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Co-expression of the transcription factors CEH-14 and TTX-1 regulates AFD neuron-specific genes *gcy-8* and *gcy-18* in *C. elegans*

Hiroshi Kagoshima [a,b], Yuji Kohara [a,*]

[a] Genome Biology Laboratory, Center for Genetic Resource Information, National Institute of Genetics, Yata 1111, Mishima, Shizuoka 411-8540, Japan
[b] Transdisciplinary Research Integration Center, Research Organization of Information and Systems (ROIS), Toranomon 4-3-13, Minato-ku, Tokyo 105-0001, Japan

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ABSTRACT

A wide variety of cells are generated by the expression of characteristic sets of genes, primarily those regulated by cell-specific transcription. To elucidate the mechanism regulating cell-specific gene expression in a highly specialized cell, AFD thermosensory neuron in *Caenorhabditis elegans*, we analyzed the promoter sequences of guanylyl cyclase genes, *gcy-8* and *gcy-18*, exclusively expressed in AFD. In this study, we showed that AFD-specific expression of *gcy-8* and *gcy-18* requires the co-expression of homeodomain proteins, CEH-14/LHX3 and TTX-1/OTX1. We observed that mutation of *ttx-1* or *ceh-14* caused a reduction in the expression of *gcy-8* and *gcy-18* and that the expression was completely lost in double mutants. This synergy effect was also observed with other AFD marker genes, such as *ntc-1*, *nlp-21*and *cng-3*. Electrophoretic mobility shift assays revealed direct interaction of CEH-14 and TTX-1 proteins with *gcy-8* and *gcy-18* promoters *in vitro*. The binding sites of CEH-14 and TTX-1 proteins were confirmed to be essential for AFD-specific expression of *gcy-8* and *gcy-18 in vivo*. We also demonstrated that forced expression of CEH-14 and TTX-1 in AWB chemosensory neurons induced ectopic expression of *gcy-8* and *gcy-18* reporters in this neuron. Finally, we showed that the regulation of *gcy-8* and *gcy-18* expression by *ceh-14* and *ttx-1* is evolutionally conserved in five *Caenorhabditis* species. Taken together, *ceh-14* and *ttx-1* expression determines the fate of AFD as terminal selector genes at the final step of cell specification.

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Introduction

Neurons are highly specialized cells that animals use to process environmental and cellular information. Given the complexity and diversity of their functions, neurons have a wide variety of morphological, connectivity, and electrochemical properties. Cell-specific transcription factors (TFs) temporally and spatially regulate their target gene expression, which determines individual cell fates during development and confers specific functions to mature neurons. The model organism *Caenorhabditis elegans* provides unique opportunities to investigate how TFs regulate neuronal identities (Hobert, 2008, 2010). Numerous mutations have been screened for abnormal neuronal differentiation and function, and several TFs have been identified that coordinate cell-specific gene expression and determine neuronal identities in *C. elegans* (Hobert, 2008, 2010; Lanjuin et al., 2003). CHE-1 is a GLASS-zinc finger TF exclusively expressed in ASE gustatory neurons (Chang et al., 2003; Uchida et al., 2003). This TF alone can control the terminal differentiation of ASE. However, most other neurons require combinatorial expression of multiple TFs for their specification. For example, a LIM-class homeodomain (LIM-HD) protein, TTX-3, and a Paired-class homeodomain (PRD-HD) protein, CEH-10, are co-expressed in the cholinergic AIY interneurons (Altun-Gultekin et al., 2001; Wenick and Hobert, 2004). Their heterodimer cooperatively binds to their cognate site, the “AIY motif,” and activates downstream gene batteries required for the function of AIY interneurons. Neuronal identity is also determined by transcriptional cascades that consist of a set of upstream and downstream TFs. For instance, in AWB olfactory neurons, an OTX-class homeodomain (OTX-HD) protein, CEH-37, initiates the expression of a LIM-HD protein, LIM-4, required for AWB-specific gene expression (Lanjuin et al., 2003).

Thermosensation is an important neuronal function for *C. elegans* to sense environmental cues that affect its metabolism and behavior. *C. elegans* memorizes temperature in association with its past cultivation conditions and migrates toward preferable temperatures and escapes starvation temperatures (Hedgecock and Russell, 1975; Mori and Ohshima, 1995). Temperature is sensed by the major thermosensory neuron AFD that are present in bilateral amphid organs in the head of *C. elegans*. The thermal

* Corresponding author.
E-mail address: ykohara@nig.ac.jp (Y. Kohara).

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stimuli received at the cilia of AFD are transmitted through a family of guanylate cyclase genes of high sequence homology, such as *gcy-8*, *gcy-18*, and *gcy-23* (hereafter referred to as *gcy-8* family genes) (Inada et al., 2006; Wasserman et al., 2011). All three *gcy-8* family genes have been identified to be AFD-enriched genes (Colosimo et al., 2004), and all are exclusively expressed in AFD (Inada et al., 2006; Yu et al., 1997). Although any single mutation in *gcy-8* family genes shows almost normal responses to thermal stimuli, animals carrying double or triple mutants of *gcy-8* family genes fail to show normal thermal responses (Inada et al., 2006). The abnormal phenotypes of the multiple mutants were partially rescued by the expression of any one of three *gcy-8* family proteins in AFD, suggesting functional redundancy of the *gcy-8* family genes. Given the similarities in the sequence homology (Fig. S1A and B), expression pattern, and function of *gcy-8* family genes, they have likely originated from a single ancestral gene by gene duplication (Inada et al., 2006), suggesting that their upstream regulatory mechanisms would be conserved among *gcy-8* family genes.

A homeodomain gene pair, *ceh-14* (LIM-HD) and *ttx-1* (OTX-HD), confers neural identity to AFD (Cassata et al., 2000a; Hedgecock and Russell, 1975; Satterlee et al., 2001). Mutants of these TFs resulted in failure of AFD fate specification, causing abnormal morphology in the ciliary structure at the tip of AFD dendrites, and mutant animals could not properly respond to temperature. *ceh-14* mutant animals show athermotactic (no response to temperature) behavior, and *ttx-1* mutant animals show cryophilic behavior (Cassata et al., 2000a; Hedgecock and Russell, 1975; Satterlee et al., 2001). The *ceh-14*::GFP (green-fluorescent protein) reporter is widely expressed in neurons and other organs, including AFD, ALA, and BDU neurons in the anterior body, several tail neurons, hypodermal syncytia, and spermatheca (Cassata et al., 2000a; Kagoshima et al., 2000, 2013). The *ttx-1*::GFP reporter is expressed in AFD and marginal cells of the pharynx (Satterlee et al., 2001). The expression of *ceh-14* and *ttx-1* overlaps only in AFD. *gcy-8*::GFP reporter expression has been observed to be reduced under a *ttx-1* mutant background (Satterlee et al., 2001); however, the expression remained at a low level, suggesting that a second upstream factor is required to regulate *gcy-8* expression.

In this study, we showed that co-expression of *ceh-14* and *ttx-1* plays a key role in AFD-specific expression of *gcy-8* and *gcy-18*. AFD-specific expression of their reporters was downregulated by mutation of either *ceh-14* or *ttx-1* and was completely lost under the double mutant background. We confirmed that both CEH-14 and TTX-1 proteins can directly bind to *gcy-8* and *gcy-18* promoters, and we subsequently identified the binding sites of these proteins *in vitro*. Furthermore, the binding sites of these factors are essential for gene expression in the AFD *in vivo*. We also demonstrated that forced expression of both CEH-14 and TTX-1 proteins in AWB chemosensory neurons could induce ectopic expression of *gcy-8*::GFP and *gcy-18*::GFP in this neuron. Finally, we showed evolutionary conservation of the regulation of *gcy-8* and *gcy-18* expression by *ceh-14* and *ttx-1* in five *Caenorhabditis* species. Based on these findings, we discuss the mechanisms of the regulation of *gcy-8* and *gcy-18* expression by CEH-14 and TTX-1.

Materials and methods

Strains and maintenance of Caenorhabditis species

Caenorhabditis strains were maintained by standard methods established for *C. elegans* (Sulston and Hodgkin, 1988). The *C. elegans* strains used in the present study were as follows: Bristol strain (N2), CB3775: *dpy-20*(*e2017*)*IV*, TB522: *dpy-20*(*e2017*)*IV*; *ceh-14*(*ch3*)*X*, YK108: *dpy-20*(*e2017*)*IV*; *ttx-1*(*p767*)*V*, YK113: *dpy-20*(*e2017*)*IV*; *ttx-1*(*p767*)*V*; *ceh-14*(*ch3*)*X*, YK203: *dpy-20*(*e2017*)*IV*, *msIs408*[*pHK143*(*Pstr-1*::*TTX-1*), *pRF4*(*rol-6*(*su1006*))], YK205: *dpy-20*(*e2017*)*IV*, *msIs411*[*pHK143*(*Pstr-1*::*CEH-14*), *pRF4*(*rol-6*(*su1006*))], YK175: *dpy-20*(*e2017*)*IV*, *msIs553*[*pHK143*(*Pstr-1*::*CEH-14*), *pHK146* (*Pstr-1*::*TTX-1*), *pRF4*(*rol-6*(*su1006*))], JZ924: *Ex*[*cng-3*::*GFP*, *odr-1*::*RFP*, *ofm-1*::*GFP*], HA444: *lin-15*(*n765*)*X*; *rtEx330*[*nlp-21*::*GFP*, *lin-15*(+)], PY1263: *lin-15*(*n765*)*X*; *Is*[*tax-2*::*GFP*, *lin-15*(+)]*IV*, LSC314: *Ex*[*ntc-1*::*GFP*, *elt-2*::*mCherry*]. The *Caenorhabditis* strains *C. briggsae* (AF16), *C. remanei* (PB4641), and *C. brenneri* (PB2801) were provided by the CGC (Caenorhabditis Genetic Center), and *C. japonica* (DF5081) was kindly provided by Drs. E. Schwarz and P. W. Sternberg. The heterozygous animals with *ceh-14* and *ttx-1* mutations (*ceh-14*/+; *ttx-1*/+) carrying *gcy-8* and *gcy-18*::GFP reporters were generated by crossing the following two strains: *msEx*[*gcy-8*(*gcy-18*)::*GFP*, *pMH86*]; *dpy-20*(*e2017*)*IV*; *ttx-1*(*p767*)*V*; *ceh-14*(*ch3*)*X* males and *dpy-20*(*e2017*)*IV* hermaphrodites. The resulting non-Dpy F1 hermaphrodites were examined.

Transgenes

gcy-8 promoter deletion constructs

pHK180 was generated by deleting the *Hind*III–*Hind*III fragment from the original GFP reporter construct pPD95.77-*gcy-8* (kindly provided by Dr. Garbers), containing *gcy-8* promoter sequences from −702 bp to +6 bp (−702:+6) of the translational initiation codon ATG of the GCY-8 protein coding sequences (Yu et al., 1997). Further deletion constructs, pHK424 (−300:-1), pHK422 (−254:+6), and pHK425 (−103:+6), were generated by amplifying promoter sequences by PCR (polymerase chain reaction) using pHK180 as a template and cloning the sequences into the *Kpn*I–*Sph*I sites of the pPD95.75 vector (http://www.addgene.org/Fire_Lab). pHK183 (−173:+6) was generated by deleting the *Hind*III–*Hind*III fragment from pHK180.

gcy-18 promoter deletion constructs

pHK371 (−796:−1) was generated by cloning the PCR fragments of the *gcy-18* promoter sequences using *C. elegans* genomic DNA as a template into the *Kpn*I–*Sph*I sites of the pPD95.75 vector. pHK380 (−300:−1) and pHK373 (−100:−1) were generated by amplifying the promoter sequences by PCR using pHK371 as a template and inserting the sequences into the *Kpn*I–*Sph*I sites of the pPD95.75 vector. pHK183 (−210:−1) was generated by deleting the *Sph*I–*SnaB*I fragment from pHK371.

Caenorhabditis GFP constructs

To identify *gcy-8* and *gcy-18* orthologs in four *Caenorhabditis* species, BLAST searches were conducted against the genome assemblies of *Caenorhabditis briggsae* (CBR), *Caenorhabditis remanei* (CRE), *Caenorhabditis brenneri* (CBN), and *Caenorhabditis japonica* (CJA) using the TBLASTN program (Altschul et al., 1990; Stein et al., 2003). Orthology was confirmed on the basis of amino acid sequence identity and reciprocal BLAST best hits. Genomic sequence information was obtained from WormBase version WS234 (http://www.wormbase.org/), with the exception of *C. japonica gcy-8*, for which the sequence data in WormBase were incorrect, probably because of misassembly. We thus obtained *C. japonica* genomic sequence data from the Genome Institute, Washington University (http://genome.wustl.edu/genomes/detail/caenorhabditis-japonica). The promoter sequences used were as follows: *CBR_gcy-8* from −11,567,054 to −11,566,469 of Chromosome IV; *CBN_gcy-8.1* from −459,271 to −458,759 of Cbre_Contig92; *CBN_gcy-8.2* from 188,037 to 188,554 of Cbre_Contig235; *CRE_gcy-8* from −18,826 to −18,421 of Crem_Contig18; *CJA_gcy-8* from 31,509 to 31,880 of Contig105 (Caenorhabditis_japonica-4.0.1-supercontig); *CBR_gcy-18* from 10,132,634

to 10,133,933 of Chromosome IV; *CBN_gcy-18* from 5962 to 6973 of Cbre_Contig157; *CRE_gcy-18* from 5074 to 5766 of Crem_Contig1360; and *CJA_gcy-18* from 438,483 to 440,627 of Cjap_Contig18163. (*CJA_gcy-18* in WormBase was not accurately annotated to CJA16650 (CJA-GCY-18 isoform a); instead, this gene should start from the upstream ORF, CJA35028.) Genomic DNA of *C. briggsae* (AF16), *C. remanei* (PB4641), *C. brenneri* (PB2801), and *C. japonica* (DF5081) was purified using the Gentra Puregene Kit (Qiagen). Promoter fragments were amplified using PCR and cloned into the pPD95.79 GFP reporter vector, except for *CJA_gcy-18*. Because genomic sequences around *CJA_gcy-18* locus were not correctly assembled, we could not construct the *CJA_gcy-18*::GFP reporter due to the failure in PCR amplification of the promoter.

GFP reporter constructs with potential CEH-14 and TTX-1 binding site mutations

We generated *gcy-8* and *gcy-18* promoter constructs with base pair substitutions at potential CEH-14 and TTX-1 binding sites, which we named ΔCEH and ΔTTX, respectively. pHK424ΔCEH, pHK424ΔTTX, and pHK424ΔCEHΔTTX were generated by cloning PCR fragments using pHK424 (*gcy-8* promoter, −300:−1) as a template and the oligonucleotide primers with the sequence substitutions. The *gcy-18* promoter constructs with binding site mutations were generated by the same protocol, using pHK380 (*gcy-18* promoter, −300:−1) as a template. Substitution sequences of the 300-bp promoter::reporter constructs were as follows (substituted sequences are shown in capital letters, and the sequence positions are indicated in brackets): pHK424ΔCEH, tattttcTGCCGCagtaGGCCgatataaag (from −230 to −201 of the *gcy-8* promoter); pHK424ΔTTX, gttgagaagTCCGaaagGCC-Catgactacc (from −180 to −151 of the *gcy-8* promoter); pHK380ΔCEH, ccttcggaaaaacCGGCcaaactaatgcat (from −195 to −179 of the *gcy-18* promoter); and pHK380ΔTTX, cagtagtCGGCct-taaattCGGAcagctca (from −120 to −91 of the *gcy-18* promoter).

AWB ectopic expression construct

The pHK143 and pHK146 constructs for ectopic expression of CEH-14 and TTX-1 in AWB chemosensory neurons were generated by amplifying the full-length cDNAs of *ceh-14* (F46C8.5) and *ttx-1* (Y113G7A.6a) and inserting them into the AWB-specific *str-1* promoter construct pHK140 (Cassata et al., 2000a; Troemel et al., 1997). PCR primer sequences used in the present study are presented in Table S1. All the constructs were confirmed by sequencing, and their sequence information is available upon request.

Transgenic worms

Plasmids containing target DNA were injected into the syncytial gonad of young adult hermaphrodite worms at a concentration of 20–100 ng/μl, as described previously (Mello and Fire, 1995), along with pRF4 [*rol-6*(*su1006*)] or pMH86 [*dpy-20*(+)] plasmids for transformation markers (also at 20–100 ng/μl). For all analyses in the present study, we generated and examined the expression of multiple transgenic lines, and we rarely found any qualitative differences among them. Where appropriate, transgenic arrays were integrated using UV irradiation (Gengyo-Ando and Mitani, 2000). Integrated strains were backcrossed more than twice with N2 wild-type animals.

Image analysis

Expression analyses were performed using a Zeiss Axioplan 2 microscope equipped with differential interference contrast (DIC) and fluorescence optics as appropriate. Worms were anesthetized on 2% agarose pads using 50 mM NaN_3, as described previously (Sulston and Hodgkin, 1988).

Protein purification

LIM-HD of CEH-14 was fused with N-terminal glutathione-S-transferase (GST) and N- and C-terminal hexahistidine (6xHis)-tags. The coding sequences of CEH-14 (39–247 aa of F46C8.5) were amplified by PCR using a plasmid containing *ceh-14* cDNA (pKS-*ceh-14*) as a template (Cassata et al., 2000a) together with the primer pair CEH-14-LIM5 and CEH-14-HD3. The PCR product was cloned into the *Pst*I-*Xho*I site of a T7-expression vector pHIT12 derived from pTAG2K (Keefe et al., 2001). HD of TTX-1 was fused with N-terminal GST and C-terminal 6xHis-tags. The coding sequences of TTX-1 (194–267 aa of Y133G7A.6a) were amplified by PCR using the cDNA clone yk345e3 (ACC#: AF381627) as a template together with the primer pair TTX-1-HD5 and TTX-1-HD3. The PCR product was cloned into pDEST15 using the Gateway System (Life Technologies). The proteins were expressed in the BL21 strain of *Escherichia coli* and purified on a nickel-nitrilotriacetic acid (Ni-NTA) resin, according to the manufacturer's instructions (Qiagen). Purified proteins were quantified by the intensities of the bands on SDS-PAGE visualized by Coomassie Brilliant Blue staining. All the constructs were confirmed by sequencing. See supplemental data for the primer sequences used in the present study (Table S1). All the constructs and their sequence information are available upon request.

Electrophoretic Mobility Shift Assay (EMSA)

For the preparation of probes for EMSA, the 3'-OH ends of synthetic oligonucleotides were labeled with a biotin-11-dUTP nucleotide analog using the Biotin 3'-End DNA Labeling Kit (Pierce). The double-stranded probes and competitors were prepared by annealing appropriate pairs of oligonucleotides at 10 pmol/μl. The DNA binding reaction was performed with 7.80 ng of CEH-14 (LIM-HD) and/or 1.50 ng of TTX-1 (HD) per reaction (final volume, 10 μl) in EMSA buffer (20 mM HEPES-KOH, 200 mM KCl, 200 μM $ZnSO_4$, 1 mM EDTA, 1 mM DTT, 6% glycerol, 100 ng poly (dI-dC), and 10 fmoles labeled probe; pH 7.6) with or without non-labeled competitors of the appropriate concentration at 25 °C for 30 min. Before electrophoresis, 2 μl of loading dye (0.025% bromophenol blue and 30% glycerol) was added to the reaction mixture. The reaction mixture (6 μl) was subsequently subjected to gel electrophoresis at 120 V for 60 min on a 6% native polyacrylamide gel (0.25 × TBE) at 4 °C and transferred to a nylon membrane (Biodyne Plus, PALL). The biotin end-labeled DNA was detected using the streptavidin–horseradish peroxidase conjugate and the chemiluminescent substrate (luminol), following the LightShift Chemiluminescent EMSA Kit protocols (PIERCE), using the fluorescent scanner ImageQuant LAS 4000 Mini System (GE Healthcare Life Sciences). Sequence information of the oligonucleotide probes and competitors used in the present study is listed in Table S2.

Results

Dissection of the gcy-8 and gcy-18 promoters

It has been shown that *gcy-8*, *gcy-18*, and *gcy-23* constitute a subfamily (*gcy-8* family) of 27 receptor-type guanylyl cyclase genes by phylogenetic analysis in *C. elegans* and that they are exclusively expressed in AFD thermosensory neuron (Inada et al., 2006; Yu et al., 1997). The high sequence similarity among the *gcy-8* family genes suggested that they arise from a common

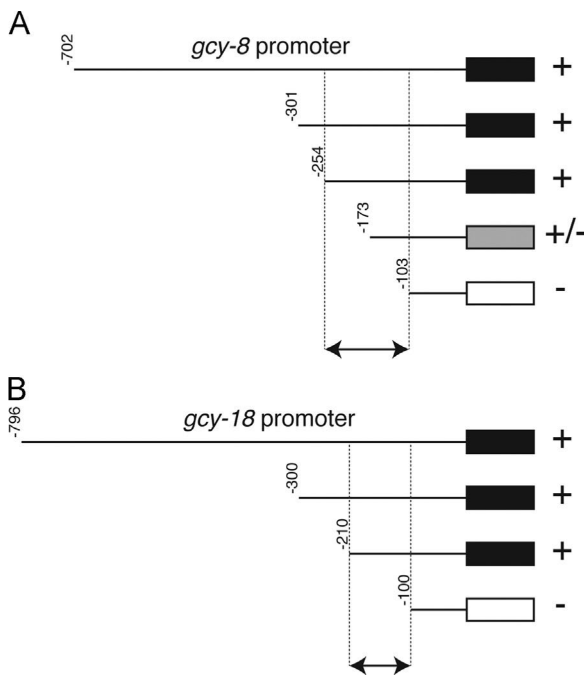

Fig. 1. Expression analysis of *gcy-8* and *gcy-18* promoter::GFP constructs in AFD thermosensory neurons. Deletion analysis of *gcy-8* (A) and *gcy-18* (B) promoter::GFP constructs. Numbers indicate the extent of the assayed fragment (in bp) relative to the translational start site. The constructs used for the analysis are (A) −702: pHK180, −301: pHK424, −254: pHK422, −173: pHK183, −103: pHK425, (B) −796: pHK371, −300: pHK380, −210: pHK183, −100: pHK373. GFP reporter expression is shown as (+) consistent strong expression, (+/−) inconsistent weak expression, or (−) no expression. The regions indicated by two-way arrows are those required for AFD-specific expression.

ancestral gene and that they share upstream regulatory mechanisms including TF binding sequences within their promoters. We examined the expression pattern of the *gcy-8* family genes using the promoter::GFP reporter and confirmed AFD-specific expression of *gcy-8*, *gcy-18*, and *gcy-23* reporters. The *gcy-23* reporter showed considerably weaker expression with low penetrance (data not shown); therefore, we further analyzed only the promoter sequences of *gcy-8* and *gcy-18* genes. A series of promoter deletion constructs was generated and tested for expression in transgenic *C. elegans*. The *gcy-8* reporters containing 254-bp promoter sequences showed strong expression in AFD; however, the 173-bp construct was only faintly and rarely expressed, while the 103-bp construct was not expressed in AFD (Fig. 1A). The *gcy-18* reporter constructs containing the 210-bp sequence showed strong expression exclusively in AFD, while the 100-bp construct showed no expression (Fig. 1B). These results indicate that upstream regulatory sequences for AFD-specific expression are located within the regions from −254 bp to −103 bp of the *gcy-8* promoter and from −210 bp to −100 bp of the *gcy-18* promoter.

ceh-14 and ttx-1 mutations synergistically affect AFD-specific expression of gcy-8 and gcy-18

The OTX-class homeodomain (OTX-HD) TF gene *ttx-1* has been reported to regulate the expression of *gcy-8* (Satterlee et al., 2001). Although the *gcy-8*::GFP expression was reduced in *ttx-1* null mutants, partial expression remained in AFD, indicating that a second factor is involved in AFD-specific expression of *gcy-8*. Candidate TFs controlling AFD-specific expression of *gcy-8* and *gcy-18* include the following four AFD-specific TF genes: *ceh-14* (LIM-HD), *ceh-23* (DLX-HD), *dac-1* (SKI/SNO/DAC), and *nhr-38* (nuclear hormone receptor) (Altun-Gultekin et al., 2001; Cassata et al., 2000a; Colosimo et al., 2004; Miyabayashi et al., 1999).

Table 1
Expression of AFD terminal markers in *ceh-14* and *ttx-1* mutants.

Construct	Background	GFP expression in AFD			
		Strong (%)	**Weak (%)**	**None (%)**	***N***
gcy-8::GFP	Wild-type	94	6	0	171
	ceh-14	74	26	0	247
	ttx-1	40	60	0	214
	ceh-14; ttx-1	0	0	100	244
	+/*ceh-14*; +/*ttx-1*	97	3	0	94
gcy-18::GFP	Wild-type	95	0	5	112
	ceh-14	9	27	64	160
	ttx-1	9	24	67	162
	ceh-14; ttx-1	0	0	100	152
	+/*ceh-14*; +/*ttx-1*	100	0	0	30
nlp-21::GFP	Wild-type	84	9	7	54
	ceh-14	65	23	12	52
	ttx-1	72	23	5	66
	ceh-14; ttx-1	26	34	40	50
ntc-1::GFP	Wild-type	97	0	3	62
	ceh-14	90	0	10	68
	ttx-1	32	36	32	88
	ceh-14; ttx-1	6	9	85	70
cng-3::GFP	Wild-type	92	1	7	108
	ceh-14	87	4	9	82
	ttx-1	54	21	25	102
	ceh-14; ttx-1	25	26	49	120
tax-2::GFP	Wild-type	81	14	5	150
	ceh-14	53	26	21	94
	ttx-1	46	22	32	108
	ceh-14; ttx-1	51	37	12	172

Expression level was categorized as follows; strong: GFP signal was observed in dendrites, axons and cell bodies; weak: observed only in cell bodies; none: no signal detected. Reporter genes in wild-type are expressed only in AFD neuron (*gcy-8* and *gcy-18*) (Inada et al., 2006; Yu et al., 1997), in AFD and 5 head neurons (*nlp-21*) (Nathoo et al., 2001), in AFD, NSM, AVK and M5 neurons (*ntc-1*), in AFD, AWB, AWC, ASE and ASI neurons (*cng-3*) (Wojtyniak et al., 2013), and in AFD, AWB, AWC, ASE, ASG, ASI, ASJ, ASK, AQR, PQR, URX and BAG neurons (*tax-2*) (Coburn and Bargmann, 1996).

Among these TFs, only *ceh-14* and *ttx-1* mutants showed severe defects in thermal responses mediated by AFD, whereas *ceh-23* and *nhr-38* mutants were normal in thermotaxis, and the behavioral defect observed in *dac-1* mutants was relatively subtle. It was also shown that *gcy-8* expression in AFD was not altered in *ceh-23* and *dac-1* mutants. Therefore, we selected *ceh-14* as a candidate TF that may regulate *gcy-8* and *gcy-18* expression together with *ttx-1*.

We confirmed that the expression of *gcy-8*::GFP was severely reduced in *ttx-1* mutants, which is consistent with the previous report (Satterlee et al., 2001) (Table 1). We observed that *ceh-14* mutants also showed a reduced *gcy-8*::GFP expression, although this effect was not as strong as that in *ttx-1* mutants. In contrast to either of the single mutants, we observed that the expression of *gcy-8*::GFP was completely lost in the double mutants. The expression of *gcy-18*::GFP was strongly reduced by either of the single mutations, however, still 36% and 33% of AFD showed GFP expression in *ceh-14* and *ttx-1* mutants, respectively (Table 1). In contrast to the single mutants, the expression of *gcy-18*::GFP was completely abolished in the double mutants. The AFD cell bodies were present in their typical locations in the double mutants, and the neurons retained the bipolar sensory morphology, as determined by DIC microscopy, indicating that loss of expression of *gcy-8* family reporters are not due to failure in generating AFD. We crossed the double mutants with wild-type animals and observed that they recovered the reporter expression to wild-type levels, confirming the intactness of reporter constructs in transgenic animals. These results demonstrate that *ceh-14* and *ttx-1* are important for the expression of *gcy-8* and *gcy-18*, and more specifically, co-expression of these two factors may play a key role for their AFD-specific expression.

ceh-14 and *ttx-1* affect the expression of other AFD marker genes

We further examined the effect of *ceh-14* and *ttx-1* mutations on the expression of other terminal marker genes for AFD: vasopressin-like peptide (*ntc-1*), neuropeptide-like protein gene (*nlp-21*) and cyclic nucleotide gated channel genes (*cng-3* and *tax-2*). *ntc-1* reporter is expressed in AFD, NSM, AVK and M5 in wild-type (Wojtyniak et al., 2013). The expression was unaffected by *ceh-14* and weakly affected by *ttx-1*; however, the double mutation caused almost complete loss of expression in AFD (Table 1). *nlp-21* and *cng-3* reporters are expressed in several head neurons including AFD in wild-type (Nathoo et al., 2001; Wojtyniak et al., 2013). The expression of these reporters in AFD was slightly reduced by either *ttx-1* or *ceh-14* single mutation, and the double mutation resulted in strong reduction of the expression (Table 1). These synergy effects are similar to those of the *gcy-8* family genes, though the extent of the effect was variable. *tax-2* reporter is expressed in eight amphid sensory neurons including AFD and other sensory neurons, AQR, PQR, URX and BAG(Coburn and Bargmann, 1996). The expression was slightly affected by *ceh-14* or *ttx-1* single mutations. In contrast to the other reporters, the double mutation did not enhance the expression (Table 1). All the expression in other neurons was unaffected in any combination of *ceh-14* and *ttx-1* mutations. These results suggest that synergistic activity of *ceh-14* and *ttx-1* is important for many distinct aspects of AFD identity.

CEH-14 and TTX-1 bind to promoter sequences of *gcy-8* and *gcy-18*

Our results suggested that CEH-14 and TTX-1 proteins are upstream TFs of the *gcy-8* and *gcy-18* promoters. We next tested the DNA binding of CEH-14 and TTX-1 proteins to *gcy-8* and *gcy-18* promoter sequences using EMSAs. We used LIM-HD of CEH-14 and HD of TTX-1 in the analysis. We first screened a series of 50-bp oligonucleotides with a 25-bp overlap in the 300-bp sequences of the *gcy-8* and *gcy-18* promoters (Fig. 2A and B). CEH-14 bound to probe #8-3 (from −250 bp to −200 bp of the translation initiation codon (−250:−200)) of *gcy-8* (Fig. 2A and C) and to probe #18-5 (−200:−150) of *gcy-18* (Fig. 2B and D). Although CEH-14 reactions resulted in multiple bands, these bands disappeared in the presence of excess of unlabeled specific competitors, thereby showing a specific protein–DNA interaction (Fig. 2K and L). Weak shift bands were observed in the binding reactions between CEH-14 and probes #18-2, #18-7, and #18-8, however, the proportions of bound probe were only 7%, 3%, and 13%, respectively, which were remarkably lower than the 40% shift observed with probe #18-5 (Fig. 2D). These weaker binding sites might contribute transcriptional regulation; however, their binding was too weak to judge their specificity (data not shown). Therefore, we chose probe #18-5 for the CEH-14 binding region for further analysis. TTX-1 bound to probes #8-5 (−200:−150) and #8-6 (−175:−125) of *gcy-8* (Fig. 2A and E) and to probe #18-8 (−125:−75) of *gcy-18* (Fig. 2B and F).

We subsequently carried out competition assays to narrow down the binding sequence of CEH-14 and TTX-1 using a series of 20-bp oligonucleotides with a 10-bp overlap (data not shown, Supplemental Table S2) and identified 30-bp probes as the minimal binding sequences for CEH-14 and TTX-1 (8-CEH, 18-CEH, 8-TTX, 18-TTX in Fig. 2A, B, G–J). For further mapping of CEH-14 and TTX-1 binding sites, we designed a series of 30-bp oligonucleotides containing 4- or 2-bp substitutions and used them as competitors in EMSA reactions (Fig. 2G–J). The competition assays showed that CEH-14 required the sequences centered at ATTA (C-I site, −222:−219) of the *gcy-8* promoter (Fig. 2G and K) and TAAT (C-II site, −182:−179) of the *gcy-18* promoter (Fig. 2H and L), while TTX-1 required the sequences around CTTA (T-1 site, −182:−179) of the *gcy-8* promoter (Fig. 2I and M) and TAAT (T-II site, −113:−110) as the major site and TAAG ("T-III site," −101:−98) as the minor site of the *gcy-18* promoter (Fig. 2J and N). These results were consistent with the results obtained from standard EMSA using competitor oligonucleotides with 4- or 2-bp substitutions as labeled probes (data not shown).

Consequently, we identified consensus binding sequences CTAAT for CEH-14 and TTAA(T/G)CTT for TTX-1 (underlined TAAT sequences represent the general binding sequence for homeodomain proteins, Fig. 2O and P), which were consistent with previously identified consensus binding sequences for their vertebrate orthologs: AATTAATTAA for LHX3/LIM3 and (C/T)TAATCC for CRX/PTX/p-OTX (Bridwell et al., 2001; Corbo et al., 2010; Yaden et al., 2006).

CEH-14 and TTX-1 binding sites are essential for *gcy-8* and *gcy-18* expression in AFD

We then investigated whether the binding sites for CEH-14 and TTX-1 identified by *in vitro* analysis are essential *in vivo*. We generated *gcy-8* and *gcy-18* promoter::GFP constructs with three types of base substitution mutations: potential CEH-14 binding site, TTX-1 site and both of them were eliminated by base pair substitutions, which we named ΔCEH, ΔTTX and ΔCEHΔTTX, respectively. They were tested in transgenic *C. elegans*. The mutation in the CEH-14 binding site, *gcy-8*(ΔCEH), resulted in a reduction in GFP expression in AFD (Fig. 3B). The intensity of GFP expression with *gcy-8*(ΔTTX) was severely reduced, however, we could still detect faint GFP signals (Fig. 3C). In contrast to single mutants, we did not observe GFP signals from the transgenics carrying the *gcy-8*(ΔCEHΔTTX) reporter at all, even in digitally enhanced images (Fig. 3D). Similarly, the expression of *gcy-18* (ΔCEH) or *gcy-18*(ΔTTX) was reduced, while that of the *gcy-18* (ΔCEHΔTTX) was completely lost (Fig. 3E–H). These results clearly demonstrate that the CEH-14 and TTX-1 binding sites identified by *in vitro* analysis are also important for the expression of *gcy-8* and *gcy-18* in AFD *in vivo*. The constructs harboring the double site mutation (ΔCEHΔTTX) exhibited more severe effects on the expression of *gcy-8* and *gcy-18* than either of the single site mutations (ΔCEH or ΔTTX). This is consistent with the results obtained from the expression of *gcy-8* and *gcy-18* wild-type reporters; reduction in either the *ceh-14* or *ttx-1* single mutant and complete loss in the double mutant background (Table 1).

Forced expression of *ceh-14* and *ttx-1* causes ectopic expression of *gcy-8* and *gcy-18* in the chemosensory neuron AWB

We have shown the necessity of *ceh-14* and *ttx-1* for AFD-specific expression of *gcy-8* and *gcy-18*, but how about the sufficiency? We subsequently assessed the sufficiency by forcing the expression of these TFs in another neuronal type. We chose to use AWB olfactory neurons for the following reasons: first, neither *gcy-8* nor *gcy-18*::GFP reporter constructs showed expression in AWB in wild-type *C. elegans* (Fig. 4A and C). Second, AFD and AWB sensory neurons are morphologically similar, with enlarged surfaces at the dendritic endings (White et al., 1986). Lastly, AWB are not required for thermal responses; however, forced expression of *ceh-14* in AWB can partially suppress the athermotactic phenotype of *ceh-14* null mutants (Cassata et al., 2000a). We generated transgenic strains harboring chromosomally integrated constructs containing *ceh-14* and *ttx-1* cDNAs under the AWB-specific *str-1* promoter (P_{AWB}::*CEH-14* and P_{AWB}::*TTX-1*, respectively) and crossed them with the reporter strains carrying either *gcy-8*::GFP or *gcy-18*::GFP. The result was striking. The *gcy-8*::GFP construct was not ectopically expressed in AWB of the P_{AWB}::*CEH-14* animals and only 2% of AWB showed a faint signal in the P_{AWB}::*TTX-1*

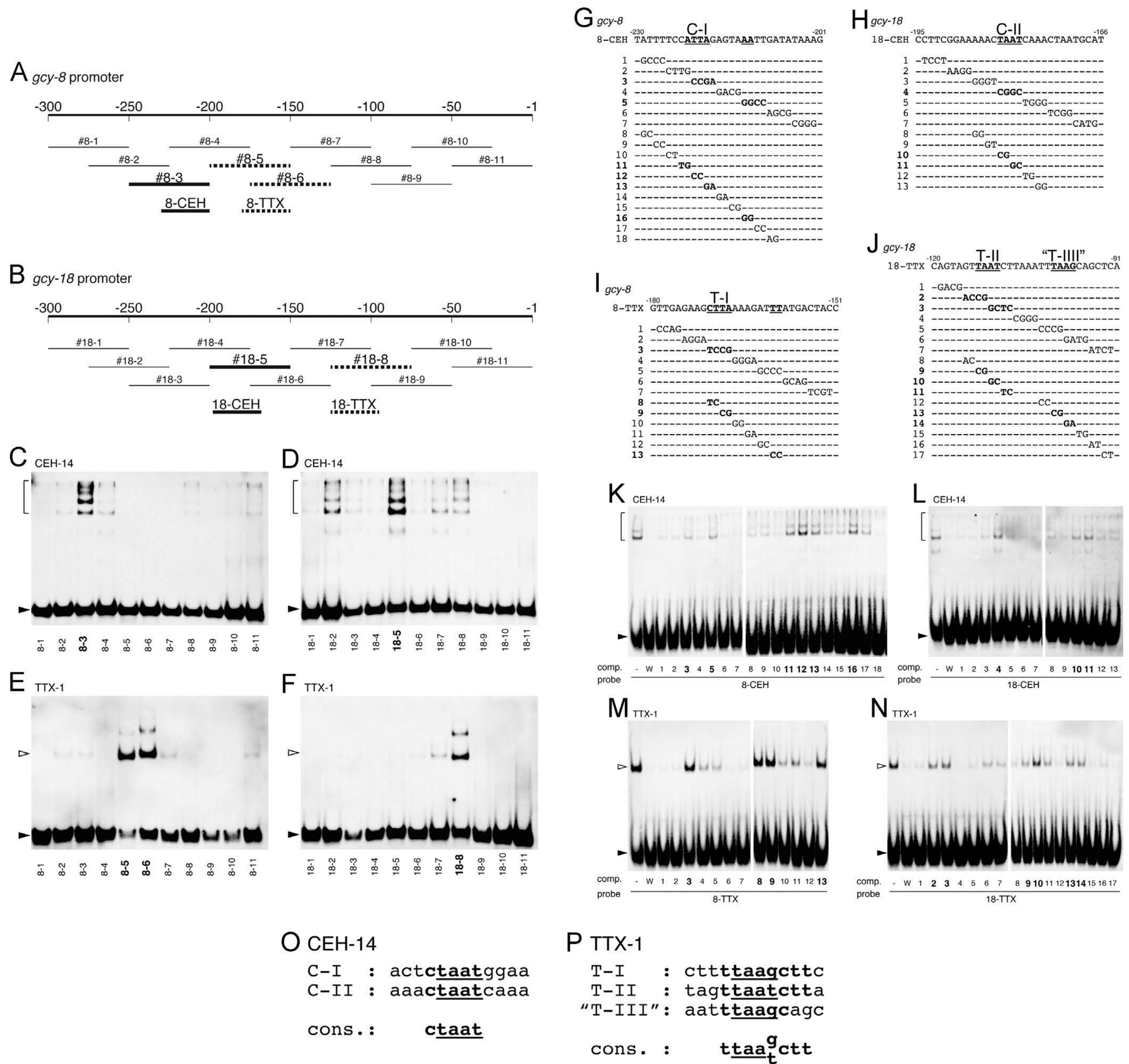

Fig. 2. DNA-binding of CEH-14 and TTX-1 proteins on *gcy-8* and *gcy-18* promoters by EMSA. (A and B) Schematic representation of the positions of the series of 50-bp (#8-1∼11, #18-1∼11) and 30-bp (8-CEH, 8-TTX, 18-CEH, 18-TTX) probes/competitors of the *gcy-8* promoter (A) and *gcy-18* promoter (B) used for EMSA. Numbers indicate the position of the oligonucleotide probes (in bp) relative to the translational start site. Thick lines and dotted lines represent probes/competitors that interact with CEH-14 and TTX-1 proteins, respectively. (C–F) The results of EMSA using 50-bp probes, which covered 300-bp promoter sequences of *gcy-8* (C, E) and *gcy-18* (D, F), with a 25-bp overlap, and the CEH-14 (C, D) and TTX-1 (E, F) proteins. Black triangles: free DNA probes, brackets and white triangles: CEH-14- and TTX-1–DNA complex, respectively. (G–J) Sequences of the 30-bp probes and mutated competitors with 4-bp and 2-bp nucleotide substitutions. The nucleotides in the mutated competitors were substituted by changing G to A, A to G, C to T, and T to C. (G, I) *gcy-8* and (H, J) *gcy-18* promoter sequences containing CEH-14 (G, H) and TTX-1 (I, J) binding sites. Nucleotide sequences required for DNA-protein interactions are underlined. More specifically, the core sequences for protein–DNA interactions are indicated by C-I and C-II for CEH-14 and by T-I, T-II, and T-III for TTX-1. (K-N) EMSA analysis using CEH-14 (K, L) and TTX-1 (M, N) proteins and labeled 30-bp probes with 100× (for CEH-14) and 50× (for TTX-1) concentrations of non-labeled 30-bp mutated competitors with 4-bp and 2-bp nucleotide substitutions (Fig. 2G–J). (O, P) Alignment and consensus sequences of CEH-14 (O) and TTX-1 (P) binding sequences. Complementary sequences are used for C-I and T-I for alignment.

animals. In contrast, in the animals carrying both ectopic constructs (P_{AWB}::*CEH-14* and P_{AWB}::*TTX-1*), 17% of transgenic animals exhibited ectopic *gcy-8*::GFP expression in AWB (Fig. 4B, Table 2). Similarly, ectopic expression of the *gcy-18* construct in AWB was remarkably increased from 1% in P_{AWB}::*CEH-14* animals and 24% in P_{AWB}::*TTX-1* animals to 69% in the animals carrying both constructs (Fig. 4D, Table 2). Taken together, our results indicate that the co-expression of *ceh-14* and *ttx-1* in AWB could induce ectopic expression of *gcy-8* and *gcy-18* in this neuron.

gcy-8 and gcy-18 promoters of five Caenorhabditis species are regulated by ceh-14 and ttx-1 in C. elegans

We examined the genomic sequence data of four *Caenorhabditis* species, *C. briggsae*, *C. brenneri*, *C. remanei* and *C. japonica*, and identified single orthologs of *ceh-14* and *ttx-1* from each species. Within the DNA-binding homeodomain, five *Caenorhabditis* CEH-14 and TTX-1 orthologs shared 92% and 98% identical amino acid sequences, respectively, and the four amino acids responsible for

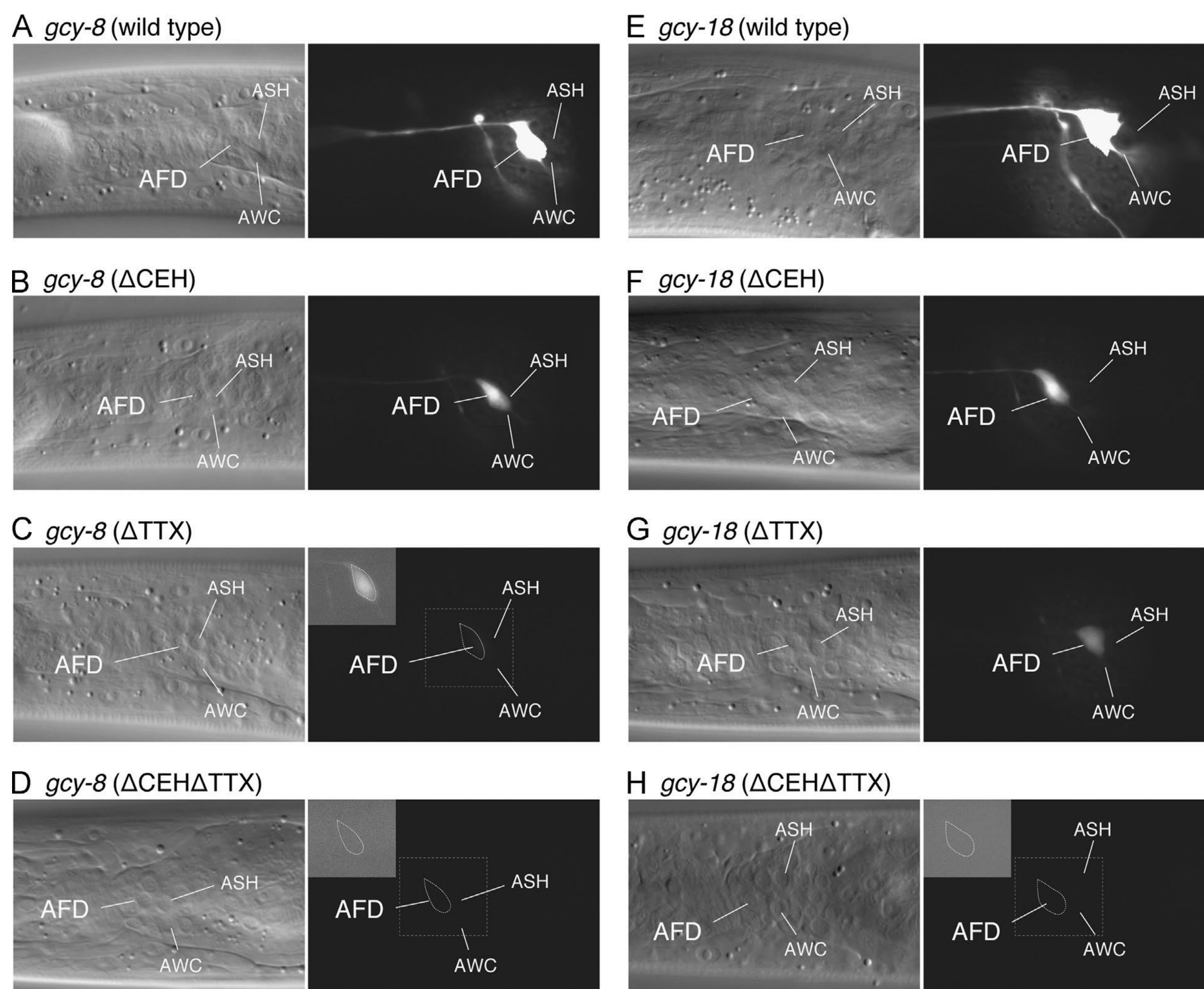

Fig. 3. Expression analysis of *gcy-8* and *gcy-18* promoter GFP constructs with CEH-14 and/or TTX-1 binding site mutations. GFP reporter expression of *gcy-8* (A–D) and *gcy-18* (E–H) promoter sequences with CEH-14 and/or TTX-1 binding site mutations; (wild-type: A, E) wild-type 300-bp promoter, (ΔCEH: B, F) CEH-14 binding site mutation, (ΔTTX: C, G) TTX-1 binding site mutation, and (ΔCEHΔTTX: D, H) CEH-14 and TTX-1 binding site mutation (see *Materials and methods*). Images were acquired using an exposure time of 0.2 s (A, E) and 1 s (B–D, F–H). The inset shows digitally enhanced images of the AFD cell body (shown by the dotted line).

direct base recognition were perfectly conserved (Fig. S2A and B), suggesting that CEH-14 and TTX-1 orthologs of all the *Caenorhabditis* species recognize very similar (probably the same) sequences. We also identified single orthologs of *gcy-8* and *gcy-18* from each species, with the exception that *C. brenneri* has two orthologs of *gcy-8*. We observed that *gcy-8* and *gcy-18* are highly conserved not only in amino acid sequence (*gcy-8*: 96% similarity, *gcy-18*: 94% similarity, Fig. S1A and B) but also in their syntenic relationships (Fig. 5A and B). In *C. elegans*, the upstream neighboring genes of *gcy-8* and *gcy-18* are C49H3.12 and *nstp-5* (ZK896.9), respectively, and these orthologs are also the upstream neighbors of the orthologs of *gcy-8* and *gcy-18* in other species (Fig. S1C and D). Furthermore, their relative directions of transcription are also conserved (Fig. 5A and B), suggesting that the regulatory elements are conserved in the upstream sequences.

To investigate whether the upstream sequences of *gcy-8* and *gcy-18* orthologs from the *Caenorhabditis* species drive AFD-specific expression in *C. elegans*, we generated promoter::GFP reporter constructs by inserting upstream sequences of the *gcy-8* and *gcy-18* orthologs and examined their expression in transgenic *C. elegans* carrying the reporters (Fig. 5, Table 3). Similar to *C. elegans* constructs, all *gcy-8*::GFP and *gcy-18*::GFP constructs examined were exclusively expressed in AFD, indicating that all the non-*elegans* constructs were functional in *C. elegans*. Furthermore, we confirmed that the upstream 300-bp sequences of the *gcy-8* and *gcy-18* orthologs were sufficient to drive the AFD-specific expression (data not shown). We subsequently examined the expression of the *gcy-8* and *gcy-18* ortholog reporters in the *C. elegans ceh-14* and *ttx-1* double mutant and observed that expression levels were strongly reduced to the level that only 0–8% of AFD showed GFP expression (Table 3). We further tested whether forced expression of *ceh-14* and *ttx-1* in AWB could also induce ectopic expression of the ortholog reporters in AWB. All the examined strains showed ectopic GFP expression in AWB, with the exception that *C. remanei gcy-8*::GFP showed extremely weak expression in only 1% of AWB (Table 3). In conclusion, the promoters of the *gcy-8* and *gcy-18* orthologs drove the exclusive expression in AFD in *C. elegans* and their expression was dependent on a set of homeobox TFs, *ceh-14* and *ttx-1*, suggesting evolutionary conservation of transcriptional regulation in the five *Caenorhabditis* species.

Discussion

We identified two CEH-14 binding sites in *gcy-8* and *gcy-18* promoters using EMSA, from which we deduced the consensus sequence CTAAT (Fig. 2O). This CEH-14 binding consensus possessed TAAT sequences, which is the binding core for

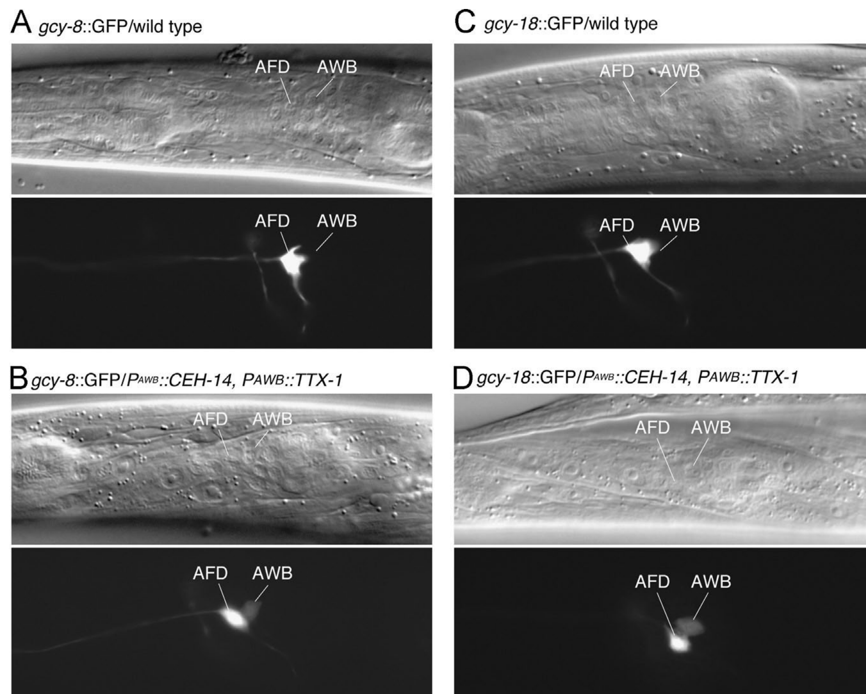

Fig. 4. Ectopic expression of *gcy-8* and *gcy-18*::GFP in AWB by forced expression of CEH-14 and TTX-1. (A, B) Wild-type expression of *gcy-8* (A) and *gcy-18* (B) promoter::GFP constructs. (C, D) Forced expression of CEH-14 and TTX-1 in AWB using the AWB-specific *str-1* promoter induced ectopic *gcy-8*::GFP (C) and *gcy-18*::GFP (D) expression in AWB in addition to intrinsic expression in AFD.

Table 2
Ectopic expression of *gcy-8* and *gcy-18*::GFP in AWB by CEH-14 and TTX-1.

Construct	Background	Ectopic expression in AWB
gcy-8::GFP	Wild-type	0% (N=154)
	P_{AWB}::*CEH-14*	0% (N=172)
	P_{AWB}::*TTX-1*	2% (N=107)
	P_{AWB}::*CEH-14*,P_{AWB}::*TTX-1*	17% (N=129)
gcy-18::GFP	Wild-type	0% (N=136)
	P_{AWB}::*CEH-14*	1% (N=128)
	P_{AWB}::*TTX-1*	24% (N=143)
	P_{AWB}::*CEH-14*,P_{AWB}::*TTX-1*	69% (N=199)

The expression from pHK424 (300 bp of *gcy-8* promoter) and pHK381 (300 bp of *gcy-18* promoter) were examined in wild-type and in the transgenic animals expressing CEH-14 and TTX-1 in AWB. The numbers of the animals are indicated in brackets.

homeodomain proteins. The ideal binding sequence for LHX3/LIM3, the vertebrate homolog of CEH-14, has been determined to be AATTAATTAA (overlapping ATTA/TAAT motifs) using selected and amplified binding site analysis with purified LHX3 protein *in vitro* (Bridwell et al., 2001). However, LHX3 bind to rather relaxed AT-rich sequences containing TAAT motifs *in vivo* (Yaden et al., 2006). The C-I and C-II sites and their neighboring sequences were highly conserved among *Caenorhabditis* (Fig. 6A). The interaction between CEH-14 and its binding site may be influenced by the surrounding sequences or by local DNA conformations affected by nucleotide composition. In particular, the 2-bp substitution from AA to GG at −213 and −212 near the C-I site on the *gcy-8* promoter showed considerable effect on the DNA binding of CEH-14 (Fig. 2G and K), possibly because of alterations in the AT-rich environment near the TAAT sequence, which was also conserved in *Caenorhabditis* (Fig. 6A).

The consensus binding site for TTX-1 was identified as TTTAA(T/G)CT in the present study (Fig. 2O). This sequence is in agreement with the consensus binding site (C/T)TAATC(C/T) obtained for CRX and PTX1/p-OTX, vertebrate homologs of TTX-1 (Corbo et al., 2010). The cytosine (C) nucleotide at the 3′-flanking site of TAAT in the OTX-binding consensus is important for the DNA binding specificity of OTX-HD proteins (Furukawa et al., 1997), and this residue is conserved in the T-I and T-II sites (Fig. 6). In the *gcy-18* promoter, TTX-1 primarily bound to the T-II site, however, the "T-III site" also had a considerable influence on TTX-1 binding (Fig. 2J and N). Although EMSA analysis revealed that the DNA binding affinity of TTX-1 to the "T-III site" is lower than that to the T-II site, the "T-III site" seemed to be required for full binding activity of TTX-1 to the T-II site. The higher affinity T-II site was less conserved than the lower affinity "T-III site," suggesting that the "T-III site" plays a major role in the regulation of *gcy-18* expression in non-*elegans* species (Fig. 6B).

In general, TTX-1 exhibited higher specificity than CEH-14 (Fig. 2K–N), and the mutations in *ttx-1* or mutations in the binding sites resulted in more severe effects on *gcy-8* and *gcy-18* expression than those in *ceh-14* (Table 1, Fig. 3). These results imply that TTX-1 may play a major role and CEH-14 may play minor roles in the regulation of *gcy-8* and *gcy-18* expression in AFD. Although the CEH-14 binding site and its surrounding sequences were more conserved than the TTX-1 site in *Caenorhabditis* species, this could be explained by the possibility that CEH-14 requires an interacting partner to achieve sufficient specificity to its binding site *in vivo*, whereas TTX-1 by itself has high specificity to DNA. It has been previously shown that CEH-14 requires cofactors, such as LIM domain binding protein, LDB-1, (Bach et al., 1997; Cassata et al., 2000b), and CEH-14 interacts with different TF partners for transcriptional regulation (see below). If so, it is natural that the CEH-14 binding site and neighboring sequences are conserved; on the other hand, TTX-1 binds to short sequences with high specificity that can be easily substituted by another position in the promoter, thereby weakening the need for sequence conservation.

In this study, we demonstrated that the AFD-specific expression of *gcy-8* and *gcy-18* was regulated by two TFs, CEH-14 and TTX-1, and that the double mutants of *ceh-14* and *ttx-1* caused more severe effect than the single mutants. Often, when two TFs coregulate a single target gene, either of single mutants would result in high penetrant effect, which is usually indicative of

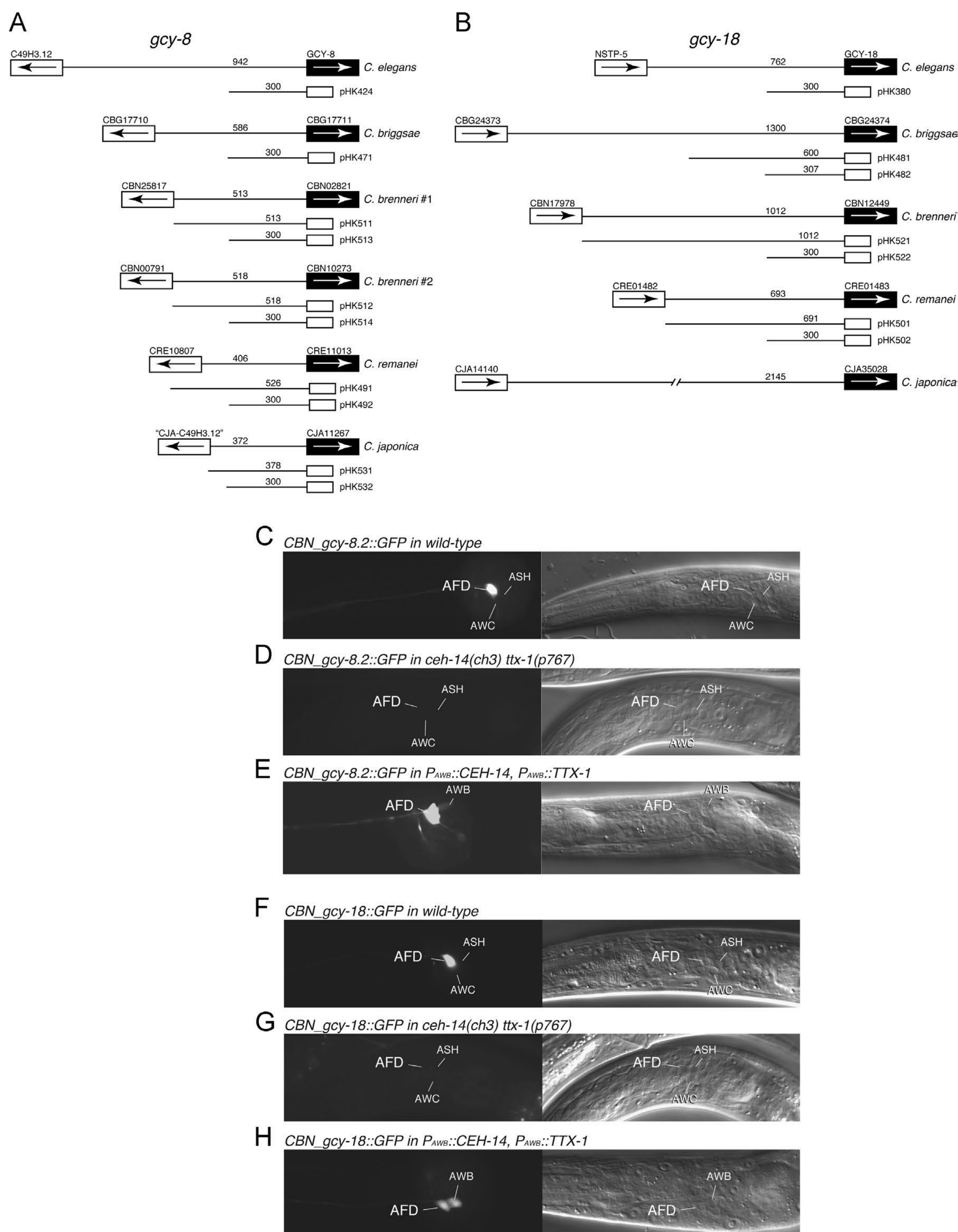

Fig. 5. Promoter::GFP expression of *gcy-8* and *gcy-18* from five *Caenorhabditis* species. (A, B) Genomic structures and reporter constructs of *gcy-8* (A) and *gcy-18* (B) from *Caenorhabditis* species. Genomic structures of *gcy-8* and *gcy-18* (black box) and their upstream genes, C49H3.12 and NSTP-5 (white box), are represented. The arrows within the boxes indicate the direction of transcription. The coding sequence name for each gene and the lengths of the intergenic sequences (bp) are shown above the genomic structure. The names of the GFP reporter constructs used for expression analysis are shown underneath the genomic structures, and the small white boxes represent the GFP reporter. The lengths of the intergenic sequences are shown above the reporter constructs. (C–H) Expression pattern of *C. brenneri gcy-8.2*(*CBN10273*)::GFP (C–E) and *gcy-18* (*CBN12449*)::GFP (F–H) constructs in wild-type (C, F), *ceh-14* and *ttx-1* double mutant (D, G), and P_{AWB}::*CEH-14*, P_{AWB}::*TTX-1* transgenic (E, H) *C. elegans*.

cooperative DNA binding of two TFs (Duggan et al., 1998; Wenick and Hobert, 2004). However, we could not observe the cooperative binding of CEH-14 and TTX-1 to the *gcy-8* family promoters by *in vitro* analysis (data not shown). This result suggested that these two TFs have no direct interaction and seemed to act independently, whereas they showed synergy. There are not many reports showing this type of transcriptional synergy, but recently, notable precedents for such regulation have been published. One paper shows that several terminal identity markers for serotonergic neuron NSM are either partially affected or unaffected in *unc-86* (POU-HD) and *ttx-3* (LIM-HD) single mutants, and are more strongly affected in the double mutant (Zhang et al., 2014).

Table 3
Expression of *gcy-8* and *gcy-18* promoter constructs of five *Caenorhabditis* species.

Species	***Promoter***	**AFD expression**		
		In wild-type [a]	**In double mutant** [b]	**In ectopic strain** [c]
C. elegans	*CEL_gcy-8*	94% (*N*=171)	0% (*N*=244)	17% (*N*=129)
	CEL_gcy-18	95% (*N*=112)	0% (*N*=152)	69% (*N*=199)
C. briggsae	*CBR_gcy-8*	91% (*N*=140)	2% (*N*=228)	12% (*N*=98)
	CBR_gcy-18	91% (*N*=116)	8% (*N*=90)	87% (*N*=178)
C. brenneri	*CBN_gcy-8.1*	93% (*N*=110)	1% (*N*=204)	15% (*N*=132)
	CBN_gcy-8.2	91% (*N*=138)	1% (*N*=238)	31% (*N*=150)
	CBN_gcy-18	88% (*N*=112)	1% (*N*=94)	75% (*N*=152)
C. remanei	*CRE_gcy-8*	90% (*N*=120)	0% (*N*=158)	1% (*N*=122)
	CRE_gcy-18	92% (*N*=112)	0% (*N*=86)	36% (*N*=100)
C. japonica	*CJA_gcy-8*	91% (*N*=108)	2% (*N*=64)	8% (*N*=76)
	CJA_gcy-18	NA	NA	NA

The expression of *gcy-8* and *gcy-18* reporter constructs of five *Caenorhabditis* species in AFD and AWB was examined in *C. elegans*. The numbers of the animals examined are indicated in brackets. NA: not analyzed (No reporter construct was obtained for *CJA_gcy-18::GFP*. see *Materials and methods*.)

[a] Wild-type animals.
[b] *ceh-14 ttx-1* double mutants.
[c] transgenics expressing CEH-14 and TTX-1 in AWB.

Another paper shows that combinatorial expression of transcription factors, including *ast-1* (Ets) and *ceh-43* (Dlx-HD), regulates terminal identity of dopaminergic neurons, and that single mutant of either TFs had little or no effect on the specific marker expression, such as *cat-1* (synaptic vesicular monoamine transporter), but the double mutant completely lost the expression (Doitsidou et al., 2013). However, the mechanism of the synergistic effect has not been clarified.

The synergistic mechanism by CEH-14 and TTX-1 is also unclear at present, but we think as one possibility that they might work as components in a complex with unidentified interacting proteins. This could be a conventional TF complex or much larger complex, for example, enhanceosome (Carey, 1998). Such a complex consists of multiple components, thus it might enable the preservation of its function (at least partly) even if one component is removed; whereas it loses the integrity for the function once two key components are removed. There might be more key components other than CHE-14 and TTX-1. Indeed, in the ectopic expression analysis, *gcy-8* and *gcy-18*::GFP in AWB was not as strong as endogenous AFD expression (Fig. 4B and D), and *gcy-8* and *gcy-18*::GFP reporters were exclusively expressed in AFD, whereas either of TFs was expressed in many other neurons. This presumably reflects a role of additional unknown factors that may be required for full activity of the promoters and it would not be present in other cells. The synergy effect was also observed with other AFD marker genes, such as *ntc-1*, *nlp-21* and *cng-3*, though the extent was variable. Combinatorial expression of multiple transcription factors might perform fine regulation through this kind of synergistic effects.

We were able to ectopically express *gcy-8* and *gcy-18* in AWB by forced co-expression of *ceh-14* and *ttx-1*. In transgenic animals, we did not observe morphological abnormalities or alternations in the characteristic winged structure of AWB cilia (data not shown). However, we observed a dye-filling defect in AWB of transgenic animals. In the wild-type, AWB takes up the lipophilic dye DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine), whereas AFD does not (data not shown). Moreover, it has been shown that forced expression of *ceh-14* in AWB can suppress the athermotactic phenotype of *ceh-14* (*ch3*) mutants (Cassata et al., 2000a). These results suggest that forced expression of *ceh-14* and *ttx-1*, at least partially, transform AWB into AFD. It has been proposed that the sensory neurons AWA, AWB, and ASG share a common AWC olfactory neuron-like developmental default fate because the combinatorial expression of specific TFs represses this default fate and promotes the expression of cell type-specific characteristics (Lanjuin et al., 2003; Sagasti et al., 1999; Sarafi-Reinach et al., 2001; Sarafi-Reinach and Sengupta, 2000; Sengupta et al., 1996). It has also been shown that mutations in *ttx-1* result in the expression of the AWC marker *str-2*, suggesting partial adoption of the AWC-like fate by AFD (Satterlee et al., 2001). Taken together, AWC may be the ground state for AFD and other similar sensory neurons, AWA, AWB, and ASG, and these sensory neurons may share a common sensory feature despite their different functions.

ttx-1 is expressed in AFD and nine pharyngeal marginal cells, but not in any other neurons (Satterlee et al., 2001); this suggests its function as an AFD-dedicated TF similar to ASE chemosensory neuron-specific TF *che-1* (Chang et al., 2003; Uchida et al., 2003). In contrast, *ceh-14* is expressed in different tissues such as the hypodermis, spermatheca, and various neurons (the anterior body neurons AFD, ALA, and BDU; the tail sensory- and interneurons PHA, PHB, PHC, DVC, PVC, PVN, PVQ, PVT, PVW, and PVR; and the touch neurons ALM, AVM, PVM, and PLM) (Cassata et al., 2000a; Kagoshima et al., 2000, 2013). *ceh-14* has been shown to regulate terminal differentiation of neurons through different TF partners in different cells. In ALA interneurons of the head, together with the PRD-HD protein CEH-17, CEH-14 regulates ALA-specific gene batteries, including *let-23*/EGFR and *plc-3*/PKCγ, which mediates lethargus (sleep-like) behavior of *C. elegans* (Van Buskirk and Sternberg, 2010). In either *ceh-14* or *ceh-17* mutants, the expression of ALA genes is slightly reduced, however, in double mutants, the expression is completely abolished (Van Buskirk and Sternberg, 2010). In the PVT interneurons of the tail ganglion, *ceh-14* and another LIM-HD TF-encoding gene, *lim-6*, are co-expressed, and they regulate the expression of *zig* genes (*zig-1*, *zig-2*, *zig-3*, *zig-4*, and *zig-8*), which are required for maintaining ventral nerve cord (VNC) organization in *C. elegans* (Aurelio et al., 2002). Single mutations of *ceh-14* or *lim-6* have only a slight effect on the initiation of *zig* expression. However, in the double mutants, the expression of *zig* is significantly affected in PVT, if not completely abolished, resulting in the disorganization of the left and right axonal tracts (Aurelio et al., 2002). In DVC tail interneurons, CEH-14 induces *ceh-63*/HOX3 expression and both homeobox TFs are critical for the expression of a further downstream helix-turn-helix type TF, *mbr-1*/MBlk-1 (Feng et al., 2012). In PHA, PHB, PHC and DVC neurons, *ceh-14* alone can regulate *eat-4*/vesicular glutamate transporter (VGULT), and it also co-regulates *eat-4* with *ttx-1* in AFD and with *unc-86*/POU class-HD in PVR neuron (Serrano-Saiz et al., 2013). These results suggest that CEH-14 itself has low binding specificity and rather supportive roles in activation of the downstream genes. This does not mean CEH-14 is

A *gcy-8* promoters

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CEL  ---------------TGTGAATA-----------GA----GTTCTC-----GTAAATCGACAATGGAGTTTTCA---------AAA---AATACATAACG
CJA  TAGTGTAATCTTGTCGATAAATAATCTCTTGTCAAGTAATTTCTCTTTCGATTCAATTCAAAAAAAAGT--------------AAGGTTAGAAAAAAGAG
CBR  --------------AAGAAAATG--------TTAAA----CTCCTACACAGTCACAC--ATCTTAACATCTATATCTTTTTTTCAATGTAGTGAACGAAT
CRE  ---------ACTTTAAAAAAATAACATTTTCTCAAT----ATTATCAGCCTGTAAAT--ACAAT----CCTCCCACTCGAAAAAAATCTTCTGAATATCT
CBN1 -----------TCTTTTATAATA------CTTCAGA----ATCCTT----ATAAAAT--ATAATAAAACTCCCAC--------AAATCTGATGAATA---
CBN2 ---------AATGTTCTATAATA------CTTCAGA----ATCCTT----AAAAAAT--ATAATAAAACTCCCAC--------AAATCCGATGAATA---
                . .   . ***:        :.:       *..:        ::*:  * :::...  .  ..         :*:. .  :.:*.:

                                  C-I                                                             T-I
CEL  ATG---AGTAATCCTTGTTATATTTTCCATTAGAGTAAATTGA------TATAAAGAAAACAGAGTTA------TATCTGAAGTTGAGAAGCTTAAAAGA
CJA  GTGTTTAGTAATC-------CACATTCCATTAGAGCGAATTGACTTCATGGAAAACAT--------------------CCATCCAGAACGGATTA-----
CBR  ATG---AGTAATC-----T-TGCATTCCATTAGAGTAGATTGA------TAAAAACATAACAAAATTGAGCTATCGGAAATATATGAGAAGCTTA-----
CRE  ATG---AGTAATC-----TACGCATTCCATTAGAGTTAATTGA------TAAAAAGAAAACATAGTTG-------TCGGGAAGAGAGGAAGCTTA-----
CBN1 -TG---AGTAATC-----GAAGCAACCCATTAGAGTAAATTGA------TAAAAAGAAAACAGGATTGAGCTGTCTTCAGAAGA--GGAAGCTTA-----
CBN2 -TG---AGTAATC-----GAAGCAACCCATTAGAGTAAATTGA------TAAAAAGAAAACAGGATTGAGCTGTCTTCAGAAGA--GGAAGCTTA-----
      **   *******      . .::..*********:.:*****      :::***.*.::::   ::.           .::..   :::*:***

CEL  TTTAT---------GACTACCGTAAG-ATAT----CC-GGATGAGCTGATACAGATG-G---------CTCATGAAGAGACGCAACGGGTTTCATTTCGT
CJA  -----TCGGCTGACAGCTACCGTAGACGGGACGATCCGGGATGAGCCAGAGA-GCCGCGAGATGGGGTCT--------------AAAAACTTCATTTCGT
CBR  -----TCGGCTGACGACTACAGTAGGAATATT-ATCCGAGATGATCCACAAC-G-CG-CAGA------CTCGTCGCTCAA--GGCACGACTTCATTTCAT
CRE  -----TCGGATGACGACTACAGTAGGAATATG-ATCCGAGATGATCCGGAAC-GACG-G---------CTCTCCGCGCGACAACGACTGCCTCATTTCGT
CBN1 -----TCGGCTGACGAATACCGTAGGAATATG-ATCCGAGATGATCCGATTC-GACG-GAGAGGGGCTCCTTCCAGTCGACAC-ACATACTTCATTTCGT
CBN2 -----TCGGCTGACGAATACCGTAGGAATATG-ATCCGAGATGATCCGATTC-GACG-GAGAGGGGCTCCTTCCAGTCGA---AATTAACTTCATTTCGT
          ::::.::::::.***.***::.::::  ::**:.*****.*:.   : *.:* :...      *.    .   ..:    .  .::*******:*

CEL  ACATTTTTATCAACTCATAT---TCGATCCACGGTAACTATCGTGAGAAGCTGTAAAAGGATTTTCGATTCTACCTTTTCCACATCAAA
CJA  ACATTTTCTTATTCTTGCAAACACCGCTTCTT-----CCTTTTTTTCGCGTCGTC------------AACTGGCTTTTTCAAC------
CBR  TCTTATTCTGTGTGGACGAC--GACGACTCAT----CGAATTCTCAATTTTTGTT-------------TCTTTCTA--TCGTGATC---
CRE  ACATTTTCATTGCTTCCGACTAGTCGACTCAT--------ATTTTGAAAGTAATA-------------ACTTATTTCTTCGTA------
CBN1 ACATTTTCTTCGATCCAAACCAGACGACTCAT----CTCTTCCCGAGGAATCGTT-------------TTCTAAAAATTCAAAGTA---
CBN2 ACATTTTCTTCGACCCAAACCAGACGACTCAT----CTCTTTCCGAGGAATCGTT-------------TTCTAAAAATTCAAAGTA---
     :*:*:**:.: .   .  *.  . **:::*::        :.   .  . : :*              .  :.    :**  .
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B *gcy-18* promoters

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CEL  -----TAAAATGTTT--------------------------TTTATCTCC--GGGTTTCTATTGTTTTCTCTGTTTTGTTAATAAGAA-------CGAAT
CJA  ----CCGATGGGCCC--------------------------CCGGT------GGAACTTTCTGGGATT------------AATAATAGAACTACCTGTAT
CBR  AGCACTAATGTGATTGAACAA---------ACATCCCTTGATTTAT------GAATTC-------ATTTTCCAGACTGCAAACCGAAACTTTGTTCTTAT
CRE  -----TAAATTGTCTGAATAACATGAAGCAAGGTCCATC--TTAAT------GAAAGTTTTCTAGTTTTTAGGAGTTTCGAAATATAAATTTG----TAT
CBN  ---ATCTATGTGAGTGCACTACTATTGGAGGGACACTTCAACACCTCACTTGGAATGTTTACGGTATATTGGGTTCTATT-----------------TGT
          ..*..:*  :. .  .               . .  .. .*      *.:. :.:   . .*:.:  .   :   ::  . :.    .    ::*

                                                                                                 C-II
CEL  TTTATAAGTAAATATAAGTAATAGT-------------------------------------AGTACGTAACACATGAAAATCCTTCGGAAAAACTAATC
CJA  TTTGATTGAAACTTTTTAGAAACTGGTTGAATTGAAATCCGCTCTGCGGTTCGGATATACTAATCTGACGCAGTAGTTAGTTTATTCCAAAAACTCATT
CBR  TTCGTAACGACATCTTATTCATTTTTGT---TTTGATGT----------CGTCGAACC----ACTACGTAACACA-------------AGAAAACTAATC
CRE  TTCAAAACAA----TTATTCATCTCTGTAAATCCAAAGA----------TTTTCTCTA----AATACGTAGTATC-------------GGAAAACTAATC
CBN  TTCG--------TTTTATTAATTTCTCCTGAATTGAACTGTGACTCTTCATTCGATAA----GATACGTAACATC--------------GAAAACTAATC
     **.. ..   : : *:::: *: .....  . ...:.             .:..    .   :.*::*::.:*                .******:**:

                                                                                T-II         "T-III"
CEL  AAACTAATGCATATTGTACGGATGGATAATCTAGTA--------CTGACGTATTTTGAGTAGACACAGTAGTTAATCTT------AAATTTAAGCAGCTC
CJA  AAAGTAATGCACAG----ATGGGAGATAATCTTGAATTCGGTTTGTGACGCC----AAAAGAGGATTTCCGATTTT---------AGAGATAAGTAG---
CBR  AAACTAATGCACAT----CTCGCAGATAATCTCAGA-----TTTCTGACGCA-----AAACGCGGATACGAATAGAA----TACGGTCACTAAGCGG---
CRE  AAACTAATGCACAT----CTCAAAGACAATCTGGGA-----TGTTTGACGTA-----AAATACGGATACAAATAAGAAGGATACGGTGTCTAAGCGG---
CBN  AAAATAATGGATAT----GTGGAAGATAATCCAGGA-----TCTGTTACGTA-----AACTATGGATACGAATAATACT------GTAGTTAAGCGG---
     ***.*****:*.*:    .:.. :**:****: :.*     : : *:***.:     *:.  . :..:.: .:*:...        ...  ****:.*

CEL  AAATCAGATTTCAAGTGAAGCGGCAACGTGGAAGAGCTCAGTTGGAAGAAATAGATATTTCGCAGCTACTTTGCAGTCGG--AGCATCAGAAA-------
CJA  ---------GAGCTCAGTCAGTTGGA-------------ATTCAGTCACAATTCATCGTAC------------CAGTCGATTGAAATCTGAAAACCCAAC
CBR  -------ATAAGTTGTGAGCCGACGATGGCTGAGATGCAATTCAGACAGCATCAGTCTTTGAAAG--AAGTCGTCGGCAG------TC------------
CRE  -------ATGGCGAGCGTCGTCGAGAT------------ATCCAGAAGAATTCATTCAAAGACAG--AAGCTGTCGGCAA--GTAACC------------
CBN  -------ATAAGATGAGAGACGGACACGAATGA------ATTCAGAATATATCAGTCTGAG------AGTTTGTTGGCGGACAGAGTC------------
               :: .. .: *.  ... .*      .      *::::*:. ..:*.. *:....   ..  :  ..:.  *.*..    ..:*

CEL  --
CJA  GA
CBR  --
CRE  --
CBN  --
```

Fig. 6. Alignment of *gcy-8* and *gcy-18* promoters from five *Caenorhabditis* species. Promoter sequences (300 bp) of *gcy-8* (A) and *gcy-18* (B) orthologs from five *Caenorhabditis* species (CEL: *C. elegans*, CBR: *C. briggsae*, CRE: *C. remanei*, CBN: *C. brenneri*, CJA: *C. japonica*) were aligned by multiple sequence comparison by log-expectation: (http://www.ebi.ac.uk/Tools/msa/muscle/) (MUSCLE) and manually adjusted by eye. *C. brenneri* has two copies of *gcy-8* (CBN1 and CBN2, probably derived from gene duplication in the *C. brenneri* sublineage). The nucleotides that are conserved in orthologous promoters with zero, one, and two mismatched residues are indicated by an asterisk (*), a colon (:), and a period (.), respectively. The binding core sequences are indicated by C-I and C-II for CEH-14 and by T-I, T-II and, “T-III” for TTX-1, as determined by EMSA analysis.

dispensable, but CEH-14 might serves as a hub molecule interacting with different TF partners in different neurons to regulate the expression of gene batteries as “terminal selector” (Hobert, 2008) genes for the specification of neuronal fate.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ydbio.2015.01.010.

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