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

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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 EGF receptor (for a review, see Moghal and Sternberg, 2003). 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 Q50 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). PS6528 syIs197[hsl LIN-3-3C, myo-2 dsRed], him-5[e1490]. PS301 let-23[syIs10];rol-6[e187] unc-4[e120]. IB16 ceh-17(np1)]. TB528 ceh-14(ch3). BW506 ceh-10[c78]. VC444 ver-3(gk227). VC226 ida-1(oka49). RIB1990 flp-7(ok2625). TU174 deg-3(u662). RB762 alr-1(ok545). PST7 mab-9(e1245); him-5(e1490). PS9873 ceh-17(np1); ceh-14(ch3). PS5043 +/ht2[qs48]; ceh-10(gm333)ht2]. PS6589 ceh-17(np1); syIs197. him-5(e1490). PS9388 ceh-17(np1); deg-3(u662). PS9564 ceh-10[c78]; syIs197. him-5(e1490). PS961 syIs197. him-5(e1490); ceh-14(ch3). PS9835 deg-3(u662); ceh-14(ch3). The following strains were raised at 15°C: PS1217 pcha-1(e1212ts); syIs197. him-5[e1490]. PS8850 ceh-17(np1); pcha-1(e1212ts). PS9563 pcha-1(e1212ts); ceh-14(ch3)]. PS5538 erv-1(mg366); syIs197. him-5[e1490]; lin-15[b744]. Strains not starting with a ‘PS’ designation were obtained from the spontaneous integrant) 2.1 kb ida-1:YFP (S. Xu, personal communication); plc-3 B isoform): syEx234 3.6 kb let-23:LET-23:GFP pk7GL44.2 (Chang et al., 1999); syEx1157 3.0 kb pch-3:GFP (S. Xu, personal communication); syEx1080 3.0 kb ver-3:GFP (Popovic et al., 2002); syEx1084 3.3 kb ida-1:IDAD:GFP (Cai et al., 2004); ynl666 3.4 kb flp-7:GFP (Kim and Li, 2004); syEx1145 2.0 kb ceh-14:GFP JRH/A9H (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-19:YFP (Schindelman et al., 2006); syEx1146 1.2 kb rab-3:GFP pAB100 (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; and 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 1-2A09 (ceh-17), Open Biosystems 11052G9 (ceh-10) and 11077F2 (ceh-14). These genes share sufficient 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 EcoRV-linked primers 5'-GGATATCTGAAAATCGCTGAAATGG-3' and 5'-GGATATCGAAATCGCTGAAATGG-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 EGFR-induced sleep; a mutation disrupting ALA axon growth, van-8(e1017), does not disrupt the sleep response (Wightman et al., 1996; Van Buskirk and Sternberg, 2007). By contrast, mutation of CEH-17, a Q0 Paired-like class homeodomain transcription factor, the function of which is known only in axon migration (Pujol et al., 2000), renders animals
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), 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

<table>
<thead>
<tr>
<th>+ hs::LIN-3/EGF</th>
<th>% EGF-resistant</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0</td>
<td>&gt;100</td>
</tr>
<tr>
<td>let-23(sy10)</td>
<td>94</td>
<td>18</td>
</tr>
<tr>
<td>ceh-17(np1)</td>
<td>100</td>
<td>75</td>
</tr>
<tr>
<td>ceh-14(ch3)</td>
<td>100</td>
<td>90</td>
</tr>
<tr>
<td>ver-3(gk227)</td>
<td>0</td>
<td>30</td>
</tr>
<tr>
<td>ida-1(ok409)</td>
<td>5</td>
<td>84</td>
</tr>
<tr>
<td>flp-7(ok2625)</td>
<td>0</td>
<td>64</td>
</tr>
</tbody>
</table>

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 (Pujo et al., 2000). As neither let-23 nor plc-3 are expressed in the SIA neurons, 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

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

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).

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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), t.r.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.
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-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).

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., 1995) by examining the expression of unc-119 and rab-3::GFP transgenes in ceh-17 and ceh-14 mutants. Expression levels of unc-119 and rab-3 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.
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),
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 Q0 Prd-like protein CEH-10/Chx10, which has roles in the specification of the A1Y, 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.
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(n178) 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(n178) 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 RNAi-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.

**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 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.

In ceh-10(rf) mutant animals, ALA axons often fail to 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 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 morphometry 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 AIG neuron differentiation

One well characterized example of LIM and Prd HD function comes from *C. elegans* AIG interneuron differentiation, which depends on the cooperative binding of TTX-3 (LIM) and CEH-10 (Prd-like) proteins to a motif found near many AIG-expressed genes (Wenick and Hobert, 2004). By contrast with ALA, severe reduction of ceh-10 function does not interfere with generation of the AIG interneurons (Forrester et al., 1998); indeed, CEH-10 is not expressed until after cleavage of the AIG mother (Bertrand and Hobert, 2009). CEH-10 is required for TTX-3 expression in AIG, and these factors autoregulate their expression, locking in the differentiation program. Differentiation of the ALA neuron is similar to that of AIG 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 AIG. However, we cannot detect any effect of the reduction-of-function mutation *ceh-10(ok178)* on the expression of *ceh-17* or *ceh-14* reporter genes in ALA, and it 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(ok178)* 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(ok178)* 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(ok178)* 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.

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### Competing interests statement

The authors declare no competing financial interests.

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