Development 137, 2065-2074 (2010) doi:10.1242/dev.040881

Paired and LIM class homeodomain proteins coordinate differentiation of the *C. elegans* ALA neuron

Cheryl Van Buskirk and Paul W. Sternberg*

SUMMARY

The ancient origin of sleep is evidenced by deeply conserved signaling pathways regulating sleep-like behavior, such as signaling through the Epidermal growth factor receptor (EGFR). In *Caenorhabditis elegans*, a sleep-like state can be induced at any time during development or adulthood through conditional expression of LIN-3/EGF. The behavioral response to EGF is mediated by EGFR activity within a single cell, the ALA neuron, and mutations that impair ALA differentiation are expected to confer EGF-resistance. Here we describe three such EGF-resistant mutants. One of these corresponds to the LIM class homeodomain (HD) protein CEH-14/Lhx3, and the other two correspond to Paired-like HD proteins CEH-10/Chx10 and CEH-17/Phox2. Whereas CEH-14 is required for ALA-specific gene expression throughout development, the Prd-like proteins display complementary temporal contributions to gene expression, with the requirement for CEH-10 decreasing as that of CEH-17 increases. We present evidence that CEH-17 participates in a positive autoregulatory loop with CEH-14 in ALA, and that CEH-10, in addition to its role in ALA differentiation, functions in the generation of the ALA neuron. Similarly to CEH-17, CEH-10 is required for the posterior migration of the ALA axons, but CEH-14 appears to regulate an aspect of ALA axon outgrowth that is distinct from that of the Prd-like proteins. Our findings reveal partial modularity among the features of a neuronal differentiation program and their coordination by Prd and LIM class HD proteins.

KEY WORDS: EGFR, Axon migration, Gene battery, Homeodomain, Sleep, *C. elegans*

INTRODUCTION

Many crucial events in metazoan development and physiology are governed by diffusible signals that trigger specific responses in highly restricted subsets of cells. This exquisite specificity of intercellular signaling requires precisely controlled expression of receptors and downstream signaling components that effect appropriate responses. The nematode *Caenorhabditis elegans* has proven a valuable model for the study of signaling specificity, notably for mechanisms of signaling through the Epidermal growth factor (EGF) receptor (for a review, see Moghal and Sternberg, 2003). The sole EGF-like ligand and EGF receptor in the *C. elegans* genome are encoded by the genes *lin-3* and *let-23*, respectively (Hill and Sternberg, 1992; Aroian et al., 1990) (Wormbase WS210). Recently we described a role for LET-23 in the regulation of *C. elegans* behavior (Van Buskirk and Sternberg, 2007). *Caenorhabditis elegans* develops through four larval stages before adulthood, and each larval molt is preceded by lethargus, a period of cuticle restructuring during which feeding and locomotion are suppressed. Behavioral quiescence at lethargus satisfies several criteria that define it as a sleep-like state (Raizen et al., 2008). *Caenorhabditis elegans* lethargus behavior is regulated in part by EGF signaling, a conserved mechanism of sleep regulation across species (for a review, see Zimmerman et al., 2008), and sleep-like behavior can be elicited at any time during development or adulthood by expression of LIN-3/EGF from a heat-shock-inducible promoter (Van Buskirk and Sternberg, 2007).

Howard Hughes Medical Institute, Division of Biology 156-29, California Institute of Technology, 1200 E. California Boulevard, Pasadena, CA 91125, USA.

*Author for correspondence (pws@caltech.edu)

Accepted 20 April 2010

The behavioral effects of ubiquitous LIN-3/EGF expression are mediated by EGFR activity within a single neuron, ALA. Thus EGF-dependent sleep in *C. elegans* provides a model for dissection of the regulatory mechanisms governing signaling specificity. EGF-resistant mutants comprise not only signaling components that allow a unique response to EGFR activity, but also factors that control the expression of EGFR and its effectors within the nervous system. Mutants of the first class have been previously described (Van Buskirk and Sternberg, 2007) and implicate PLC-γ signaling through the second messenger diacylglycerol (DAG) in stimulating secretory vesicle release in response to EGFR activation. EGF-resistant mutants that define components of the transcriptional regulatory network directing the expression of EGFR and its downstream effectors within the ALA neuron have not yet been described and are expected to shed light on the mechanisms by which a single neuron adopts unique functional properties.

Accumulating evidence suggests that functional diversity within nervous systems relies on unique combinations of certain classes of transcription factors. In *Drosophila* and mammals, overlapping domains of expression of LIM subfamily homeodomain (HD) proteins, a 'LIM code', has been proposed to specify different neuron classes (for a review, see Shirasaki and Pfaff, 2002). In some cases, these patterns of expression reflect combinatorial function (e.g. Thaler et al., 2002). In other cases, the observed diversity of neuron types depends upon interaction of LIM class proteins with other HD factors, such as POU domain proteins (e.g. Certel and Thor, 2004). Homeodomain transcription factors of the Paired (Prd) class also play important roles in neuronal specification. For example, the vertebrate Prd class protein Phox2b is required for noradrenergic neuron differentiation (for a review, see Brunet and Pattyn, 2002). Relatively little is known about combinatorial codes involving Prd-HD proteins, although a well-characterized example comes from *C. elegans* AIY interneuron

differentiation, which depends on interaction of Prd and LIM class proteins CEH-10 and TTX-3 (Wenick and Hobert, 2004). It is of debate whether combinatorial codes generally govern all aspects of the neuronal differentiation program, as inferred from several cases (Hobert, 2008), or whether certain characteristics can be adopted in a piecemeal fashion.

Here we describe three EGF-resistant mutants defective in ALA-specific gene expression. One corresponds to the LIM class homeodomain protein CEH-14, previously shown to contribute to the differentiation of the AFD thermosensory neurons (Cassata et al., 2000). The other two, CEH-10 and CEH-17, belong to the Q_{50} Prd-like subgroup of the Paired class of HD proteins. CEH-17 function had been previously thought to be limited to the control of axon migration in the posteriorly projecting ALA and SIA neurons (Pujol et al., 2000). CEH-10 functions in the differentiation of the AIY interneurons, and a role in ALA has not been described (Forrester et al., 1998; Altun-Gultekin et al., 2001). Here we show that these three HD proteins contribute to the expression of a common ALA-specific gene battery, but that the Prd and LIM class proteins direct different aspects of ALA axon outgrowth. We present evidence that CEH-10 plays a role in ALA generation, and that CEH-10 and CEH-17 contribute similarly to ALA function but act with distinct temporal profiles.

MATERIALS AND METHODS

Strains

The following strains used in this study were cultured under standard conditions at 20°C (Brenner, 1974). PS5628 *syIs197*[hs:LIN-3C, *myo-2*:dsRed], *him-5*(*e1490*). PS301 *let-23*(*sy10*)/*rol-6*(*e187*)*unc-4*(*e120*). IB16 *ceh-17*(*np1*). TB528 *ceh-14*(*ch3*). BW506 *ceh-10*(*ct78*). VC444 *ver-3*(*gk227*). VC226 *ida-1*(*ok409*). RB1990 *flp-7*(*ok2625*). TU1747 *deg-3*(*u662*). RB762 *alr-1*(*ok545*). PS73 *mab-9*(*e1245*); *him-5*(*e1490*). PS5873 *ceh-17*(*np1*); *ceh-14*(*ch3*). PS5043 *+/hT2[qIs48]*; *ceh-10*(*gm133*)/*hT2*. PS5689 *ceh-17*(*np1*); *syIs197, him-5*(*e1490*). PS5838 *ceh-17*(*np1*); *deg-3*(*u662*). PS5964 *ceh-10*(*ct78*); *syIs197, him-5*(*e1490*). PS5961 *syIs197, him-5*(*e1490*); *ceh-14*(*ch3*). PS5835 *deg-3*(*u662*); *ceh-14*(*ch3*). The following strains were raised at 15˚C: PS2172 *pha-1*(*e2123ts*); *him-5*(*e1490*). PS5830 *ceh-17*(*np1*); *pha-1*(*e2123ts*). PS5963 *pha-1*(*e2123ts*); *ceh-14*(*ch3*). PS5538 *eri-1*(*mg366*); *syIs197, him-5*(*e1490*); *lin-15B*(*n744*). Strains not starting with a 'PS' designation were obtained from the Caenorhabditis Genetics Center.

Behavioral assays

To assay EGF-induced (ALA-dependent) sleep, animals carrying the integrated hs:LIN-3/EGF transgene s*yIs197* were hand-selected at the L2 or young adult stage and transferred to NGM plates with a thin lawn of OP50 bacteria. The plates were sealed with parafilm and placed in a 33°C water bath for 30 minutes, returned to 20°C for 2 hours, and scored for feeding, which is completely suppressed during EGF-induced sleep. Feeding behavior was assessed by monitoring each animal for 5 seconds under a dissecting microscope for full contractions of the posterior pharyngeal bulb (pumping); animals showing pharyngeal pumping were scored as EGF-resistant. In the case of *ceh-10* embryonic RNAi, animals were selected for normal feeding behavior before the heat-shock assay, as a fraction of these animals are sickly and fail to feed.

Reporter genes and expression analysis

Each reporter is listed here with a transgene number, the length of sequence upstream of the start ATG, the construct name (and number if available) and a reference to its construction (in the case of *unc-53*, the ATG is that of the B isoform): syEx234 3.6 kb *let-23*:LET-23:GFP pK7GL44.2 (Chang et al., 1999); syEx1157 3.0 kb *plc-3*:YFP (S. Xu, personal communication); syEx1080 3.0 kb *ver-3*:GFP (Popovici et al., 2002); syEx1084 3.3 kb *ida-1*:IDA-1:GFP (Cai et al., 2004); ynIs66 3.4 kb *flp-7*:GFP (Kim and Li, 2004); syEx1145 2.0 kb *ceh-14*:GFP JRH/AH9 (Dupuy et al., 2004); syIs1158 (spontaneous integrant) 2.1 kb *ceh-17*:GFP pNP69 (Pujol et al., 2000); syEx1147 3.4 kb *des-2*:DES-2:GFP (Treinin et al., 1998); syEx1081 3.4 kb *unc-53*:GFP pNP21 (Stringham et al., 2002); syEx1029 2.2 kb *unc-119*:YFP (Schindelman et al., 2006); syEx1146 1.2 kb *rab-3*:GFP pRAB100 (http://neuroscience.wustl.edu/nonetlab/ResourcesF/seqinfo.html). While all of these reporter genes are expressed in the ALA neuron, they may not contain the regulatory sequences necessary to accurately reflect all aspects of endogenous gene expression. During analysis of *ceh-14*:GFP expression, we observed faintly detectable expression in the RID neuron, which has not been previously described.

With the exception of the integrated *flp-7*:GFP reporter, all reporter constructs were injected into a *pha-1*(*e2123ts*) background along with *pha-1*+ DNA for selection (Granato et al., 1994). Multiple lines were analyzed for each reporter and a representative line was crossed to *ceh-17*(*np1*) and *ceh-14*(*ch3*) mutants. Reporter gene expression in ALA was scored as detectable or undetectable on a Zeiss Axioskop with wild-type and mutant animals scored in the same session. The intensity of fluorescence in ALA was quantified using Openlab software (Improvision) as follows. Fluorescence images of ALA and a reporter-expressing control neuron were taken under identical exposure. The mean pixel intensity (m.p.i.) over the brightest area of each cell was recorded, and the relative fluorescence intensity (r.f.i.) was calculated by dividing the m.p.i. of ALA by the m.p.i of the control neuron. The control neurons used were as follows: for *ida-1*:GFP, HSN; for *flp-7*:GFP, SAA; for *ceh-14*:GFP, AFD; for *plc-3*:YFP, the posterior of the two unidentified ventral head neurons expressing the reporter. Approximately 20 animals were scored to determine the mean r.f.i. for each genotype.

RNA-mediated interference

An RNAi-sensitized strain carrying the hs:LIN-3 transgene was used in RNAi experiments. For next-generation RNAi, PS5538 hermaphrodites were hand-selected at the L4 stage and transferred to NGM plates seeded with dsRNA-expressing bacteria (Timmons et al., 2001) and allowed to lay eggs at 15°C. The parental hermaphrodites were removed 2 days later and plates were transferred to 20°C. Young adult animals were assayed for the ALA-dependent sleep response. For postembryonic RNAi, PS5538 L1 animals were synchronized from an axenized egg prep (www.wormbook.org), aliquoted onto RNAi plates, grown at 20°C and assayed as young adults. RNAi library clones used in this study: Geneservice I-2A09 (*ceh-17*), Open Biosystems 11052G9 (*ceh-10*) and 11077F2 (*ceh-14*). These genes share insufficient identity to produce cross-target effects (Rual et al., 2007). A GFP RNAi clone was used as a control, and the *let-23* RNAi insert was amplified using *Eco*RV-linked primers 5′-GGATATCTGAAAATCGCTGAAATGG-3′ and 5′-GGATATCGAGA-ACTTGGCATACTTGG-3′.

Statistics

Two-tailed *P*-values were calculated using InStat software (GraphPad). Means were compared using an unpaired t-test, with Welch's correction in the case of unequal variances. Data sets displaying non-Gaussian distributions were compared using a Mann-Whitney U-test. Categorical data (detectable versus undetectable) were compared using Fisher's exact test.

RESULTS

CEH-17/Phox2 is required for EGFR and PLC-γ expression in ALA

Activation of LET-23/EGFR within the ALA interneuron mediates *lin-3*/EGF-induced sleep behavior in *C. elegans* (Van Buskirk and Sternberg, 2007). The ALA cell body lies in the head and sends two bilaterally symmetrical axons along lateral nerve bundles to the tail (White et al., 1986). Complete ALA axon migration is dispensable for EGF-induced sleep: a mutation disrupting ALA axon growth, *vab-8*(*e1017*), does not disrupt the sleep response (Wightman et al., 1996; Van Buskirk and Sternberg, 2007). By contrast, mutation of CEH-17, a Q_{50} Paired-like class homeodomain transcription factor, the function of which is known only in axon migration (Pujol et al., 2000), renders animals

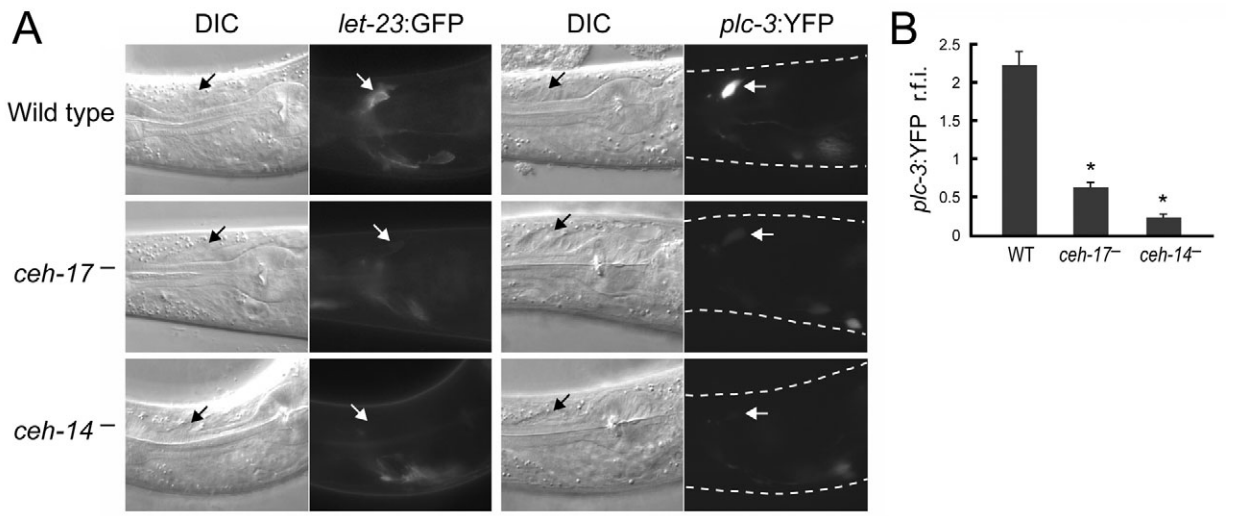

Fig. 1. CEH-17 and CEH-14 are required for expression of EGFR pathway components in ALA. (**A**) DIC images show that the ALA nucleus (arrows) is present in *ceh-17* and *ceh-14* null mutant animals, whereas fluorescence images show decreased expression of *let-23*/EGFR and *plc-3*/PLC-γ reporter genes in ALA. All images are taken from animals at the final larval stage (L4). Anterior is to the left, dorsal is up. (**B**) Among animals in which *plc-3*:YFP expression could be detected (Table 2), r.f.i. in ALA was quantified (Methods), showing that *plc-3*:YFP expression is severely compromised in L4 animals lacking either CEH-17 or CEH-14. **P*<0.0001 versus wild type.

resistant to the sleep-inducing effects of *lin-3*/EGF (Van Buskirk and Sternberg, 2007) (Table 1). These observations suggest that CEH-17 has targets other than genes involved in axon migration, which are crucial for ALA-dependent sleep.

We tested the possibility that CEH-17 affects the expression of LET-23/EGFR in ALA. In wild type, a *let-23*:LET-23-GFP translational reporter is expressed in several non-neuronal cells and a small number of neurons, including ALA (Van Buskirk and Sternberg, 2007). We examined the expression of this reporter in *ceh-17*(*np1*) null mutant animals and found its expression to be severely decreased in ALA, with other sites of expression intact (Fig. 1A, Table 2). Thus CEH-17 is required for EGFR expression specifically within the ALA neuron. We wished to determine if other effectors of the sleep response were under CEH-17 control. Phospholipase C-γ (PLC-γ, encoded by *plc-3*), a direct target of activated EGFR, is required for ALA-dependent sleep (Van Buskirk and Sternberg, 2007). We examined the expression of a PLC-γ transcriptional reporter that is normally expressed in ALA at all stages and detectable in a small number of other neurons (S. Xu, personal communication). We found *plc-3* expression to be specifically disrupted in ALA in the *ceh-17*(*np1*) animals (Fig. 1A,B). Thus multiple effectors of ALA-dependent sleep are regulated by CEH-17.

Table 1. Genetic suppression of EGF-induced sleep

+ hs:LIN-3/EGF	% EGF-resistant	*n*
Wild type	0	>100
let-23(sy10)	94	18
vab-8(e1017)	0	67
ceh-17(np1)	100	75
ceh-14(ch3)	100	90
ver-3(gk227)	0	30
ida-1(ok409)	5	84
flp-7(ok2625)	0	64

Animals carrying the hs:LIN-3 transgene were well fed and grown at 20°C. Young adult animals were scored 2 hours after heat shock for EGF-induced sleep behavior (see Materials and methods).

CEH-14/Lhx3 functions in parallel to CEH-17

CEH-17 is also expressed in the four SIA neurons (Pujol et al., 2000). As neither *let-23* nor *plc-3* are expressed in the SIAs, CEH-17 probably acts with other factors to regulate gene expression in ALA. CEH-14 is a LIM-class homeodomain transcription factor with expression that overlaps with CEH-17 only in one cell, the ALA neuron. CEH-14, orthologous to vertebrate Lhx3 and Lhx4, is expressed in several cell types and confers thermosensory function to the AFD neurons (Cassata et al., 2000). We investigated a potential role for CEH-14 in ALA differentiation. We found that similar to *ceh-17* animals, the ALA neuron is present and its cell body is positioned normally (Fig. 1A, DIC images) but the *ceh-14*(*ch3*) null mutant animals are completely resistant to the behavioral effects of EGF expression (Table 1). We therefore tested whether CEH-14 affects the same target genes within ALA as does CEH-17. We found that the *ceh-14*(*ch3*) mutation nearly abolished the expression of *let-23*/EGFR and severely affected the expression of *plc-3*/PLC-γ specifically within ALA (Table 2, Fig. 1A,B). Thus

Table 2. Percentage of animals showing detectable reporter gene expression in ALA

ALA reporter	Wild type	*ceh-17(np1)*	*ceh-14(ch3)*
let-23:GFP	96% (50/52)	13% (7/54)	1% (1/68)
plc-3:YFP	100% (21/21)	95% (20/21)*	16% (23/141)*
unc-119:YFP	97% (29/30)	95% (21/22)†	91% (20/22)†
rab-3:GFP	80% (36/45)	79% (22/28)	83% (20/24)
ver-3:GFP	82% (32/39)	20% (7/35)	0% (0/40)
ida-1:GFP	95% (38/40)	44% (21/48)*	0% (0/20)
flp-7:GFP	100% (20/20)	100% (20/20)*	0% (0/20)

Transgenic animals at the L4 stage (except for *ver-3*:GFP, which was analyzed at the L1 stage) were mounted and scored as + or – for reporter gene expression in the ALA neuron. In cases of undetectable expression, the presence of ALA was confirmed under DIC optics.
*ALA fluorescence was quantified and found to be significantly weaker than wild type (see Fig. 1B, Fig. 3B,C).
†ALA fluorescence was quantified and found to be not significantly different from wild type (not shown).

both Paired-like (CEH-17) and LIM-class (CEH-14) homeodomain proteins are required for expression of genes that mediate ALA-dependent sleep.

We wished to distinguish whether CEH-17 and CEH-14 act in a single pathway that activates target gene expression in ALA, or whether they function in parallel. If these factors act in parallel, we would expect to see enhancement of the *ceh-14* null mutant phenotype by the *ceh-17* null mutation. As the expression of our *let-23* reporter gene is nearly abolished in the *ceh-14*(*ch3*) single mutant (Table 2), we could not use it as a readout of target gene expression in a double-mutant analysis. We therefore examined the expression of *plc-3:*YFP, which is expressed in a fraction of each of the single mutants. We found expression of *the plc-3* reporter gene to be completely abolished in the *ceh-14*(*ch3*);*ceh-17*(*np1*) animals (0/78 show expression, $P<0.0001$ versus *ceh-14*(*ch3*) alone, *t*-test with Welch correction). Thus CEH-17 and CEH-14 act at least partially in parallel in the regulation of ALA-specific gene expression. One mechanistic basis for such parallel action could be that each of these factors directly activates target gene expression, and that loss of either impairs expression, whereas loss of both completely abrogates it.

CEH-17 and CEH-14 constitute an autoregulatory module in ALA

The capacity for EGF-dependent sleep to be induced at any stage of larval development and adulthood relies on the expression of EGFR and PLC-γ throughout the postembryonic life of the ALA neuron. As such sustained expression is often controlled by transcriptional feedback loops (Edlund and Jessell, 1999), we examined whether CEH-17 and CEH-14 affect each other's expression. Evidence that *ceh-14* expression may be regulated by CEH-17 has been found in a yeast one-hybrid screen, in which CEH-17 was found to activate transcription of a DNA bait containing 2 kb of *ceh-14* upstream sequence (Deplancke et al., 2004) (http://edgedb.umassmed.edu). We analyzed the expression of a GFP reporter driven by this same *ceh-14* promoter fragment (*ceh-14*:GFP; kindly provided by I. Hope), and found that its expression was specifically decreased in ALA in the *ceh-17*(*np1*) mutant (Fig. 2A). The defect could not be detected by eye, but quantification of fluorescence revealed a significant decrease in ALA-specific expression across larval stages. Thus CEH-17 contributes to CEH-14 expression in ALA. We then investigated whether CEH-14 regulates the expression of a *ceh-17*:GFP reporter (pNP69; kindly provided by N. Pujol) which is normally expressed in ALA and the four SIA neurons (Pujol et al., 2000). We found that ALA-specific expression of *ceh-17*:GFP was nearly abolished in the *ceh-14* mutant throughout development (Fig. 2B). Thus CEH-17 and CEH-14 positively regulate each other's expression, with CEH-14 having the greater effect.

We also tested each factor for autoregulation. We found that in *ceh-14*(*ch3*) animals, *ceh-14*:GFP expression in ALA is compromised at all stages examined, with a fraction of animals showing no detectable expression in ALA (Fig. 2C), and the remaining animals having an ALA fluorescence intensity significantly lower than wild type (not shown). This autoregulatory effect is of a magnitude that makes it unlikely to be an indirect consequence of CEH-14–CEH-17 cross-regulatory interactions, as a complete loss of CEH-17 produces relatively mild defects in *ceh-14* expression. In *ceh-17*(*np1*) mutants, *ceh-17*:GFP expression in ALA becomes compromised as development proceeds (Fig. 2D). This temporal profile argues against cross-regulatory interactions with CEH-14 being the predominant contributor to the observed CEH-17 autoregulation, as the cross-regulatory effects are consistent across larval stages (Fig. 2A,B).

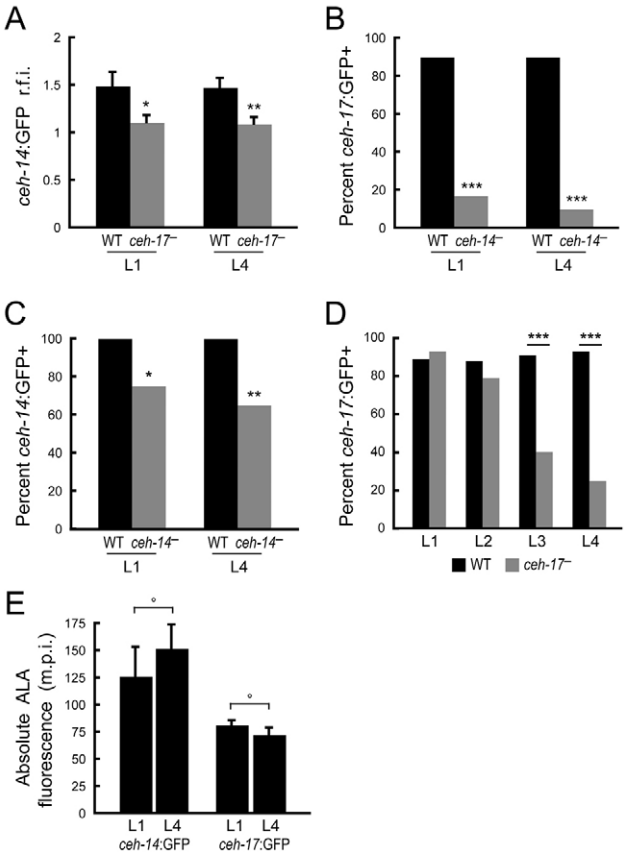

Fig. 2. CEH-17 and CEH-14 participate in cross- and autoregulation in ALA. (**A**) *ceh-14*:GFP expression in ALA is decreased in *ceh-17* null mutant animals. The fraction of animals with detectable expression is not different from wild type, but quantification of ALA fluorescence (Methods) shows a mild but significant decrease in *ceh-17*(*np1*) at the first (L1) and last (L4) larval stages. (**B**) *ceh-17*:GFP expression in ALA is severely compromised throughout development in *ceh-14* null mutant animals. Transgenic animals were scored for detectable fluorescence in ALA. (**C**) CEH-14 is required for its own expression. Transgenic animals were scored for detectable fluorescence in ALA. (**D**) CEH-17 is required for the maintenance of its own expression during later larval stages. (**E**) Expression levels of *ceh-14* and *ceh-17* are relatively constant over time, as determined by comparison of m.p.i. in wild-type L1 and L4 ALA neurons. **P*<0.05, ***P*<0.005, ****P*<0.0005 versus wild type. ° no significant difference. WT, wild type.

Thus CEH-14 contributes to its own expression and to the expression of *ceh-17* in ALA throughout larval development. CEH-17 is in turn required for wild-type levels of *ceh-14* expression throughout development and for the maintenance of its own expression in ALA during later larval stages. This CEH-14–CEH-17 regulatory module appears to maintain relatively constant expression of these genes across larval stages in wild type (Fig. 2E).

CEH-17 and CEH-14 affect ALA-specific aspects of neuron differentiation

To characterize the extent that CEH-17 and CEH-14 affect ALA differentiation, we examined the expression of the pan-neuronal genes *unc-119* (Maduro and Pilgrim, 1995) and *rab-3* (Nonet et al.,

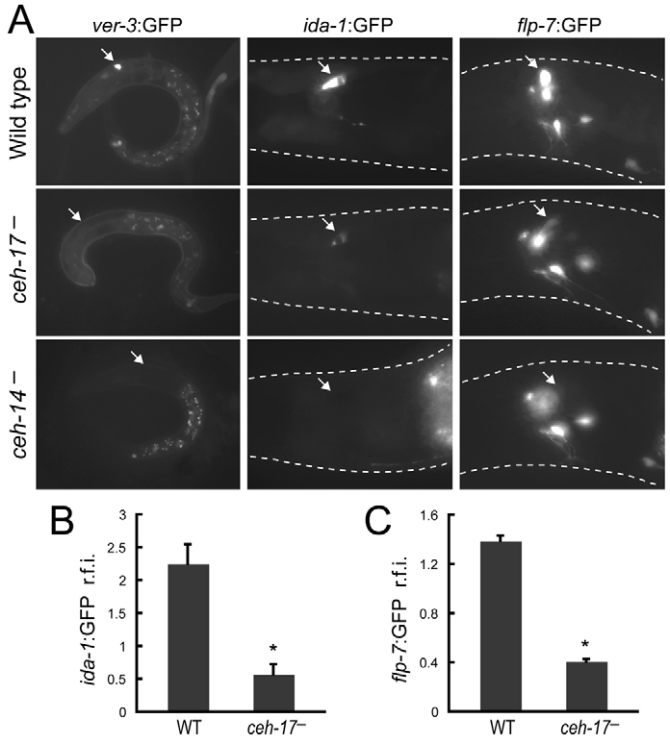

Fig. 3. CEH-17 and CEH-14 regulate the expression of *ver-3*, *ida-1* and *flp-7* in ALA. (**A**) Fluorescence images show *ceh-17* and *ceh-14* null animals defective in ALA-specific expression of several reporter genes. *ver-3*:GFP is normally expressed in ALA throughout development; L1 animals also show expression in the tail, and hence this stage was chosen for identification of transgenic animals. *ida-1*:GFP and *flp-7*:GFP reporters were examined at the L4 stage. Expression of each reporter in ALA (arrows) was decreased or completely abolished in *ceh-17*(*np1*) and *ceh-14*(*ch3*) animals. Anterior is to the left, dorsal is up. (**B**,**C**) Quantification of reporter expression in *ceh-17* L4 animals. As many *ceh-17* mutant animals showed detectable expression of the *ida-1* and *flp-7* reporter genes, ALA fluorescence intensity was quantified as described in Methods. * P<0.0001 versus wild type, *t*-test (*ida-1*:GFP) and U-test (*flp-7*:GFP). WT, wild type.

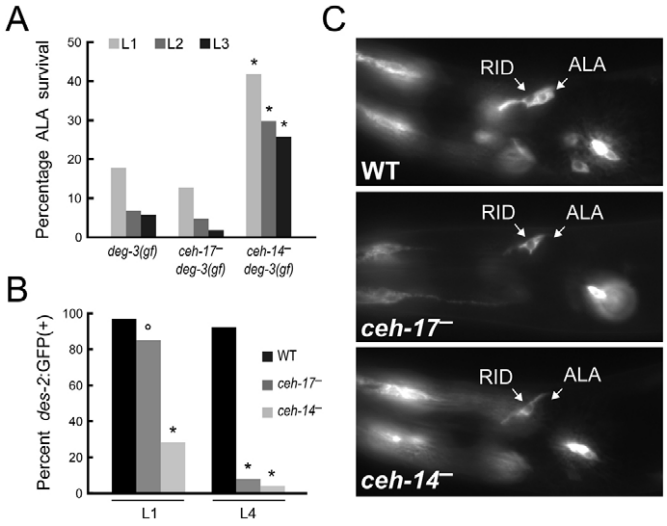

Fig. 4. CEH-17 and CEH-14 contribute to *deg-3* expression in ALA. (**A**) ALA neuron survival at the L1-L3 stages in animals carrying the *deg-3*(*gf*) mutation. The *ceh-14* null mutation suppresses the ALA degeneration phenotype of *deg-3*(*gf*), suggesting that CEH-14 contributes to early *deg-3* expression in ALA. *P<0.01 versus *deg-3*(*gf*), Fisher's exact test. (**B**) Percentage of animals expressing a *deg-3* reporter gene, *des-2*:GFP, in ALA at the first and last larval stages. *des-2*:GFP expression in ALA is dependent on *ceh-14* across larval stages but dependent on *ceh-17* only at the later stage. ˚ no significant difference and *P<0.0001 versus wild type, Fisher's exact test. (**C**) The head region of L4 stage animals showing *des-2*:GFP expression. In wild type, *des-2*:GFP is expressed in the M1 head muscles and several neurons, including ALA and RID. In *ceh-17* and *ceh-14* null mutant L4 animals, *des-2*:GFP is undetectable in ALA. Anterior is to the left, dorsal is up. WT, wild type.

1997). We found no defects in the expression of *unc-119*:YFP or *rab-3*:GFP reporter genes in either *ceh-17* or *ceh-14* mutant animals (Table 2), indicating that ALA retains neuronal character in these mutants. We then examined several ALA-expressed genes that have more restricted patterns of expression. *ver-3* encodes a VEGF receptor-related tyrosine kinase with postembryonic expression that is restricted to the ALA neuron and a few muscle cells (Popovici et al., 2002). *ida-1* encodes a tyrosine phosphatase-like transmembrane protein that is associated with dense core vesicles and is expressed in several secretory cell types, including ALA (Zahn et al., 2001; Cai et al., 2004). *flp-7* encodes an FMRFamide neuropeptide that is expressed in ALA and several other neurons (Kim and Li, 2004). Using GFP reporters, we examined the expression of these genes in *ceh-17*(*np1*) and *ceh-14*(*ch3*) mutant animals, and in each case found ALA-specific gene expression to be impaired (Table 2, Fig. 3). We then tested whether any of these genes are required for EGF-induced sleep: only the *ida-1* mutation had an effect, and a mild one (Table 1). Thus CEH-17 and CEH-14 affect the expression of an array of genes in ALA, some of which are required for sleep and others that are of unknown function.

Another gene that is known to function in ALA is *deg-3*, encoding a nicotinic acetylcholine receptor subunit. Expression of DEG-3 in ALA has not been noted in studies of *deg-3* reporter genes, but we have observed DEG-3 activity in ALA using a functional readout (Van Buskirk and Sternberg, 2007). In this analysis, we used a gain-of-function mutation, *deg-3*(*u662*), that causes constitutive channel activity and cell-autonomous neurodegeneration (Treinin and Chalfie, 1995). ALA degenerates in *deg-3*(*gf*) animals, with only 8% of ALA neurons surviving at the L2 stage. To determine whether CEH-17 and/or CEH-14 regulate *deg-3* expression in ALA, we tested the ability of null mutations in these transcription factors to rescue the ALA degeneration phenotype of *deg-3*(*gf*), reasoning that impaired expression of the mutant receptor might allow neuronal survival. We found that *ceh-14*(*ch3*), but not *ceh-17*(*np1*), enhances ALA survival in *deg-3*(*gf*) mutant animals (Fig. 4A). Thus CEH-14, but not CEH-17, appears to contribute to *deg-3* expression in ALA, at least during the early stage of ALA development measured by this assay. We wished to confirm that this effect on DEG-3 activity reflects alterations in *deg-3* transcription. *deg-3* is the downstream gene in an operon comprising two functionally dependent alpha subunits of a heteromeric nAchR (Treinin et al., 1998). Accordingly, sequences upstream of the first gene, *des-2*, drive expression of GFP in a pattern similar to that observed with DEG-3 antibodies (Treinin et al., 1998; Yassin et al., 2001). We analyzed the expression of *des-2*:GFP in the ALA neuron of *ceh-14* and *ceh-17* null mutant animals and found that at the L1 stage, reporter expression is dependent on CEH-14 but not on CEH-17 (Fig. 4B),

Table 3. Genetic suppression of EGF-induced sleep at mid-larval and adult stages

+ hs:LIN-3/EGF	% EGF-resistant at L2	% EGF-resistant as adults
Wild type	0	0
ceh-14(ch3)	96	100
ceh-17(np1)	66*	100
alr-1(ok545)	0	0
mab-9(e1245)	0	0
ceh-10(ct78)	61	49

All animals were well fed, grown at 20°C and carried the hs:LIN-3 transgene. Hand-selected L2 and young adult animals were scored 2 hours after heat shock for feeding as a measure of resistance to the sleep-inducing effects of LIN-3/EGF expression. At least 50 animals of each genotype were scored at each stage. *P<0.0001, versus adult stage, Fisher's exact test.

consistent with our observed genetic results with *deg-3*(*gf*). At the L4 stage, however, *deg-3* expression is dependent on both factors (Fig. 4B,C), revealing different temporal requirements for CEH-17 and CEH-14 in *deg-3* expression.

CEH-10/Chx10 is required for ALA function

Our observation that CEH-17 is dispensable for *deg-3* expression early in development led us to examine whether this might be true for multiple CEH-17 targets. If so, the *ceh-17* null mutation might confer a lower level of EGF-resistance during early larval stages than in adults. We therefore assayed L2 stage animals for EGF-induced sleep, and found that, in contrast to *ceh-14* mutants, the EGF-resistance of *ceh-17* mutants is significantly lower at the L2 stage than in adults (Table 3). This observation suggested the involvement of an unidentified factor contributing to early ALA function. We investigated a potential redundancy between *ceh-17* and the most closely related Prd-like gene in the *C. elegans* genome, *alr-1* (Wormbase WS210). However, the deletion mutation *alr-1*(*ok545*) has no effect on ALA-dependent sleep (Table 3). MAB-9 is a T-box transcription factor that has been observed to be expressed in ALA (Appleford et al., 2008), but the null mutation *mab-9*(*e1245*) has no discernable effect on ALA function (Table 3). Another transcription factor expressed in ALA is the Q_{50} Prd-like protein CEH-10/Chx10, which has roles in the specification of the AIY, RMED, RID and CAN neurons (Forrester et al., 1998). CAN function is required for viability, and animals lacking CEH-10 activity die as first-stage larvae (Forrester et al., 1998). To examine CEH-10 in ALA function throughout development, we used a viable reduction-of-function mutation, *ceh-10*(*ct78*). We found the *ceh-10*(*rf*) mutation conferred partial EGF-resistance (Table 3), consistent with a role for CEH-10 in ALA function.

CEH-10 is required for ALA generation and differentiation

To investigate the role of CEH-10 in ALA neuron development, we first examined whether the ALA neuron was present in *ceh-10*(*rf*) and *ceh-10* null mutant animals. In wild type, the ALA neuron is identifiable by its position in the dorsal head ganglion alongside the RID neuron, which is not the sister cell of ALA but also expresses CEH-10. By DIC optics the ALA and RID nuclei can be seen in a region flanked by hypodermal nuclei, along the dorsal midline (Fig. 5A). We examined *ceh-10*(*gm133*) null mutant animals (at the early L1 stage before necrosis) for the presence of the ALA neuron, and found that while 34% of these animals possess ALA (and RID) neurons, the majority appear to lack both ALA and RID, while others show a single neuron in this region. (Fig. 5B). Thus a severe loss of CEH-10 function impairs either the generation or positioning of the ALA and RID neurons. In *ceh-10*(*ct78*) reduction-of-function mutant animals, these neurons can be found in their wild-type positions in 75% of cases, and in the remaining animals a single cell can be found where the ALA and RID normally reside (Fig. 5A,B). Our examination of ALA-specific reporters below reveals that approximately 90% of *ceh-10*(*rf*) animals possess an ALA neuron, and we infer that in the majority of cases in which only one of these neurons is detectable, it is the RID that is missing.

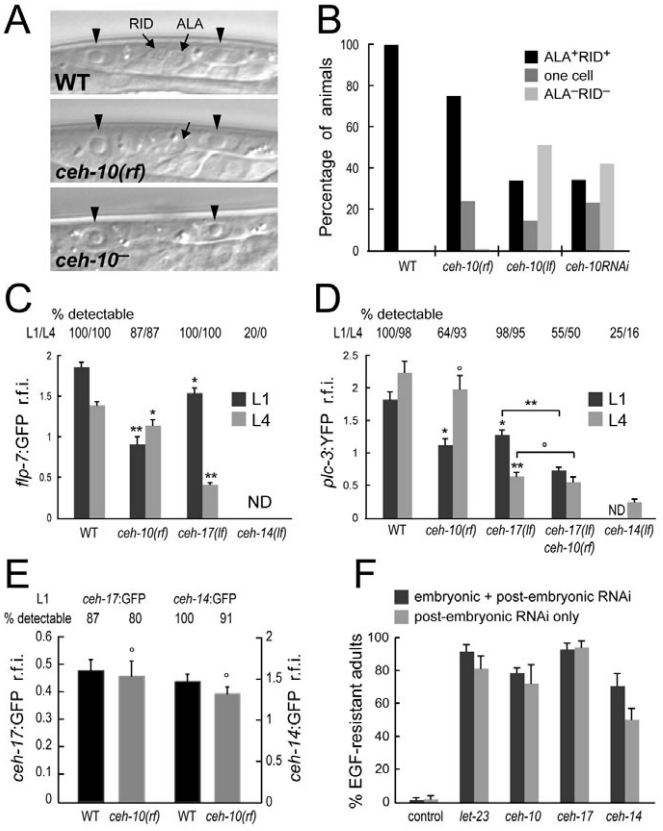

Fig. 5. CEH-10 functions in ALA generation and differentiation. (**A**) DIC images showing wild-type RID and ALA nuclei in the dorsal ganglion, flanked by hypodermal nuclei (arrowheads), a *ceh-10*(*ct78*) animal harboring only one neuron in this region (arrow) and a *ceh-10*(RNAi) animal lacking both RID and ALA. (**B**) Percentage of animals in which the ALA and RID neurons can be detected. (**C**) *flp-7*:GFP and (**D**) *plc-3*:YFP expression during the first (L1) and last (L4) larval stages, showing that the contribution of CEH-10 to ALA-specfic gene expression decreases over time, while that of CEH-17 increases. Among the animals with detectable expression, fluorescence intensity in ALA was quantified. *P*-values were calculated as described in Methods and are shown versus wild-type animals of the same stage unless otherwise indicated. *P<0.005, **P<0.0001, ° no significant difference. (**E**) ALA expression of *ceh-17*:GFP and *ceh-14*:GFP in *ceh-10*(*ct78*) animals is not significantly different from wild type at the first larval stage (shown) or at later stages (data not shown). (**F**) RNAi against *let-23*/EGFR or any of the three *ceh* genes can interfere with the ability of the the ALA neuron to mediate EGF-induced sleep, whether initiated embryonically or postembryonically. Mean EGF resistance was averaged from at least three RNAi trials. Error bars: s.e.m. WT, wild type. rf, reduction of function; lf, loss of function. *ceh-10*(*rf*)=*ct78*; *ceh-10*(*lf*)=*gm133*; *ceh-17*(*lf*)=*np1*; *ceh-14*(*lf*)=*ch3*.

To examine the state of ALA differentiation in animals with reduced CEH-10 function, we examined the expression of pan-neuronal and ALA-specific reporter genes in *ceh-10*(*ct78*) mutant animals. We observed wild-type expression of the pan-neuronal reporters *unc-119*:YFP and *rab-3*:GFP in both the ALA and RID neurons (not shown). However, expression of the ALA-specific reporters *flp-7*:GFP and *plc-3*:YFP are impaired, and more at the L1 stage than at L4 (Fig. 5C,D). Thus the *ceh-10*(*rf*) mutation impairs ALA-specific gene expression, but this defect is ameliorated as development proceeds. Conversely, the effects of *ceh-17*(*lf*) on ALA-specific gene expression are more pronounced at later larval stages. Consistent with CEH-10 contributing to early gene expression in ALA, we found *plc-3*:YFP expression in *ceh-17*(*lf*);*ceh-10*(*rf*) animals to be more severely impaired compared with *ceh-17*(*lf*) alone at the L1 stage but not at the L4 stage (Fig. 5D). *ceh-14*(*ch3*) animals show a severe impairment of reporter gene expression across larval stages (percentage detectable, Fig. 5C,D). We also examined whether *ceh-10*(*ct78*) impairs the expression of *ceh-17*:GFP or *ceh-14*:GFP reporter genes in ALA, and found no difference from wild type, either at the L1 stage (Fig. 5E) or later in development (not shown).

To further investigate the temporal requirements for each of these transcription factors, we performed RNA-mediated interference (RNAi) using an RNAi-sensitized strain (Materials and methods). For comparison, we performed RNAi against *let-23*/EGFR, which is expressed in ALA and mediates the EGF-induced sleep response. Animals were exposed to RNAi continuously from either the start of embryogenesis or beginning at the L1 stage. Young adults were then tested for the ALA-dependent response to EGF expression. We found that RNAi against each of the three transcription factors or *let-23* could produce severe defects in ALA function when initiated either embryonically or postembryonically (Fig. 5F). Thus CEH-14, CEH-17 and CEH-10 each have postembryonic roles in ALA differentiation. Also, when *ceh-10* RNAi was initiated embryonically, a fraction of the resulting animals lacked a detectable ALA neuron, similar to the *ceh-10* null mutant (Fig. 5A,B). Thus CEH-10 is required during embryogenesis for the generation or placement of the ALA neuron, and again postembryonically for ALA function.

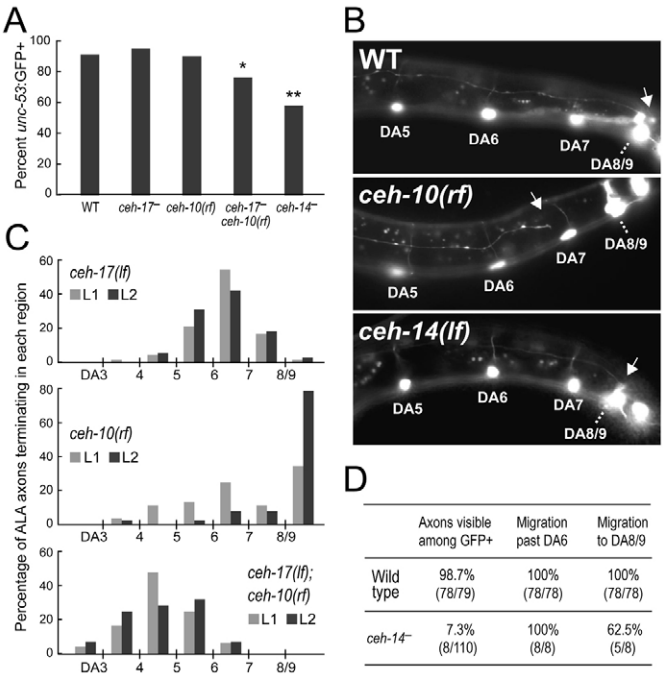

Fig. 6. Prd and LIM proteins differentially affect ALA axon outgrowth. (**A**) Percentage of animals expressing an *unc-53*:GFP reporter in the ALA cell body. **P*=0.0033 versus WT and *P*=0.0159 versus *ceh-10*(*rf*) alone, ***P*<0.0001 versus WT, Fisher's exact test. (**B**) *unc-53*:GFP labels ALA and the DA motoneurons, among others. In wild type, ALA axons are seen to extend past all DA commissures to reach the tail, while in *ceh-10*(*ct78*), ALA axons often fail to complete their migration, similar to *ceh-17*(*lf*) animals. In *ceh-14* null animals in which ALA axons are detectable, the majority reach the tail. Anterior is to the left, dorsal is up. Arrows indicate axon end points. (**C**) Distribution of ALA axon end points with respect to DA commissures at the L1 and L2 stages in *ceh-17*(*np1*), *ceh-10*(*ct78*), and doubly mutant animals. ALA axons can be seen to reach the tail between the L1 and L2 stages in *ceh-10*(*ct78*) in a CEH-17 dependent manner. (**D**) Among *ceh-14*(*ch3*) animals with bright *unc-53*:GFP expression in the ALA cell body, very few ALA axons can be detected; those that are visible extend past the mid-body and the majority reach the tail (DA8/9).

Prd and LIM class proteins differentially affect ALA axon outgrowth

In wild-type embryogenesis, the ALA cell body extends two axons that enter lateral cords and migrate to the tail (White et al., 1986). In *ceh-17*(*np1*), ALA axons fail to complete their migration, with the majority stopping just past the midbody (Pujol et al., 2000). To determine whether CEH-10 and CEH-14 also play roles in ALA axon migration, we examined *unc-53*:GFP (pNP21; N. Pujol), which labels several neurons including ALA and the DA neurons of the ventral cord that extend commissural axons, marking body length (Stringham et al., 2002). In wild-type animals this reporter labels the ALA axons during the L1-L2 stages. We first examined *unc-53*:GFP in ALA in *ceh-17*(*null*) and *ceh-10*(*rf*) L1-L2 animals and found that expression of the reporter was not detectably impaired (Fig. 6A). We then scored the extent of ALA axon migration and found that the *ceh-10*(*rf*) animals showed a *ceh-17*-like truncation of the ALA axons, albeit less severe (Fig. 6B,C). While the *ceh-17*(*lf*) axon migration phenotype is similar when scored in either L1 or L2 stage animals, we found that a greater fraction of *ceh-10*(*rf*) ALA axons reach the tail by the L2 stage than at L1. This was surprising, as wild-type ALA axons complete their migration before hatching (Pujol et al., 2000). To determine if the postembryonic ALA axon migration seen in *ceh-10*(*rf*) mutant animals might be dependent on CEH-17 activity, we examined ALA axon migration in *ceh-17*(*np1*);*ceh-10*(*ct78*) animals. We found the extent of axon migration to be similar between the L1 and the L2 stages, and more severe at each stage than in either single mutant (Fig. 6C). These observations suggest a role for both CEH-10 and CEH-17 in ALA axon migration, and to a capacity of stalled ALA axons to migrate after hatching in a CEH-17-dependent manner.

We then examined *unc-53*:GFP expression in *ceh-14* null mutant animals, and observed a mild but significant decrease in reporter expression, indicating that CEH-14 affects *unc-53* transcription in ALA (Fig. 6A). To analyze axon migration, we therefore chose only animals in which *unc-53*:GFP intensity in the ALA cell body was comparable with wild type. Unexpectedly, ALA axons could not be identified in the vast majority of these *ceh-14*(*ch3*) *unc-*

53:GFP+ animals (Fig. 6D), indicating that CEH-14 functions at an early stage of axon projection. Consistent with this interpretation, short neurites of varying morphology have been observed to extend ventrally from the ALA cell body in *ceh-14* null mutant animals (H. Kagoshima and T. Bürglin, personal communication), but these fail to enter the lateral bundles that project to the tail. In the few *ceh-14*(*ch3*) animals in which we did observe lateral ALA axons, they extended well past the midbody, and the majority completed their migration to the tail (Fig. 6B,D). This observation suggests that although CEH-14 is required for the initial pathfinding of the ALA axons, it is largely dispensable for axon migration per se. CEH-17, by contrast, is dispensable for initial pathfinding but plays a major role in posterior axon migration, and does so in all neurons in which it is expressed (Pujol et al., 2000). As CEH-14 is required for wild-type levels of CEH-17 in ALA, we would expect to see a *ceh-17*-like axon migration defect among the rare axons that do extend in the *ceh-14* mutant; and indeed, some of the ALA axons in *ceh-14*(*ch3*) stop short of the tail (Fig. 6D).

DISCUSSION

Differentiation of a sleep-inducing neuron

Here we have shown that mutations in the *C. elegans* homeodomain transcription factors CEH-10, CEH-14 and CEH-17 confer resistance to the sleep-inducing effect of EGF, which is mediated by the ALA neuron. A model for how these factors control ALA differentiation is outlined in Fig. 7. CEH-14 and CEH-17 are not required for the generation of ALA, nor for its adoption of neuronal character; the EGF-resistance of these mutants arises from defects in the expression of an ALA-specific battery of genes that includes components of the sleep response such as the EGF receptor *let-23* and its downstream effector phospholipase C-γ (*plc-3*). These genes are normally expressed in the ALA neuron throughout larval development and adulthood, allowing ALA to function in EGF-dependent sleep throughout the life of the animal. CEH-17 and CEH-14 are themselves maintained at relatively constant levels in the ALA neuron by a transcriptional autoregulatory loop in which each factor contributes to the expression of the other and to its own expression. We have observed that even mild reductions in ALA-specific gene expression are correlated with a high degree of EGF-resistance. We infer that there are additional components of the sleep response besides *let-23* and *plc-3* that are impaired in the transcription factor mutants, and that small reductions in the expression of many genes can produce penetrant defects in ALA function.

Several lines of evidence suggest that the Paired-like HD factor CEH-10 overlaps functionally with CEH-17 in ALA. First, the *ceh-17* null mutant phenotype is enhanced by a *ceh-10*(*rf*) mutation, with respect to its effects on gene expression (*plc-3*:YFP and *unc-53*:GFP) and axon migration. In addition, examination of reporter genes across larval stages shows that as development proceeds, the contribution of CEH-10 to ALA-specific gene expression decreases while that of CEH-17 increases. Lastly, a fraction of *ceh-10*(*rf*) animals hatch with incompletely migrated ALA axons that can complete their migration postembryonically only if CEH-17 is present. These observations indicate that CEH-10 and CEH-17 function similarly in ALA differentiation. One possibility is that these Prd-like class HD proteins possess similar DNA-binding specificities. A target cis-regulatory motif has been identified for CEH-10 (Wenick and Hobert, 2004), although not yet for CEH-17 for comparison.

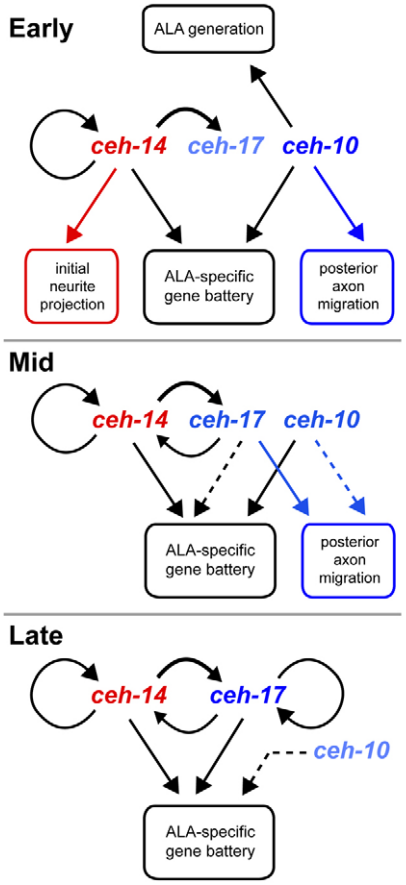

Fig. 7. Model for transcriptional regulation of ALA neuron development in *C. elegans*. Early (embryogenesis): the Prd-like homeobox gene *ceh-10* is required for the generation of ALA and several lineally related neurons. The ALA neuron extends axons along lateral cords to the tail; the LIM class homeobox gene *ceh-14* is required for the projection of the axons into the lateral cords and *ceh-10* is required for their posterior migration. Both *ceh-14* and *ceh-10* contribute to the expression of a common ALA-specific gene battery, and *ceh-14* directs the expression of the Prd-like homeobox gene *ceh-17*. Mid (late embryo and early larva): as development proceeds, the contribution of *ceh-17* to ALA-specific gene expression increases. *ceh-17* is required for ALA axons to reach the tail, and directs a similar aspect of axon migration in other neurons in which it is expressed. Unlike *ceh-10*, *ceh-17* can direct stalled ALA axons to complete their migration postembryonically. *ceh-17* also contributes to *ceh-14* expression by the L1 stage. Late (late larva and adult): *ceh-17* shows autoregulation in ALA and contributes with *ceh-14* to target gene expression. *ceh-10* contributes less significantly to ALA-specific gene expression than at earlier stages.

Re-utilization of CEH-10 at multiple steps of neuron development

In a fraction of *ceh-10* mutant animals, the ALA neuron cannot be identified at its normal location. The ALA neuron might be generated but fail to reach its correct position or express ALA-specific markers, and thus go undetected. Alternatively, loss of CEH-10 could disrupt divisions in the ALA lineage such that the ALA neuron is never generated. Consistent with this latter possibility, the sister cell of ALA, the RMED neuron, is undetectable in the majority of *ceh-10*(*gm127*) (rf) mutant animals (Forrester et al., 1998). It is therefore possible that CEH-10

functions during the embryonic cell divisions that give rise to ALA and RMED. Our results show that the function of CEH-10 in ALA development is not limited to embryogenesis, however: RNAi against *ceh-10* can impair ALA function even when initiated postembryonically. CEH-10 therefore functions at multiple steps in the neuronal differentiation program of ALA. CEH-10 may act similarly in the development of the canal-associated neurons (CANs): these neurons are undetectable in severe *ceh-10* mutants, whereas mild reductions in CEH-10 activity disrupt only CAN migration (Forrester et al., 1998). We have shown here that the CEH-10-expressing neuron RID is also undetectable in *ceh-10* mutants. Thus CEH-10 appears to affect the generation of several neurons within a given lineage (ALA, RMED, CAN and RID are all daughters of the blast cell 'ABalap'), and also acts in the differentiation of at least some of these. Similarly, the *ceh-10* ortholog Chx10 is required at multiple stages of murine retinal development, early for retinal progenitor proliferation and later for bipolar cell differentiation (Burmeister et al., 1996).

ALA versus AIY neuron differentiation

One well characterized example of LIM and Prd HD function comes from *C. elegans* AIY interneuron differentiation, which depends on the cooperative binding of TTX-3 (LIM) and CEH-10 (Prd-like) proteins to a motif found near many AIY-expressed genes (Wenick and Hobert, 2004). By contrast with ALA, severe reduction of *ceh-10* function does not interfere with generation of the AIY interneurons (Forrester et al., 1998); indeed, CEH-10 is not expressed until after cleavage of the AIY mother (Bertrand and Hobert, 2009). CEH-10 is required for TTX-3 expression in AIY, and these factors automaintain their expression, locking in the differentiation program. Differentiation of the ALA neuron is similar to that of AIY in that LIM and Prd proteins are required for neuron-specific gene expression, but in this case the task of the Prd class protein is split by CEH-10 and CEH-17, with contribution of CEH-17 to ALA-specific gene expression increasing over time while that of CEH-10 decreases. CEH-17 and CEH-14 participate in a positive-feedback circuit, maintaining the differentiated state. Given the earlier role of CEH-10 in ALA generation, we would predict that CEH-10 initiates expression of CEH-14 and/or CEH-17, and thus the positive autoregulatory circuit, in ALA. However, we cannot detect any effect of the reduction-of-function mutation *ceh-10*(*ct78*) on the expression of *ceh-17* or *ceh-14* reporter genes in ALA, and what initiates the expression of these factors is unknown. The postembryonic requirement for CEH-17 and CEH-14, along with their expression in ALA throughout development and adulthood, suggests an ongoing function for these proteins. However, their confirmation as terminally acting transcription factors will rely on the identification of the cis-regulatory elements directing gene expression in ALA.

LIM and Prd HD regulation of axon migration

Previous work suggested that CEH-17 functions specifically in axon migration (Pujol et al., 2000). We have shown here that CEH-17 affects the expression of multiple genes in ALA that have no known role in axon migration – a point that we pressed further by analyzing *let-23* and *ver-3* mutants for ALA axon defects, for which we found none (not shown). Thus CEH-17 regulates multiple aspects of ALA differentiation. The role of CEH-17 in axon migration appears to be limited to the later stages of axon elongation, as the majority of *ceh-17* null mutant ALA axons extend past the midbody but fail to complete their migration to the tail. We observe a similar phenotype in the *ceh-10*(*ct78*) mutant; however this is a partial reduction of function allele, and we would predict a complete loss of CEH-10 to produce a more severe truncation. An intriguing aspect of the *ceh-10*(*ct78*) phenotype is that the ALA axons continue to migrate after hatching. We have shown that this postembryonic migration is dependent on CEH-17. This is reminiscent of the contributions of CEH-10 and CEH-17 to other aspects of ALA differentiation: we have shown that ALA expression of a *plc-3* reporter gene is impaired by *ceh-10*(*ct78*) at the L1 stage but that *plc-3* expression improves over time in a CEH-17-dependent manner to reach near wild-type levels by the L4 stage. An analysis of Prd HD function alone would suggest that ALA axon migration is simply another aspect of neuronal identity specified by a common master regulatory machinery. However, this is not the case: CEH-14 acts in concert with the Prd HD proteins to activate an ALA-specific gene battery, but not in the regulation of axon migration. Rather, ALA axons are defective at an early stage of outgrowth in *ceh-14* mutant animals and fail to enter the lateral cord, an effect not associated with reduction of CEH-10 or CEH-17 activity. Among the rare *ceh-14* mutant ALA axons that do initiate their posterior migration, the majority reach the tail, revealing that CEH-14 is not crucial for axon migration itself. Thus while both LIM and Prd class proteins contribute to the expression of a common ALA-specific gene battery, they regulate different aspects of ALA axon development, with the Prd-like protein CEH-17 regulating posterior axon migration in all neurons in which it is expressed. Thus axon migration is a feature of ALA differentiation that appears to be adopted in a partially modular fashion, possibly reflecting a modular (and more flexible) ancestral mode of neuron differentiation.

Acknowledgements

We are grateful to N. Pujol for reporter constructs and insightful comments and to H. Kagoshima and T. Bürglin for sharing unpublished observations. We thank M. Nonet, M. Treinin, S. Xu, G. Schindelman, R. Roubin, I. Hope, C. Li and T. Cai for reporter constructs, the Caenorhabditis Genetics Center for strains, members of the Sternberg lab for helpful discussions and O. Hobert and A. Wright for critical reading of the manuscript. This work was supported by a grant from the US National Institute on Drug Abuse (DA018341) to P.W.S., an investigator of the HHMI. Deposited in PMC for release after 6 months.

Competing interests statement

The authors declare no competing financial interests.

References

Altun-Gultekin, Z., Andachi, Y., Tsalik, E. L., Pilgrim, D., Kohara, Y. and Hobert, O. (2001). A regulatory cascade of three homeobox genes, *ceh-10*, *ttx-3* and *ceh-23*, controls cell fate specification of a defined interneuron class in *C. elegans*. *Development* **128**, 1951-1969.

Appleford, P. J., Gravato-Nobre, M., Braun, T. and Woollard, A. (2008). Identification of cis-regulatory elements from the *C. elegans* T-box gene *mab-9* reveals a novel role for *mab-9* in hypodermal function. *Dev. Biol.* **317**, 695-704.

Aroian, R. V., Koga, M., Mendel, J. E., Ohshima, Y. and Sternberg, P. W. (1990). The *let-23* gene necessary for *Caenorhabditis elegans* vulval induction encodes a tyrosine kinase of the EGF receptor subfamily. *Nature* **348**, 693-699.

Bertrand, V. and Hobert, O. (2009). Wnt asymmetry and the terminal division of neuronal progenitors. *Cell Cycle* **8**, 1973-1974.

Brenner, S. (1974). The genetics of *Caenorhabditis elegans*. *Genetics* **77**, 71-94.

Brunet, J. F. and Pattyn, A. (2002). Phox2 genes-from patterning to connectivity. *Curr. Opin. Genet. Dev.* **12**, 435-440.

Burmeister, M., Novak, J., Liang, M. Y., Basu, S., Ploder, L., Hawes, N. L., Vidgen. D., Hoover, F., Goldman, D., Kalnins, V. I. et al. (1996). Ocular retardation mouse caused by *Chx10* homeobox null allele: impaired retinal progenitor proliferation and bipolar cell differentiation. *Nat. Genet.* **12**, 376-384.

Cai, T., Fukushige, T., Notkins, A. L. and Krause, M. (2004). Insulinoma-associated protein IA-2, a vesicle transmembrane protein, genetically interacts with UNC-31/CAPS and affects neurosecretion in *Caenorhabditis elegans*. *J. Neurosci.* **24**, 3115-3124.

Cassata, G., Kagoshima, H., Andachi, Y., Kohara, Y., Durrenberger, M. B., Hall, D. H. and Burglin, T. R. (2000). The LIM homeobox gene *ceh-14* confers thermosensory function to the AFD neurons in *Caenorhabditis elegans*. *Neuron* **25**, 587-597.
Certel, S. J. and Thor, S. (2004). Specification of *Drosophila* motoneuron identity by the combinatorial action of POU and LIM-HD factors. *Development* **131**, 5429-5439.
Chang, C., Newman, A. P. and Sternberg, P. W. (1999). Reciprocal EGF signaling back to the uterus from the induced *C. elegans* vulva coordinates morphogenesis of epithelia. *Curr. Biol.* **9**, 237-246.
Deplancke, B., Dupuy, D., Vidal, M. and Walhout, A. J. (2004). A gateway-compatible yeast one-hybrid system. *Genome Res.* **14**, 2093-2101.
Dupuy, D., Li, Q. R., Deplancke, B., Boxem, M., Hao, T., Lamesch, P., Sequerra, R., Bosak, S., Doucette-Stamm, L., Hope, I. A. et al. (2004). A first version of the *Caenorhabditis elegans* Promoterome. *Genome Res.* **14**, 2169-2175.
Edlund, T. and Jessell, T. M. (1999). Progression from extrinsic to intrinsic signaling in cell fate specification: a view from the nervous system. *Cell* **96**, 211-224.
Forrester, W. C., Perens, E., Zallen, J. A. and Garriga, G. (1998). Identification of *Caenorhabditis elegans* genes required for neuronal differentiation and migration. *Genetics* **148**, 151-165.
Granato, M., Schnabel, H. and Schnabel, R. (1994). *pha-1*, a selectable marker for gene transfer in *C. elegans*. *Nucleic Acids Res.* **22**, 1762-1763.
Hill, R. J. and Sternberg, P. W. (1992). The gene *lin-3* encodes an inductive signal for vulval development in *C. elegans*. *Nature* **358**, 470-476.
Hobert, O. (2008). Regulatory logic of neuronal diversity: terminal selector genes and selector motifs. *PNAS* **105**, 20067-20071.
Kim, K. and Li, C. (2004). Expression and regulation of an FMRFamide-related neuropeptide gene family in *Caenorhabditis elegans*. *J. Comp. Neurol.* **475**, 540-550.
Maduro, M. and Pilgrim, D. (1995). Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. *Genetics* **141**, 977-988.
Moghal, N. and Sternberg, P. W. (2003). The epidermal growth factor system in *Caenorhabditis elegans*. *Exp. Cell Res.* **284**, 150-159.
Nonet, M. L., Staunton, J. E., Kilgard, M. P., Fergestad, T., Hartwieg, E., Horvitz, H. R., Jorgensen, E. M. and Meyer, B. J. (1997). *Caenorhabditis elegans rab-3* mutant synapses exhibit impaired function and are partially depleted of vesicles. *J. Neurosci.* **17**, 8061-8073.
Popovici, C., Isnardon, D., Birnbaum, D. and Roubin, R. (2002). *Caenorhabditis elegans* receptors related to mammalian vascular endothelial growth factor receptors are expressed in neural cells. *Neurosci. Lett.* **329**, 116-120.
Pujol, N., Torregrossa, P., Ewbank, J. J. and Brunet, J. F. (2000). The homeodomain protein CePHOX2/CEH-17 controls antero-posterior axonal growth in *C. elegans*. *Development* **127**, 3361-3371.
Raizen, D. M., Zimmerman, J. E., Maycock, M. H., Ta, U. D., You, Y. J., Sundaram, M. V. and Pack, A. I. (2008). Lethargus is a *Caenorhabditis elegans* sleep-like state. *Nature* **451**, 569-572.
Rual, J.-F., Klitgord, N. and Achaz, G. (2007). Novel insights into RNAi off-target effects using *C. elegans* paralogs. *BMC Genomics* **8**, 106.
Schindelman, G., Whittaker, A. J., Thum, J. Y., Gharib, S. and Sternberg, P. W. (2006). Initiation of male sperm-transfer behavior in *Caenorhabditis elegans* requires input from the ventral nerve cord. *BMC Biol.* **4**, 26.
Shirasaki, R. and Pfaff, S. L. (2002). Transcriptional codes and the control of neuronal identity. *Annu. Rev. Neurosci.* **25**, 251-281.
Stringham, E., Pujol, N., Vandekerckhove, J. and Bogaert, T. (2002). *unc-53* controls longitudinal migration in *C. elegans*. *Development* **129**, 3367-3379.
Thaler, J. P., Lee, S. K., Jurata, L. W., Gill, G. N. and Pfaff, S. L. (2002). LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. *Cell* **110**, 237-249.
Timmons, L., Court, D. L. and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in *Caenorhabditis elegans*. *Gene* **263**, 103-112.
Treinin, M. and Chalfie, M. (1995). A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*. *Neuron* **14**, 871-877.
Treinin, M., Gillo, B., Liebman, L. and Chalfie, M. (1998). Two functionally dependent acetylcholine subunits are encoded in a single *Caenorhabditis elegans* operon. *PNAS* **95**, 15492-15495.
Van Buskirk, C. and Sternberg, P. W. (2007). Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. *Nat. Neurosci.* **10**, 1300-1307.
Wenick, A. S. and Hobert, O. (2004). Genomic cis-regulatory architecture and trans-acting regulators of a single interneuron-specific gene battery in *C. elegans*. *Dev. Cell* **6**, 757-770.
White, J. G., Southgate, E., Thomson, J. N. and Brenner, F. R. S. (1986). The structure of the nervous system of the nematode *Caenorhabditis elegans*. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **314**, 1-340.
Wightman, B., Clark, S. G., Taskar, A. M., Forrester, W. C., Maricq, A. V., Bargmann, C. I. and Garriga, G. (1996). The *C. elegans* gene *vab-8* guides posteriorly directed axon outgrowth and cell migration. *Development* **122**, 671-682.
Yassin, L., Gillo, B., Kahan, T., Halevi, S., Eshel, M. and Treinin, M. (2001). Characterization of the DEG-3/DES-2 receptor: a nicotinic acetylcholine receptor that mutates to cause neuronal degeneration. *Mol. Cell. Neurosci.* **17**, 589-599.
Zahn, T. R., Macmorris, M. A., Dong, W., Day, R. and Hutton, J. C. (2001). IDA-1, a *Caenorhabditis elegans* homolog of the diabetic autoantigens IA-2 and phogrin, is expressed in peptidergic neurons in the worm. *J. Comp. Neurol.* **429**, 127-143.
Zimmerman, J. E., Naidoo, N., Raizen, D. M. and Pack, A. I. (2008). Conservation of sleep: insights from non-mammalian model systems. *Trends Neurosci.* **31**, 371-376.