Evolutionary innovation of the excretory system in *Caenorhabditis elegans*

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The evolution of complexity relies on changes that result in new gene functions. Here we show that the unique morphological and functional features of the excretory duct cell in *C. elegans* result from the gain of expression of a single gene. Our results show that innovation can be achieved by altered expression of a transcription factor without coevolution of all target genes.

Differences in gene expression patterns contribute to the structural and functional differences between species[1,2]. These differences can result from loss of a particular regulatory element from the gene in one species or gain of the element in the other. From an evolutionary standpoint, only changes that result in a gain of expression serve to increase genomic and species complexity. Although differences in gene expression patterns have been described previously (*e.g.*, refs. 3–6), evolutionary gain of gene expression has not been conclusively shown, to our knowledge.

The excretory system in nematodes mediates osmotic and ionic regulation[7,8]. *C. elegans* and *C. briggsae* have different morphologies of part of the excretory system, the excretory duct cell, including different positions of the duct opening[9]. It was not known whether this difference resulted from loss of a feature in *C. briggsae* or from gain of a feature in *C. elegans*. We characterized several strains representing six species and found that *C. elegans* is unique among *Caenorhabditis* species with respect to duct position (**Fig. 1a**). In an assay of excretory system function, *C. elegans* also showed a greater ability to survive when challenged with high salt (**Fig. 1b**). Thus, *C. elegans* has innovations in both structure and function of the excretory system.

The zinc-finger gene *lin-48* is expressed in the *C. elegans* excretory duct cell and is necessary for normal duct cell features[9] (**Fig. 2**), but it is not expressed in this cell in *C. briggsae*. One difference between the two species is the presence of enhancers in *C. elegans lin-48* that are required for correct expression of *lin-48*, including sites that respond to the bZip transcription factor CES-2 (ref. 9). We isolated *lin-48* from two additional *Caenorhabditis* species and did not find the CES-2 enhancer sequences that are present in *C. elegans lin-48* (**Fig. 1c**). We made *lin-48:gfp* reporter transgenes and found that *lin-48* from the other species was expressed at lower levels than *C. elegans lin-48:gfp* in the excretory duct cell (**Fig. 1d**). In contrast, *lin-48* cDNA from each of the four species rescued the structural and functional defects in *C. elegans lin-48* mutants (**Fig. 2a**,**b**). These results indicate that changes have occurred in the regulation of *lin-48* expression but not in the function of LIN-48 protein.

The genetic and comparative analysis shows *C. elegans* has unique excretory system features that result from the gain of expression of a transcription factor. However, a transcription factor can mediate cellular effects only if target genes respond. Are naive excretory duct cells able to respond appropriately to LIN-48 when introduced *de novo*? To test this, we used a promoter that acts in the excretory duct cell in both *C. elegans* and *C. briggsae* (*Ce_pB0303.11*; ref. 10 and data not shown). Under control of this promoter, *lin-48* cDNA rescued excretory duct

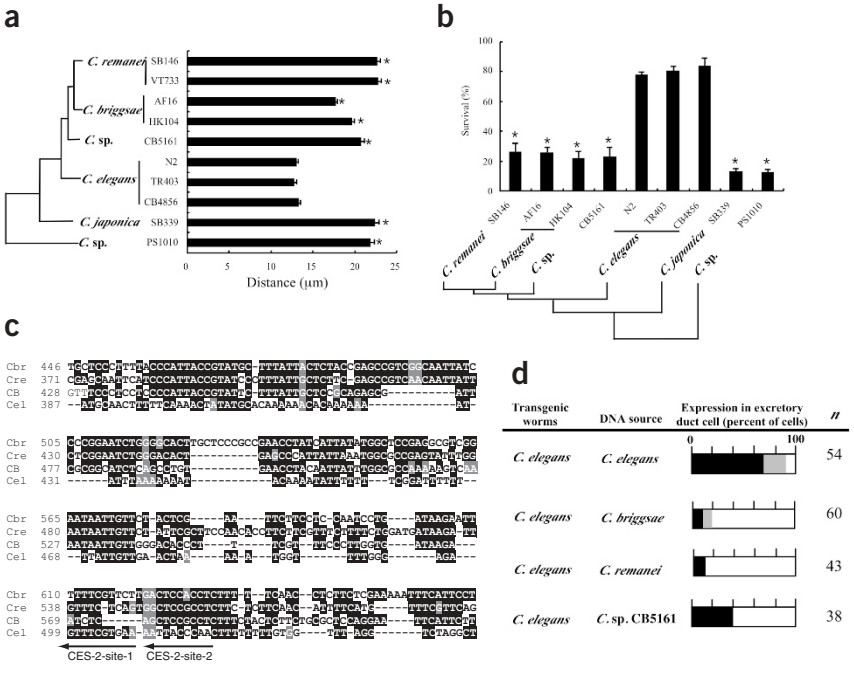

Figure 1 *C. elegans* differs from other *Caenorhabditis* species in excretory system features. (**a**) Excretory duct position in *Caenorhabditis* species. Bars (mean ± s.e.m.) show the distance between the excretory duct opening and the base of the posterior pharynx bulb (see **Fig. 2c**). Species are grouped according to a reported molecular phylogeny[11]. Our analysis of *lin-48* coding and noncoding DNA gives a similar phylogeny for the four species analyzed (data not shown). (**b**) Salt tolerance in *Caenorhabditis* species. Percent survival of L3 worms after 24 h on NGM plates made with 500 mM (rather than 50 mM) sodium chloride. (**a**,**b**) $*P < 0.001$ by treatment (strains and species other than *C. elegans* N2) versus control (*C. elegans* N2). Data were analyzed using one-way ANOVA and two-sample *t*-test with Bonferroni adjustment. (**c**) Alignment of the *lin-48* proximal upstream regulatory region from *C. briggsae*, *C. remanei*, *C.* sp. CB5161 and *C. elegans*. Black arrows indicate the position of the CES-2-response sites identified in the *C. elegans* gene[9]. Sequences were aligned using ClustalW 1.8 (ref. 12) and shaded with Boxshade 3.21. (**d**) *lin-48:gfp* transgenes from *C. elegans*[9] and *C. briggsae*[9] and comparable ones from *C. remanei* and *C.* sp. CB5161 were tested in *C. elegans*. The percentages of cells expressing green fluorescent protein (GFP; black bar), cells expressing very low but detectable levels of GFP (gray bar) and cells not expressing GFP (white bar) are indicated for each construct. *n*, number of worms scored. Transgenes were generated and assayed as described[9].

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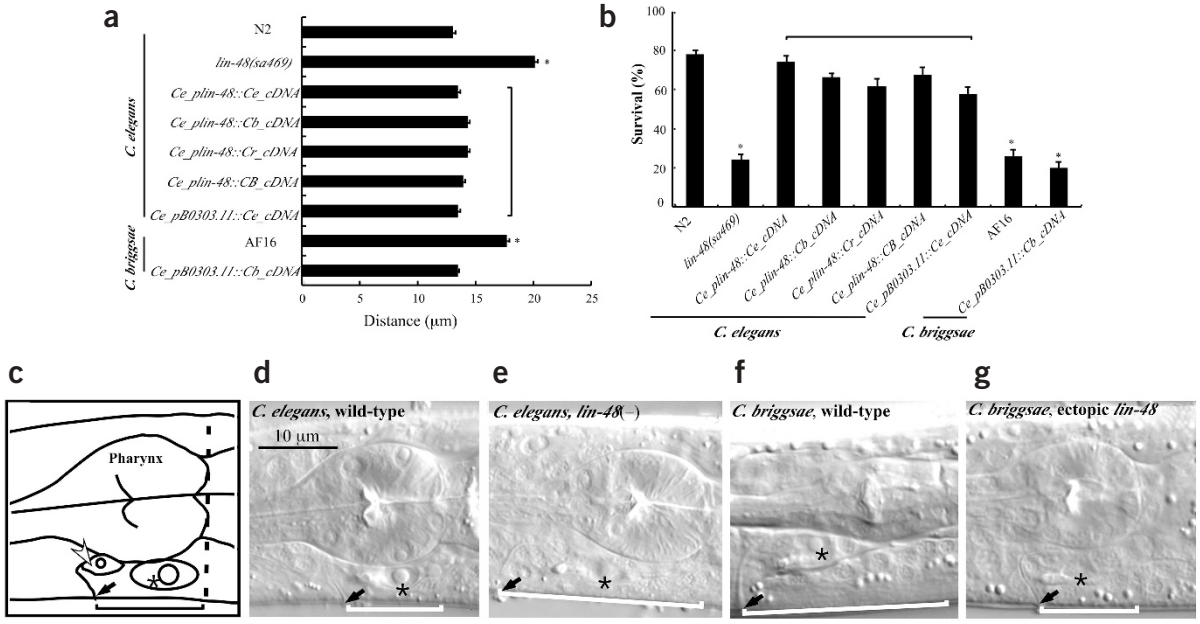

Figure 2 *lin-48* expression affects excretory system features in *Caenorhabditis*. (**a**) Excretory duct position in transgenic worms. Data presented as in **Figure 1**. Bars grouped in bracket correspond to transgene-bearing *lin-48(sa469)* worms. The source of each cDNA is indicated by the two-letter species abbreviation, except *C.* sp. CB5161 is indicated as CB. Duct distance was significantly shorter in *C. elegans* N2 and transgene-rescued worms than in *lin-48(sa469)* mutants ($P < 0.001$). Under the control of *B0303.11* promoter (*Ce_pB0303.11*), *C. briggsae lin-48* cDNA altered the *C. briggsae* duct to a morphology similar to that of *C. elegans* wild-type worms ($P = 0.56$, *t*-test). (**b**) Salt tolerance in transgenic worms. *C. elegans lin-48(sa469)* mutants were sensitive to salt and could be rescued by expression of *lin-48* cDNA. In contrast, transgenic *C. briggsae* worms were as sensitive to salt as were *C. briggsae* AF16 wild-type worms. (**a**,**b**) $^*P < 0.001$ by treatment (strains and species other than *C. elegans* N2) versus control (*C. elegans* N2). (**c**) Diagram of the excretory system. The large excretory cell nucleus (labeled with asterisk) is posterior to the excretory duct cell nucleus (open arrowhead). The excretory duct connects to an opening in the ventral cuticle (filled arrow). The distance between the duct and the base of the posterior pharynx bulb is indicated with a bracket. In the photos, the excretory duct cell nucleus is out of the plane of focus. Excretory system in (**d**) *C. elegans* wild-type worm, (**e**) *C. elegans lin-48* mutant, (**f**) *C. briggsae* wild-type worm and (**g**) *C. briggsae* worm expressing ectopic *lin-48* in the excretory duct cell. Left, anterior; top, dorsal.

features of *C. elegans lin-48* mutants (**Fig. 2a**,**b**). Notably, it also conferred *C. elegans*-like morphology on otherwise wild-type *C. briggsae* worms. This result indicates that genes downstream of *lin-48* that mediate duct cell morphogenesis can respond to LIN-48 in *C. briggsae* without any further change or coevolution. In contrast, transgenic *C. briggsae* worms were still sensitive to high salt. Thus, the role of *lin-48* in duct morphogenesis is separable from its role in salt tolerance. We propose that this is due to different LIN-48 target genes that mediate the different excretory duct cell features. Our results show that specific, gain-of-function changes in a single regulatory gene between species can result in structural and functional innovation.

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COMPETING INTERESTS STATEMENT
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