animals to form reporters for the two genes, named as *uIs215[egl-44::GFP]* and *uEx927[egl-46::GFP]*. TU#924 contains a 400 bp *mec-18* promoter inserted into pPD95.75 between HindIII and BamHI sites, and this *mec-18p::GFP* construct was used as a template to create a series promoter variants shown in Figure S10 using the Q5 site-directed mutagenesis kit from New England Biolabs (Ipswich, MA).

Transgenes *zdIs5[mec-4p::GFP]* I, *muIs32[mec-7p::GFP]* II, *uIs31[mec-17p::GFP]* III, *uIs115[mec-17p::RFP]* IV, *uIs134[mec-17p::RFP]* V, and *uIs72[mec-18p::mec-18::GFP]* were used as fluorescent markers for the TRN cell fate. *uEx1104[Pbicd-1::GFP]*, *uEx1105[dma-1p::GFP]*, *uEx1106[flp-4p::GFP]*, and *uIs232[sto-5p::GFP]* served as FLP fate markers. *zdIs13[tph-1p::GFP]* and *vsIs97[tph-1p::DsRed2]* were used as HSN fate marker. *uIs22[mec-3p::GFP]* and *uIs152[mec-3p::RFP]* were used as *mec-3* transcriptional reporter; *uEx1007[mec-*

3p::mec-3::GFP] and *wgIs55[mec-3::TY1::EGFP::3xFLAG]* were used as *mec-3* translational reporter. *wgIs83[zag-1::TY1::EGFP::3xFLAG]*, *wgIs476[unc-86::TY1::EGFP::3xFLAG]*, *wgIs200[alr-1::TY1::EGFP::3xFLAG]*, *leEx1709[ahr-1::GFP]* and *uEx1107[mec-19p::GFP]* served as the reporters for *zag-1*, *unc-86*, *alr-1*, *ahr-1*, and *mec-19* respectively. *uIs211[mec-3p::egl-44]*, *uEx926[mec-3p::zag-1]*, and *uEx1027[mec-3p::alr-1]* were used for misexpression.

To perform cell identification, we crossed *wgIs83[zag-1::TY1::EGFP::3xFLAG]* and *uIs215[egl-44::GFP]* into the *otIs518[eat-4::SL2::mCherry::H2B]*, *otIs544[cho-1::SL2::mCherry::H2B]*, and *otIs564 [unc-47::SL2::H2B::mChopti]*, labeling glutamatergic, cholinergic, and GABAergic neurons (Gendrel et al., 2016; Pereira et al., 2015; Serrano-Saiz et al., 2013), respectively. We identified *zag-1* and *egl-44* expressing neurons, based on the position and neurotransmitter identity of the cells expressing GFP.

RNAi screen

RNAi screen was performed using a modified bacteria-feeding protocol previously reported (Kamath et al., 2003; Poole et al., 2011). We used the Ahringer RNAi library from Source Bioscience (http://www.lifesciences.sourcebioscience.com/) and the list of 392 RNAi clones targeting transcription factors were generated by searching WormBase WS238 using Gene Ontology terms related to "DNA binding" and "transcription factor activity" (see the complete list in Table S1). To perform the RNAi experiments, we seeded bacteria expressing dsRNA on NGM agar plates containing 6 mM IPTG and 100 µg/ml ampicillin. One day later, eggs from TU4429, *eri-1 (mg366); lin-15B (n744); uIs134[mec-17p::RFP]* animals were placed onto these plates; the eggs hatched and grew to adults at 20°C. The F1 progeny of these worms were scored for the expression of TRN markers at the second larval stage. RNAi clones were considered to be positive if more than 15% ($n > 20$) of the treated animals failed to show RFP

expression in the ALM neurons in at least two of the three replicate plates. Three initial rounds of screens were performed on all the 392 clones, and 14 clones were found positive in all the three rounds. Two more screening rounds were then conducted on these 14 RNAi clones, which were all confirmed to be positive. We sequenced the inserts of all positive clones to confirm the identity of the target genes.

Yeast two-hybrid assay

Yeast media and plates were prepared according to recipes from Clontech (Mountain View, CA) and yeasts were grown at 30°C. The yeast strain PJ69-4a (provided by Songtao Jia at Columbia University) used for the two-hybrid assays contains GAL1-HIS3, GAL2-ADE2, and GAL7-lacZ reporters. Vectors pGAD424 and pGBT9 (Clontech) were used to express proteins fused to the yeast activating domain (AD) and binding domain (BD), respectively. cDNA fragments of *mec-3*, *zag-1*, *ldb-1*, *egl-44*, and *egl-46* were cloned into the two-hybrid vectors either using restriction enzymes or with Gibson Assembly (NEB).

Combinations of the AD or BD vectors were co-transformed into yeast using the Frozen-EZ II kit from Zymo Research (Irvine, CA) and using empty vectors as negative controls. Growth assays were performed by growing individual colonies overnight in selective media lacking tryptophan and leucine. Cultures were then diluted to let OD600 become 0.5, and 10 μl of a further 1:10 diluted culture were spotted onto plates lacking histidine to test the expression of the HIS3 reporter. Plates were imaged after 2 days of growth. Liquid β-galactosidase assays were performed using the Yeast β-Galactosidase Assay Kit (Thermo Scientific, Rockford, IL).

Electrophoretic mobility shift assay (EMSA)

Recombinant GST::EGL-44 proteins were produced in *E. coli* BL21 (DE3) using the expression vector pGEX-6p-1 (Amersham Pharmacia Biotech, UK) and purified using affinity chromatography columns filled with Glutathione Sepharose 4B beads (Amersham). EGL-44 was cleaved off the column using PreScission Protease (Amersham). EGL-46 was expressed using the pET32a vector (Novagen, Madison, WI) and purified using the S-Tag rEK purification kit (Novagen). UNC-86 and MEC-3 were produced according to previous methods(Xue et al., 1993).

Gel mobility shift assays were performed using a modified protocol previously reported (Xue et al., 1993). DNA probes were labeled with Biotin by Biotin 3' End labeling kit (Pierce, Rockford, IL) and then annealed into double strands. 100 ng of proteins were incubated with 0.005 ng probe at room temperature for 30 min, and the mixture was loaded onto a 10% TBE polyacrylamide mini gel (Bio-Rad, Hercules, CA). The gel was transferred to a 0.2 mm nylon membrane, which was then treated with UV light to crosslink DNA and proteins. Biotin-labeled DNA was detected using LightShift chemiluminescence EMSA kit (Pierce). The sequences of the probes are listed in Table S3.

smFISH, phenotypic scoring, and statistical analysis

Single-molecule fluorescence in situ hybridization (smFISH) was performed as described previously (Topalidou et al., 2011). Imaging was conducted on a Zeiss Axio Observer Z1 inverted microscope with a CoolSNAP HQ2-FW camera (Photometrics, Tucson, AZ).

To examine the expression pattern of TRN markers, we grew animals at 20° C, examined them using the same microscope and recorded the percentages of TRN cells that express the fluorescent reporter in three independent experiments. The results are presented as aggregates; no significant differences were seen between replicates. To test transgenic animals, we injected

DNA constructs (5 ng/μl for each expression vector) into the animals to establish stable lines carrying the extrachromosomal array; at least three independent lines were tested. In some cases, the transgene was integrated into the genome using γ-irradiation (Mello et al., 1991), and at least three integrant lines were outcrossed and examined.

Statistical significance was determined using the Student's t-test for the majority of comparisons of two sets of data. For multiple comparisons, the Holm-Bonferroni method was used to correct the *p* values.

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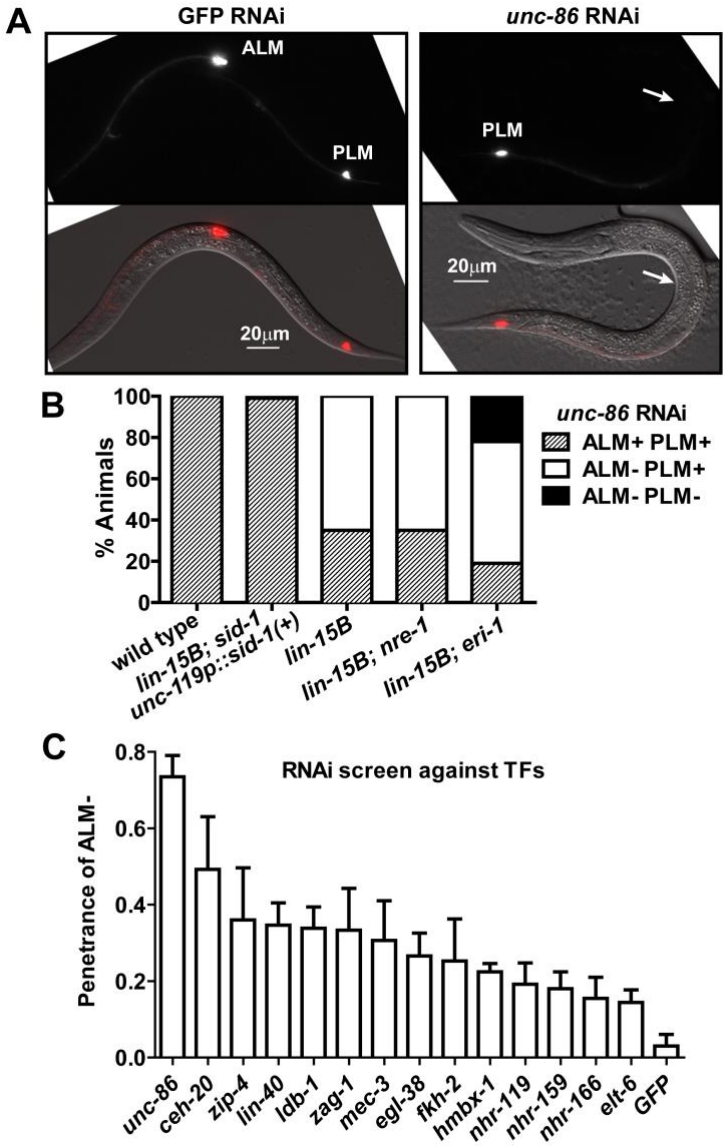
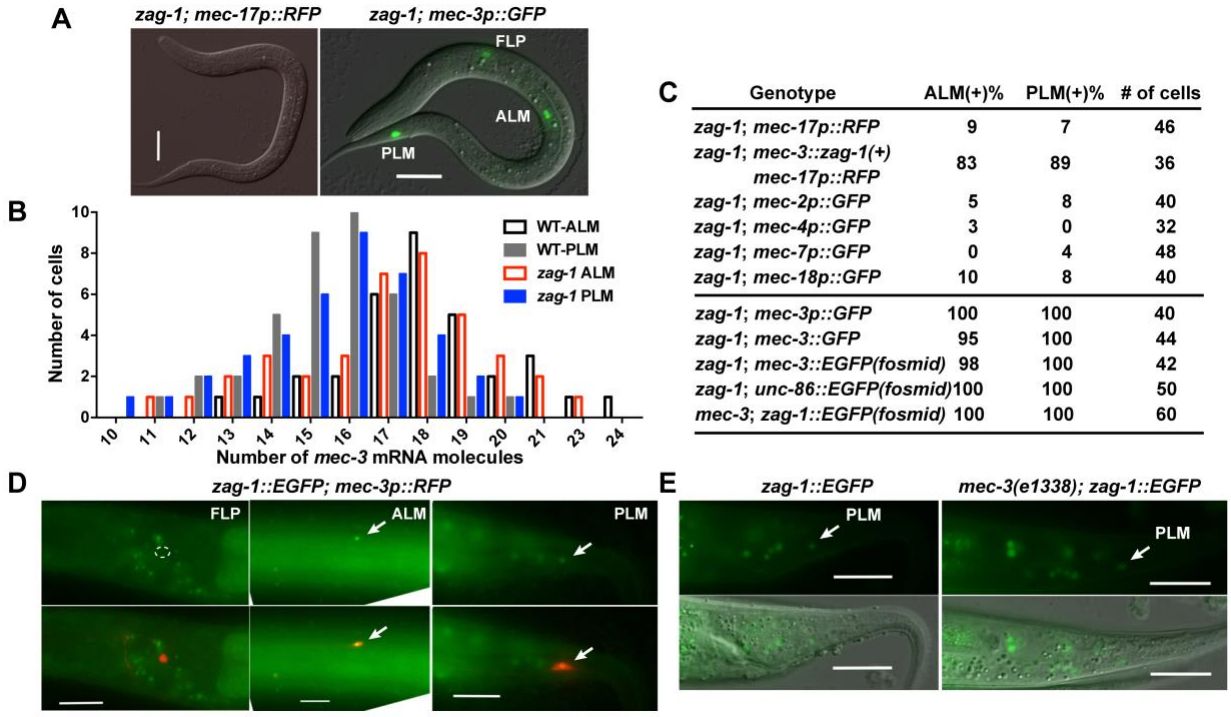


Figure 1. RNAi screen identifies positive regulators of TRN fate. (A) TU4429, *eri-1(mg366); lin-15B(n744); uIs134[mec-17p::RFP]* animals treated with RNAi against *unc-86* or GFP. (B) Percentage of animals that showed RFP or GFP expression in at least one ALM and one PLM (ALM+PLM+), in no ALM but at least one PLM (ALM-PLM+), and in no ALM and no PLM (ALM-PLM-) neurons, respectively. Strains tested for the efficiency of RNAi are TU4429, TU4396, *nre-1(hd20) lin-15B(hd126); uIs134[mec-17p::RFP]*, TU3595, *sid-1(pk3321) him-5(e1490); lin-15B(n744); uIs72[mec-18::GFP]*, and TU4301, *lin-15B(n744); uIs115[mec-17p::RFP]*. (C) The positive RNAi clones identified from the screen; $p > 0.05$ for all the positives using the data from all five rounds of screen.



Genotype	ALM(+)%	PLM(+)%	# of cells
zag-1; mec-17p::RFP	9	7	46
zag-1; mec-3::zag-1(+) mec-17p::RFP	83	89	36
zag-1; mec-2p::GFP	5	8	40
zag-1; mec-4p::GFP	3	0	32
zag-1; mec-7p::GFP	0	4	48
zag-1; mec-18p::GFP	10	8	40
zag-1; mec-3p::GFP	100	100	40
zag-1; mec-3::GFP	95	100	44
zag-1; mec-3::EGFP(fosmid)	98	100	42
zag-1; unc-86::EGFP(fosmid)	100	100	50
mec-3; zag-1::EGFP(fosmid)	100	100	60

Figure 2. *zag-1* is required for the expression of TRN markers independently of *mec-3*. (A) The expression of *mec-17p::RFP* and *mec-3p::GFP* reporters in *zag-1(hd16)* animals at L1 stage. (B) The number of *mec-3* transcripts in TRNs from wild-type and *zag-1* animals from smFISH experiments. (C) The penetrance for the expression of the TRN fate markers, and *mec-3*, *unc-86*, and *zag-1* reporters in ALM and PLM cells of *zag-1(hd16)* animals at L1 stage. Those reporters are expressed in 100% ALM and PLM cells in the wild-type animals. (D) The expression of fosmid-based reporter *zag-1::EGFP* in TRNs but not FLPs. (E) *zag-1:EGFP* expression in *mec-3* mutants. Scale bars = 20 μm.

A

Genotype	FLP(+)%	ALM(+)%	PLM(+)%	# of cells
WT; *mec-17p::GFP*	0	100	100	50
egl-44(n1080); mec-17p::GFP	84	100	100	58
egl-46(gk692); mec-17p::GFP	75	100	100	56
egl-46(n1075); mec-17p::GFP	30	100	100	60
zag-1; egl-44; mec-17p::GFP	80	90	88	40
zag-1; egl-46; mec-17p::GFP	69	84	89	36
egl-44::GFP	100	0	0	40
egl-46::GFP	100	0	0	44
egl-44; egl-46::GFP	5	0	0	42
zag-1; egl-44::GFP	100	81	78	32
zag-1; egl-46::GFP	100	76	82	38

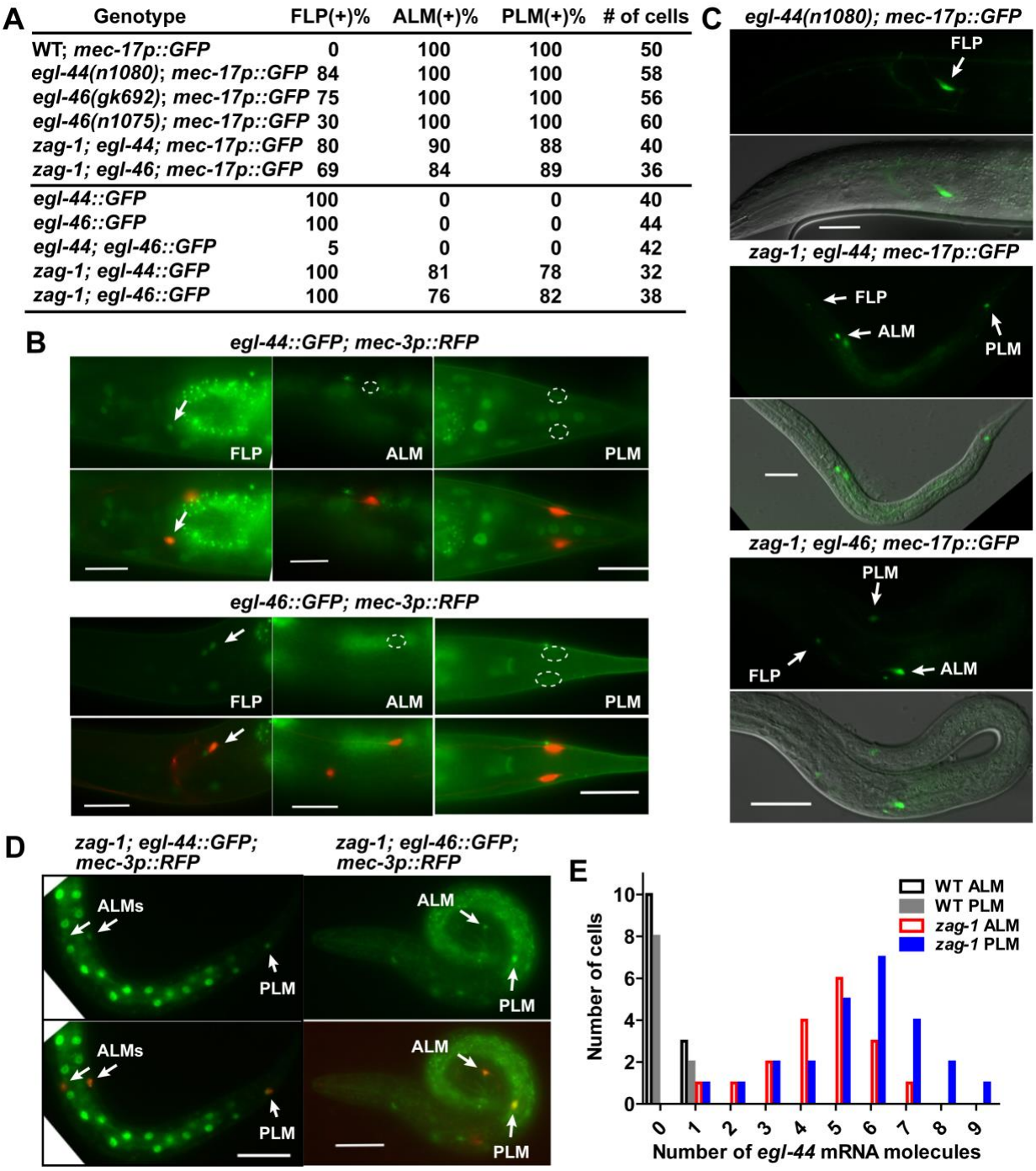
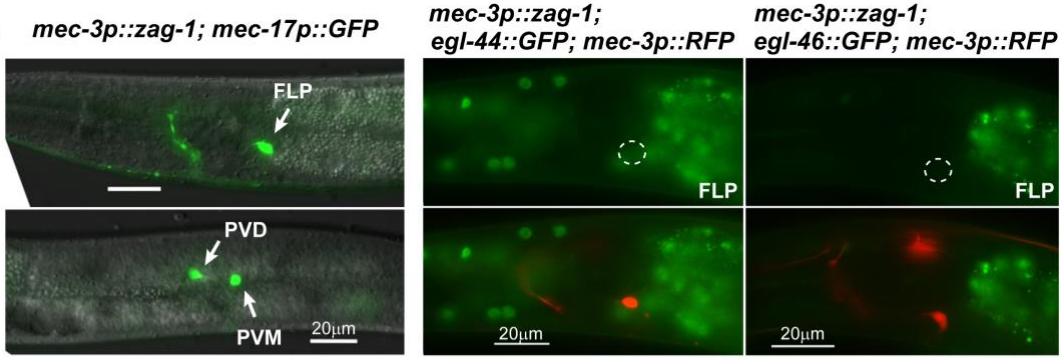


Figure 3. ZAG-1 promotes TRN fate by repressing *egl-44* and *egl-46*. (A) The expression of TRN marker *mec-17p::GFP* in FLP neurons of *egl-44* animals and in TRNs in *egl-44; zag-1* and *zag-1; egl-46* animals. (B) Penetrance of the expression of various reporters. (C-D) The expression of *egl-44::GFP* and *egl-46::GFP* reporters in FLPs but not TRNs of wild-type animals and in TRNs of *zag-1* animals. (E) The number of *egl-44* mRNA molecules in TRNs of wild-type and *zag-1* animals.

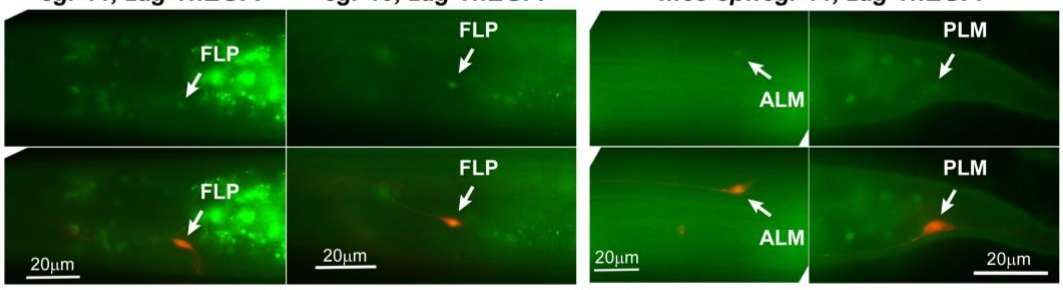
A



B

Genotype	FLP(+)%	ALM(+)%	PLM(+)%	# of cells	
mec-3p::zag-1; mec-17p::GFP	90	100	100	50	
mec-3p::ldb-1; mec-17p::GFP	0	100	100	50	
mec-3p::zag-1; egl-44::GFP	2	0	0	60	
mec-3p::zag-1; egl-46::GFP	0	0	0	52	
egl-44; zag-1::EGFP	82	100	100	60	
egl-46; zag-1::EGFP	75	100	100	56	
mec-3p::egl-44; mec-17p::GFP	0	0	0	50	
mec-3p::egl-46; mec-17p::GFP	0	100	100	54	
mec-3p::egl-44; egl-46::GFP	100	93	95	58	
mec-3p::egl-44; zag-1::EGFP	0	88	92	60	
	0	55	61	60	(%weak exp.)
mec-3p::egl-44; mec-3p::zag-1; mec-17p::GFP	87	90	96	68	
mec-3p::egl-44; mec-3p::zag-1 mec-3p::egl-46; mec-17p::GFP	2	0	0	62	
mec-3p::egl-44; mec-3p::zag-1; egl-46::GFP	3	3	0	30	
egl-46; mec-3p::egl-44; mec-17p::GFP	44	60	67	48	

C



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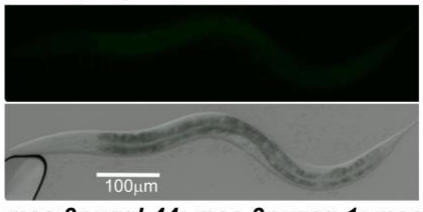

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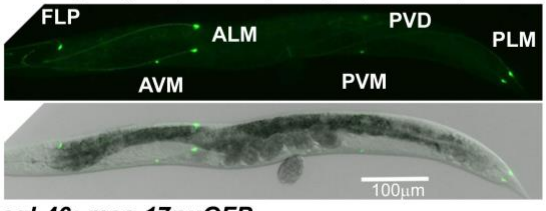

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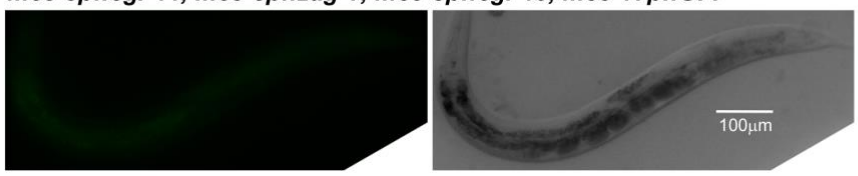
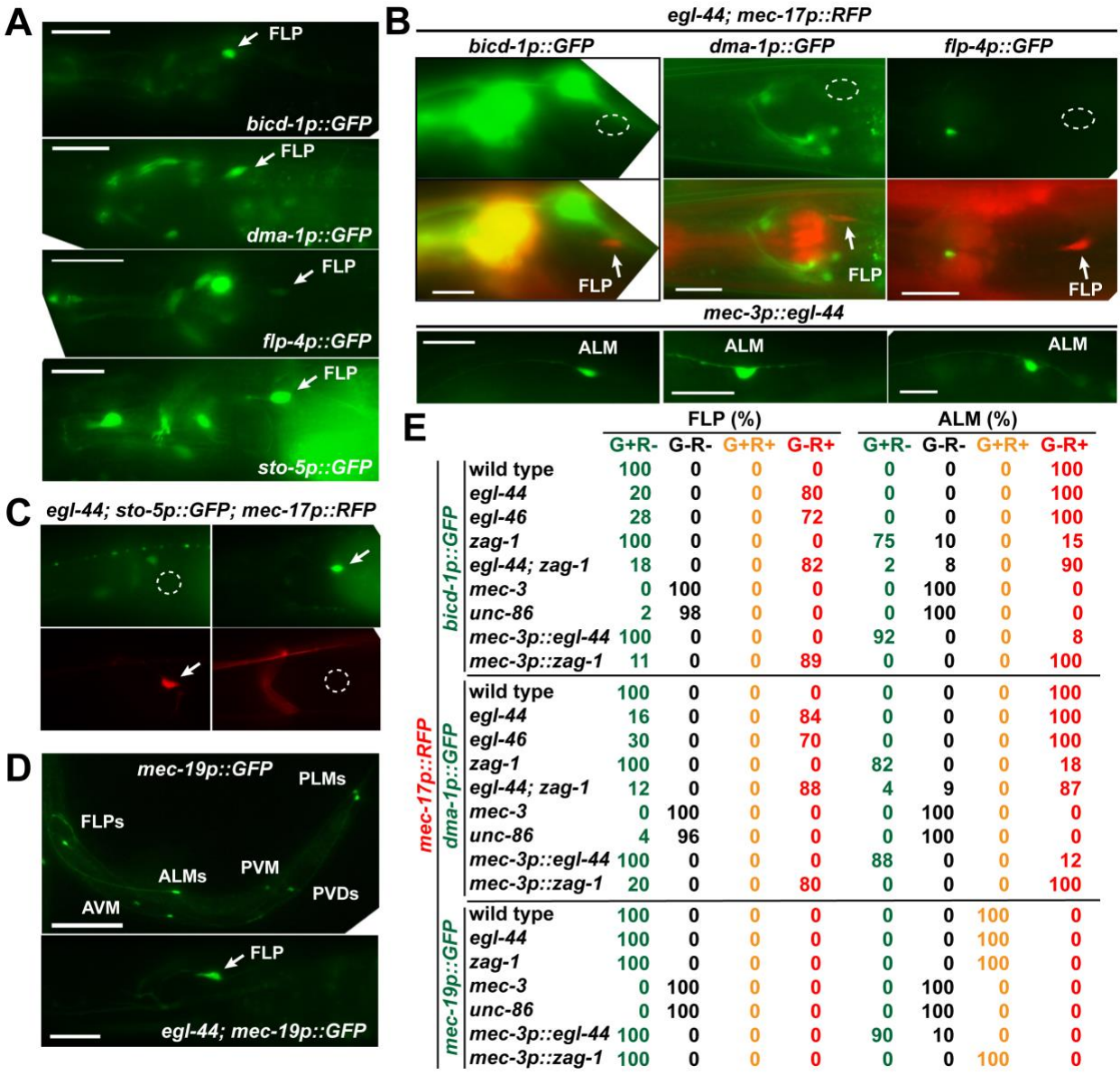


Figure 4. Mutual inhibition between *zag-1* and *egl-44* regulate TRN fate decision. (A) The activation of TRN marker and the loss of the expression of *egl-44* and *egl-46* reporters in FLP neurons of animals carrying *mec-3p::zag-1* transgene. (B) The percentage of cells expressing the indicated markers in various strains. (C) The expression of *zag-1::EGFP* in FLP neurons of *egl-44* and *egl-46* mutants, and weak expression of *zag-1* reporter in animals misexpressing *egl-44* from *mec-3* promoter. (D-F) The expression of TRN marker *mec-17p::GFP* in animals carrying various transgenes.



E

		FLP (%)				ALM (%)			
		G+R-	G-R-	G+R+	G-R+	G+R-	G-R-	G+R+	G-R+
bicd-1p::GFP	wild type	100	0	0	0	0	0	0	100
	egl-44	20	0	0	80	0	0	0	100
	egl-46	28	0	0	72	0	0	0	100
	zag-1	100	0	0	0	75	10	0	15
	egl-44; zag-1	18	0	0	82	2	8	0	90
	mec-3	0	100	0	0	0	100	0	0
	unc-86	2	98	0	0	0	100	0	0
	mec-3p::egl-44	100	0	0	0	92	0	0	8
	mec-3p::zag-1	11	0	0	89	0	0	0	100
mec-17p::RFP dma-1p::GFP	wild type	100	0	0	0	0	0	0	100
	egl-44	16	0	0	84	0	0	0	100
	egl-46	30	0	0	70	0	0	0	100
	zag-1	100	0	0	0	82	0	0	18
	egl-44; zag-1	12	0	0	88	4	9	0	87
	mec-3	0	100	0	0	0	100	0	0
	unc-86	4	96	0	0	0	100	0	0
	mec-3p::egl-44	100	0	0	0	88	0	0	12
	mec-3p::zag-1	20	0	0	80	0	0	0	100
mec-19p::GFP	wild type	100	0	0	0	0	0	100	0
	egl-44	100	0	0	0	0	0	100	0
	zag-1	100	0	0	0	0	0	100	0
	mec-3	0	100	0	0	0	100	0	0
	unc-86	0	100	0	0	0	100	0	0
	mec-3p::egl-44	100	0	0	0	90	10	0	0
	mec-3p::zag-1	100	0	0	0	0	0	100	0

Figure 5. EGL-44/EGL-46 simultaneously induces FLP genes and suppresses TRN genes. (A-B) Various FLP fate reporters were expressed in wild-type FLP neurons but not in *egl-44* mutants. (C) The expression of FLP and TRN fate markers were mutually exclusive in FLP neurons of *egl-44* mutants. (D) The expression of mec-19 was not affected by *egl-44* mutation. (E) Percentage of FLP and ALM neurons expressing the green (G) and red (R) markers in various strains. The number of cells > 40.

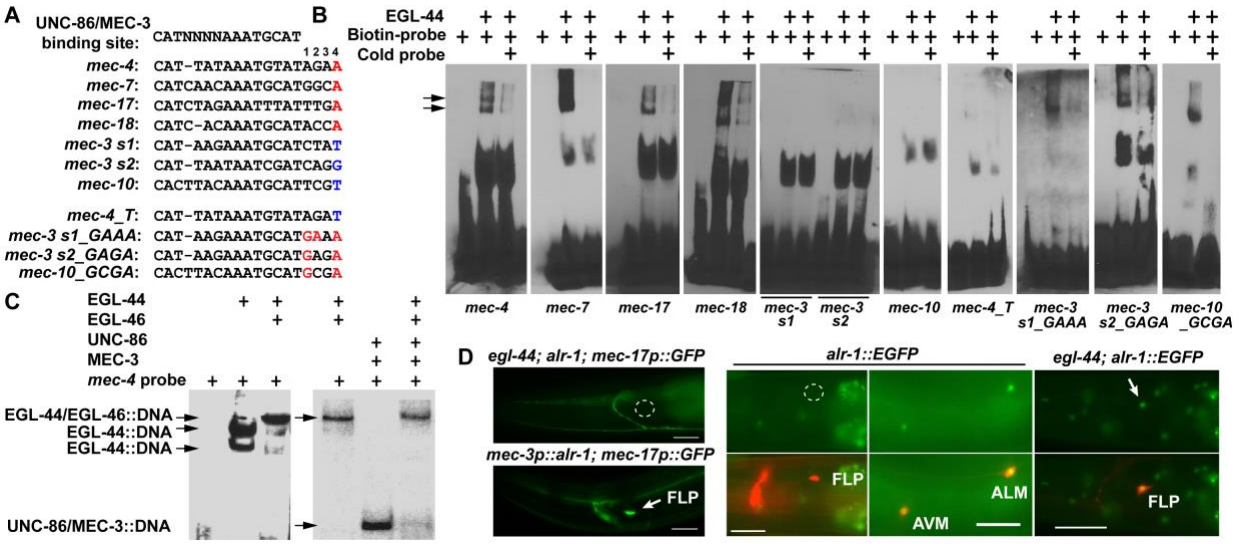


Figure 6. EGL-44/EGL-46 inhibits the expression of TRN genes by outcompeting UNC-86/MEC-3 for the binding of the same *cis*-regulatory elements. (A) The alignment of various *cis*-regulatory motifs tested in electrophoretic mobility shift (EMSA) assays. Positions 1-4 were assigned to the four nucleotides following the consensus UNC-86/MEC-3 binding site (Zhang et al., 2002). Nucleotides in red were considered important for EGL-44 binding and nucleotides in blue were responsible for the lack of EGL-44 binding. (B) The binding of recombinant EGL-44 proteins to various probes in EMSA assays. Arrows point to the band of EGL-44::DNA complexes. (C) The binding of EGL-44/EGL-46 with *mec-4* probes in the presence or absence of UNC-86/MEC-3. (D) The expression of TRN fate marker in *egl-44; alr-1* double mutants and animals misexpressing ALR-1 from the *mec-3* promoter and the expression of a fosmid-based *alr-1* reporter *wgIs200[alr-1::EGFP]* in wild-type animals and *egl-44* mutants.

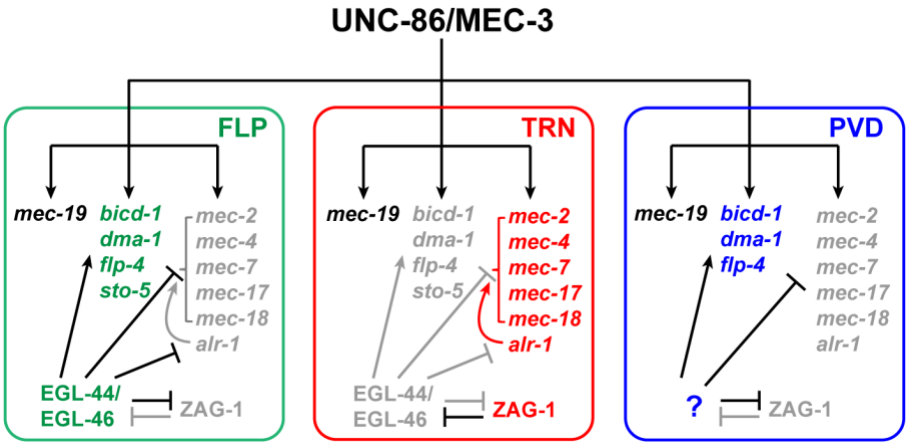


Figure 7. A model for the regulatory mechanisms controlling the cell fate specification among FLP, PVD, and TRN fates. Gene names in green, red, and blue indicate genes expressed in FLP, TRN, and PVD neurons, respectively; black names for genes commonly expressed in all three types and grey name for genes repressed.